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Considerations for the Human Health Risk Assessment of Externally Applied dsRNA-Based Pesticides

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Series on Pesticides
No. 110

Considerations for the Human Health Risk Assessment of Externally Applied dsRNA-Based Pesticides

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
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Environment Directorate
ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
Paris 2023

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Foreword

The purpose of the OECD work leading to this paper was to develop guidance on the risk assessment of pesticide products based on exogenously-applied double-stranded RNA (dsRNA). This work has been led by the OECD's *Ad Hoc* Expert Group on RNAi-based Pesticides, a sub-group of the OECD's Working Party on Pesticides (WPP); the WPP, which directs the OECD pesticide programme, helps member countries to harmonise the methods and approaches used to assess pesticides.

A significant focus of the work of the Expert Group is on issues relating to considerations for both environmental risk assessments (ERA) and human health risk assessments (HHRA) of double-stranded RNA (dsRNA) molecules used as pesticides. These two OECD documents are based on the available literature, input from experts in OECD member countries, and the results of an OECD Conference (OECD, 2019) on this topic. On 25 September 2020 the OECD published a document developed by the Expert Group, entitled "*Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides*."

The document on environmental risk assessment includes the following:

- a summary overview of the available scientific information related to the mechanism of RNA interference (RNAi)
- information about molecular structures of RNA, chemical modifications, assay methods, commercial production and formulation of RNAi-based products
- types of dsRNA-based end-use products and application methods
- effects on non-target organisms from exposure to RNAi-based products (including an overview of RNAi machinery in different organisms)
- fate of dsRNA in RNAi-based pesticides applied in the environment
- issues for consideration in conducting environmental risk assessments of dsRNA-based pesticides

A limited amount of this information is repeated in this document; thus, it is strongly recommended that the contents of two documents be considered together.

The present document, "*Considerations for the Human Health Risk Assessment of the Application of Externally Applied dsRNA-Based Pesticides*," specifically focusses on human health risk assessment. It should be read in conjunction with the environmental considerations document since that document already provides valuable information on the biological mechanisms of RNA interference, considers a range of issues directly relevant to human exposure arising from the application of externally-applied dsRNA-based pesticides, and discusses possible effects of dsRNA exposure in mammals.

RNA interference technologies used for pest control can include 1) externally applied dsRNA with an absence of genetically-modified organisms (GMO-free), 2) externally applied dsRNA produced by GMOs that are still present in the product, and 3) GMO plants which incorporate the machinery to synthesise RNAi molecules specifically directed against a pest species feeding on a crop, e.g. "plant-incorporated

protectant," (PIP) or as *in planta* RNAi). This document focuses on GMO-free externally applied dsRNA pesticides.

The initial draft of this document was developed by Les Davies, former co-chair of the Ad Hoc Expert Group on RNAi-based pesticides, who served as a consultant to the Secretariat. The document was finalised by the Expert Group members Salvatore Arpaia (Italy), Ann-Kristin Diederich (Germany), Sabrina Feustel (Germany), Achim Gathmann (Germany), Emily Hopwood (Canada), Mike Mendelsohn (Chair, USA), Nina Ortiz (USA), Amanda Pierce (USA), Wiebke Striegel (USA), and Andrea Zikova-Kloas (Germany).

The working document was approved by the Working Party on Pesticides in May 2023. This document is being published under the responsibility of the Chemicals and Biotechnology Committee (CBC), which has agreed that it be declassified and made available to the public.

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1. Introduction

1.1. Background

1. The purpose of the OECD work leading to this paper was to develop guidance on the risk assessment of pesticide products based on exogenously-applied double-stranded RNA (dsRNA). This work has been led by the OECD's *Ad Hoc* Expert Group on RNAi-based Pesticides, a sub-group of the OECD's Working Party on Pesticides (WPP); the WPP, which directs the OECD pesticide programme, helps member countries to harmonise the methods and approaches used to assess pesticides.

2. A significant focus of the work of the Expert Group is on issues relating to considerations for both environmental risk assessments (ERA) and human health risk assessments (HHRA) of double-stranded RNA (dsRNA) molecules used as pesticides. These two OECD documents are based on the available literature, input from experts in OECD member countries, and the results of an OECD Conference (OECD, 2019) on this topic. On 25 September 2020 the OECD published a document developed by the Expert Group, entitled "Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides."

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5. The present document, "*Considerations for the Human Health Risk Assessment of the Application of Sprayed or Externally Applied dsRNA-Based Pesticides*," specifically focusses on human health risk assessment. It should be read in conjunction with the environmental considerations document since that document already provides valuable information on the biological mechanisms of RNA interference, considers a range of issues directly relevant to human exposure arising from the application of externally-applied dsRNA-based pesticides, and discusses possible effects of dsRNA exposure in mammals.

6. In summary, the focus of the first document is on the environmental risk assessment of exogenously-applied dsRNA-based products, while the focus of this second document is on human health risk assessment, considering the exposure of pesticide

applicators and bystanders, as well as consumers of treated agricultural produce which may contain residues. Relevant background information is common to both documents.

1.2. Purpose of this document

7. This document aims to serve as a guide[1] to the pesticide industry developing new products using RNAi as a mode of action and to regulators conducting human health safety assessments of pesticides. This document focuses on issues which will have a bearing on data requirements for determining the toxicological hazards of dsRNA, possible exposure routes, and potential risks to the health of pesticide applicators and bystanders, as well as to consumers of agricultural produce treated with these products.

8. Different sections of this human health risk assessment document cover:

- problem formulation (i.e., what are the specific issues with dsRNA-based products that will need to be considered that are not already being considered by regulators in assessing the human safety of chemical and biological pesticides). Plant protection products containing viable genetically-modified organisms (GMO) need additional authorisations for the release of GMO into the environment according to the specific legislation of the concerned OECD country. Considerations on the problem formulation for such authorisation are outside of the scope of this document.
- impact of end-use product formulation and dsRNA modification on exposure and hazard characterization of dsRNA-based pesticides
- a brief re-consideration of the environmental stability and persistence of the dsRNA, since this will impact human exposure
- possible routes and extent of exposure of humans to dsRNA molecules applied in the environment to control pests
- possible adverse effects in exposed humans
- the overarching risk assessment
- risk management.

9. Thus, the standard risk assessment paradigm has been applied in the consideration of these novel pesticides *viz*. risk is a function of **hazard** (the intrinsic properties of the agent, and its potential adverse effect on human health) and the **extent of exposure** to that agent (where the extent of exposure is determined by a number of factors including the use pattern of the product in question, and the physicochemical properties and environmental stability of the product).

1.3. Scope of this document

10. RNAi based technologies may be used in plant production in different contexts. A major distinction is to be made between plants producing small RNA molecules due to modifications of the genome (GMO) and externally applied ds-RNA based plant protection products. In general, a relevant aspect is whether the product contains viable organisms or only purified RNA molecules. A further aspect can be the intended use of the product e.g.,

[1] As stated by Arpaia et al (2020), 'guidelines for risk assessment cannot be "cookbooks" and some flexibility should be left for risk assessors to adapt and justify the details of their assessment. It is up to regulatory agencies to judge the validity of risk assessment approaches and support applicants in delivering estimates of risk at the highest safety standard, considering the severity and the likelihood of possible impacts on human, animal and environmental safety'.

as food and feed (see Schenkel & Gathmann 2021, Dietz-Pfeilstetter et al. 2021). In case GMO are released into the environment or GMO are used as food or feed additional authorisation might be needed depending on the relevant legislations of the OECD countries. Therefore, this document does NOT address issues related to the human health risk assessment of genetically-modified organisms which incorporate the machinery to synthesise RNAi molecules specifically directed against a pest species feeding on the crop (referred to in this document either as "plant-incorporated protectant," (PIP) or as *in planta* RNAi); that is, its focus is solely on the risk assessment of the application of environmental (or 'free' [exogenous]) dsRNA. Nevertheless, some results from the human health risk assessment of these products remain relevant to the risk assessment consideration of exogenously-applied RNA pesticides. Thus, several RNAi-based PIP products[2] are discussed as regulatory examples since the dsRNA produced *in planta* in these examples has been studied as free dsRNA, separate from the GM plant.

[2] The expression of RNAi silencing constructs in plants (targeting pests and pathogens) has sometimes been referred to as host-induced gene silencing, or HIGS.

2. Overview of RNA interference

This section provides a summary overview of RNA interference. RNA interference (RNAi) is a biological process in which small ribonucleic acid (RNA) molecules inhibit gene expression, typically by causing the enzymatic destruction of specific messenger RNA (mRNA) molecules which are the templates for the synthesis of proteins. This process is commonly referred to as post-transcriptional gene silencing (PTGS); that is, mRNA is transcribed from nuclear DNA but before the message is translated into proteins by ribosomes, the mRNA is blocked or otherwise destroyed by an enzymatic process guided by a specific non-coding small interfering RNA (siRNA) or microRNA (miRNA).
For a more detailed explanation, the reader should refer to Section 2 of ENV/JM/MONO(2020)26 and the reviews cited therein.

11. Interference or suppression of gene expression by naturally-occurring dsRNA was largely unknown until the basic mechanism was elucidated in a scientific paper by Andrew Fire and Craig Mello (Fire et al, 1998); this work led to the award of the 2006 Nobel Prize in Physiology or Medicine[3].

12. Much research on modifying plants, insects, mammals and other organisms with transgenes or antisense constructs producing single-stranded RNA (ssRNA) had been published (USEPA, 2013) prior to the discovery that dsRNA was at least 10-times more potent in its effect on gene expression than ssRNA (Fire et al, 1998). This finding unleashed

[3] This was an even shorter period than that between the 1983 paper reporting the polymerase chain reaction (PCR) and Kary Mullis's award of the 1993 Nobel Prize in Chemistry (with Michael Smith).

a major research focus into the biochemical mechanism of what was termed 'RNA interference' (or RNAi).

2.1. Gene Silencing Pathways

13. The term 'gene silencing' refers to the interruption or suppression of the expression of a gene at either the transcriptional, post-transcriptional or translational level. Transcriptional gene silencing is a mechanism of epigenetically directed alterations in gene expression based on chemical modifications of nucleotides and histones without changes to DNA sequences, but with potential heritability.

14. As noted above, RNAi corresponds to a post-transcriptional gene silencing process, initiated by dsRNA molecules, that inhibits expression of specific genes by inactivation of specific mRNAs (Zamore et al, 2000).

15. The discovery of the RNAi mechanism did not occur with a single event or publication. The phenomenon had been observed in plants (called 'Post Transcriptional Gene Silencing' or 'co-suppression'; see USEPA, 2013 and references cited therein) and in fungi (called 'quelling'; Vance & Vaucheret, 2001; Mello & Conte, 2004). However, it was only after these apparently-unrelated processes were understood that it became clear that they all described the same phenomenon, called RNA interference (RNAi) by Andrew Fire and Craig C. Mello in their 1998 paper on blocking gene expression in the nematode worm *Caenorhabditis elegans* by the application of dsRNA (Fire et al, 1998). Their observation that specific and more robust gene silencing could be achieved using micro-injected dsRNA (rather than ssRNA) in this model organism led to the elucidation of the RNAi machinery as we now understand it. The initial discovery of the potency of dsRNA as an elicitor of gene silencing was quickly followed by the finding that, at least in *C. elegans*, dsRNA from the environment (in this case produced in *Escherichia coli* bacteria used as a food source for the worms) could also trigger gene specific silencing (Timmons & Fire, 1998).

16. RNAi has been demonstrated as an important endogenous pathway used in many different organisms to regulate gene expression post-transcriptionally, for example as "Not only is RNAi a vital part of the immune response of plants to viruses and bacteria and fungi (Stram & Kuznetsova, 2006; Katiyar-Agarwal et al, 2006; Obbard et al, 2009; Zhao et al., 2018)". In addition, in both juvenile and adult *Drosophila*, RNAi is important in antiviral immunity and is active against pathogens such as Drosophila X virus (Zambon et al, 2006; Wang et al, 2006a). This immune-type response, triggered by the application of exogenous long dsRNA molecules, probably resembles the response to double-stranded nucleic acid from RNA viruses. Some species, such as flies and plants, use RNAi as part of their immune system because RNA from infecting viruses triggers an RNAi response.

17. There are several variations in RNAi pathways which differ in the source of the RNA and the specific mechanism through which gene silencing is accomplished, but they all are triggered by the presence of a double-stranded RNA (dsRNA) molecule and all follow a similar order of events (Roberts et al, 2015). The dsRNA is processed into smaller RNAs (normally between 21–25 base pairs in length) by an enzyme called 'Dicer' or its homologs (part of the RNase III family of ribonucleases[4]) and incorporated into a protein complex known as RISC - the 'RNA-Induced Silencing Complex' (Figure 1) (Elbashir et al, 2001b; Tijsterman & Plasterk, 2004: Vance, 2011). RISC then uses one strand of the siRNA as a guide to find and bind to a complementary sequence on a specific mRNA, noting that some sequence mismatch may be allowed (Du, 2005); in plants, endogenously-produced forms

[4] Dicer is also known as 'endoribonuclease Dicer' or 'helicase with RNase motif', names which reflect its function. Being part of the RNase III family, it cleaves double-stranded RNA (dsRNA) and pre-microRNA (pre-miRNA) into short double-stranded RNA fragments called small interfering RNA and microRNA, respectively.

of small RNA (micro RNA, or miRNA – see Section 2.1.2 below) usually bind with perfect or near-perfect complementary to their complementary mRNA sequences (and induce direct mRNA cleavage by RISC), while miRNAs in animals tend to be more divergent in their requirements for target complementarity with some mismatching (and induce translational repression) (Saumet and Lecellier, 2006; Bartel et al., 2009). The binding of RISC to mRNA leads either to its degradation or the interruption of its translation into protein. The following sections provide more detail about several RNAi pathways, which are distinct but significantly conserved in various functions. The two main types of small RNAs are siRNA and miRNA.

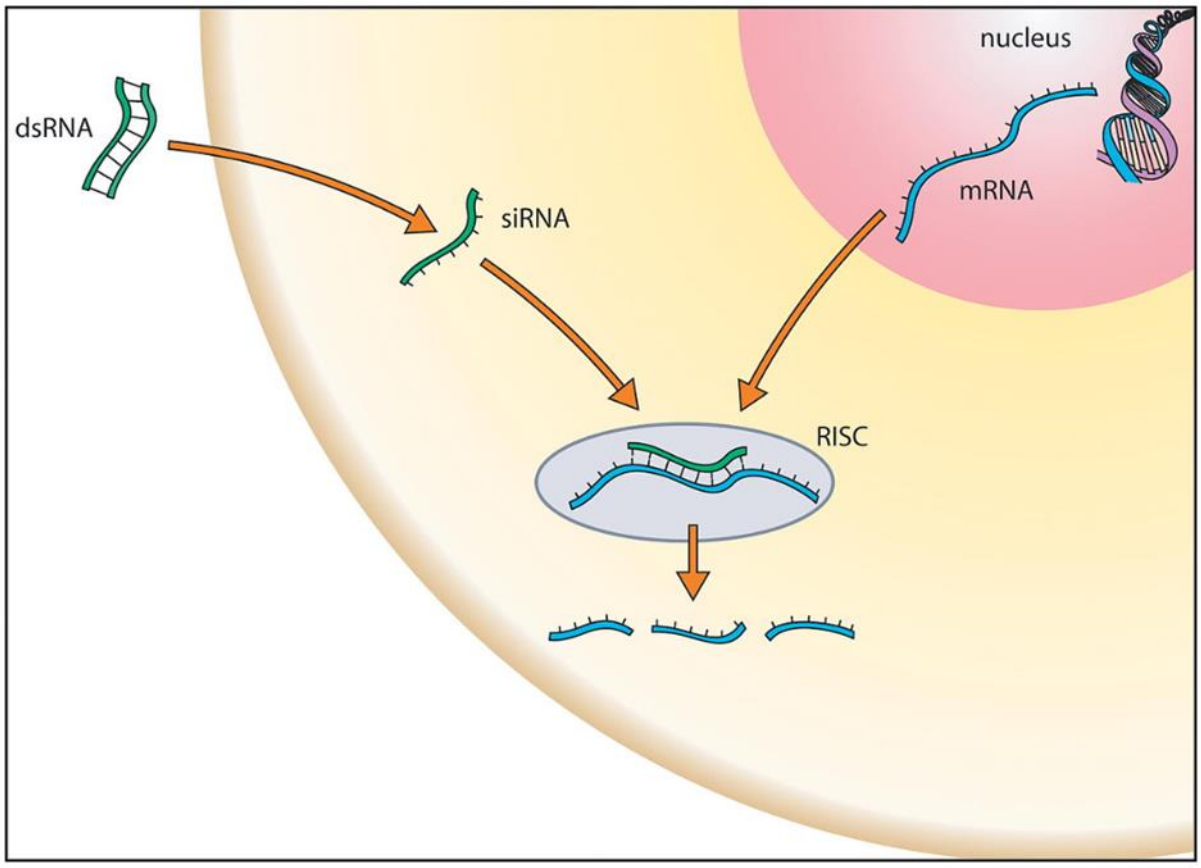

Figure 1: Simplified schematic of environmental RNAi: A double-stranded RNA is taken up by the cell and cleaved into small interfering RNAs. A guide strand from the siRNA incorporates in the RNA-Induced Silencing Complex (RISC), which undergoes binding to a complementary sequence on a messenger RNA (mRNA) and leads to its degradation. [Figure from Wikimedia Commons (https://commons.wikimedia.org/wiki/File:RNAi.jpg). Source: Robinson, 2004]

2.1.1. Small interfering RNA (siRNA)

18. An inducible RNAi pathway is triggered by the introduction of **exogenous** dsRNA. The dsRNA is cleaved by 'Dicer' into a pool of siRNA duplexes 21 to 25 nucleotides in length. The siRNAs comprise a population representing the entire length of the exogenous dsRNA; they are double stranded because they derive from the long dsRNA that triggers the process. One strand of the siRNA duplex (the so-called 'guide strand') incorporates into the RNA-induced silencing complex (RISC). The 'passenger' strand of the siRNA is degraded. Which strand becomes the guide or passenger strand is determined by the thermodynamic properties of the duplex. Once incorporated in RISC, the siRNA strand acts

as a guide to find complementary sequences in mRNAs that it can bind to by Watson-Crick base-pairing. In post-transcriptional silencing, RISC contains a ribonuclease that cleaves the target mRNA to which the siRNA guide has bound, triggering degradation of the target. In this way, targeting of mRNA degradation is sequence-specific and RNA complementary to the dsRNA trigger is silenced.

19. siRNAs generally show full complementarity to their target mRNA, and cleavage occurs 10–12 bases from the 5′ end of the guide strand binding site (Davidson & McCray, 2011).

2.1.2. microRNA (miRNA)

20. The second pathway is used by **endogenous** micro RNAs (miRNAs). These small RNAs are formed from endogenously-expressed transcripts in the nucleus of the cell. miRNAs are derived from inverted repeat sequences that form one or more stem-loop structures, where the stem consists of dsRNA and the loop is unpaired, single-stranded RNA. In plants these primary miRNAs (pri-miRNAs) are processed by DICER-LIKE 2 (DCL2) in a two-step process in the nucleus which finally produces mature miRNA of 20- to 22 nucleotides (Bologna and Voinnet, 2014; Axtell et al, 2011). In animals pri-miRNAs are processed in the nucleus into pre-miRNA by the ribonuclease III enzyme Drosha. After the pre-miRNAs are exported to the cytoplasm, Dicer processes these pre-miRNAs into mature miRNAs that are approximately 22 bp in length and function similar to siRNAs (see above). Because of the size and structure of the primary miRNA transcript and the specificity of the processing, the result is a single miRNA, which may target multiple mRNA transcripts (Siomi & Siomi, 2010). Either processed strand can mediate post-transcriptional gene silencing, but many miRNAs show asymmetry, primarily loading one strand into the RISC. The miRNA guides RISC to the mRNA target, where the miRNA typically binds to the 3′ UTR. In animals, Watson-Crick base pairing between miRNAs and their targets is usually partial, but with high complementarity from bases 2–8 of the miRNA, which is known as the 'seed' region. Base pairing can also occur between central miRNA nucleotides and target mRNAs. Data from several laboratories showed that miRNAs repress the initiation of translation, although more recent work indicates that miRNA–mRNA complexes can be transported to cytoplasmic processing bodies, after which de-adenylation, decapping and mRNA degradation occurs. It appears that some miRNA-mediated translational repression is reversible (Davidson & McCray, 2011).

21. Plant miRNAs usually have near-perfect pairing with their mRNA targets, which induces gene repression through cleavage of the target transcripts. In contrast, mammalian miRNAs can recognise their target mRNAs by using as little as 6–8 nucleotides (the seed region) at the 5' end of the miRNA, which is not enough pairing to induce cleavage of the target mRNAs but may result in translational pausing. As noted above, a given miRNA may have hundreds of different mRNA targets; conversely a given target might be regulated by multiple miRNAs.

2.1.3. siRNA vs miRNA

22. In considering the related siRNA and miRNA pathways for RNA interference, the differences between them can be summarised as follows (Lam et al, 2015):

- miRNAs are endogenously-derived from specific loci within the genome of the cell, while siRNA may be derived from transposons (Ghildiyal et al, 2008), viruses, heterochromatic DNA[5] or exogenous dsRNA.

[5] Non-coding RNAs transcribed from heterochromatic DNA repeats function in the assembly of heterochromatin and keep heterochromatic domains silent (Bühler & Moazed, 2007).

- miRNAs are processed from longer precursor hairpin transcripts, whereas siRNAs are processed from long dsRNA (bimolecular) or small hairpin RNAs.
- Each miRNA hairpin precursor molecule produces a single miRNA duplex, whereas each long dsRNA molecule produces multiple siRNA duplexes.
- siRNA sequences are rarely conserved (e.g., different plant viruses don't generate the same siRNA sequences in a common host), while many miRNA sequences are well conserved across both plant and animal species (and are thought to be a vital and evolutionarily-ancient component of gene regulation) (Carrington & Ambros, 2003).
- All bases within an siRNA generally contribute to its target specificity, whereas only the 5' half of miRNA contributes to its target specificity.
- miRNA often binds to the 3' untranslated region of target transcripts, whereas siRNAs can form a complementary duplex anywhere along a target mRNA.

2.1.4. PIWI-interacting RNA

23. PIWI-interacting RNAs (piRNAs) are a class of longer-than-average miRNAs, about 26-31 nucleotides long as compared to the more typical miRNA or siRNA of about 21 nucleotides. Found in most metazoans (multicellular animals with differentiated tissues and organs), they bind to particular Argonaute proteins called PIWI proteins; the term 'PIWI' (sometimes also 'piwi') is derived from 'P-element induced wimpy testis' proteins described in research on *Drosophila*. piRNAs have specialised roles in the nuclei of some cells. For example, they are involved in silencing sequences of DNA called 'transposable elements' or transposons. Silencing stops these sequences moving around the genome, which can eliminate the potential for transposon-induced mutations (Siomi et al, 2011).

24. PIWI proteins and their bound piRNAs are key components of a regulatory pathway that is essential for germline establishment and maintenance. Loss of PIWI proteins in *Drosophila*, mice, and zebrafish (*Danio rerio*) leads to a loss of fertility, due to a disruption in germline stem-cell formation or maintenance, arrest in meiosis, and other gametogenic defects (Juliano et al, 2014).

25. PIWI proteins are also expressed outside the germline, primarily in various kinds of stem and progenitor cells. For example, PIWI genes are expressed in the pluripotent stem cells of planarians, sponges, and tunicates and are required for epimorphic regeneration in these organisms. PIWI expression is also found in somatic stem cells in cnidarians and ctenophores, mesenchymal stem cells in mice, and hematopoietic stem cells in humans (Juliano et al, 2014).

26. However, detailed investigations have largely been confined to the function of the PIWI-piRNA pathway in the germline and gonadal somatic cells in a few model bilaterians (animals with bilateral symmetry), with a focus on transposon silencing. The potential significance of the pathway in stem cells outside the gonad and on non-transposon sequences is yet to be investigated to any significant extent Juliano et al, 2014).

27. It appears that piRNAs require less sequence complementarity than siRNAs and have been more frequently involved in translational pausing in mammals and higher organisms than direct mRNA cleavage (USEPA, 2013).

2.1.5. Deadenylation and decapping pathways

28. In plants, fungi and animals, mRNA can be degraded as part of eukaryotic RNA 'quality control'. Decapping and deadenylation pathways result in removal of the 5´ cap or 3´ poly(A) tail, respectively, thereby destabilising the mRNA. Modulating the length of the poly(A) tail of an mRNA by deadenylation is a means of controlling protein production and mRNA stability (Humphreys et al, 2005); changes in the length of mRNA poly(A) tails are

catalysed by a diverse range of deadenylase enzymes (Goldstrohm & Wickens, 2008). In plants, for example, as the tail is shortened, the mRNA typically undergoes decay by exoribonucleases present in the cytoplasm (Abassi et al, 2013).

29. The removal of the 5' cap (7-methylguanylate) exposes a 5' monophosphate cap, leading to the 5'3' degradation of mRNA sequences by 5' exonucleases such as XRN1 in eukaryotes (Poole & Stevens, 1997); decapping may or may not be linked to the 3'-5' degradation of mRNAs (USEPA, 2013). In mammals, miRNAs are known to bind to the 3' untranslated region of the mRNA, which is often followed by deadenylation and mRNA decay (Beilharz et al, 2009). miRNAs are also known to effect translational repression of mRNAs in *Drosophila* and zebrafish (*Danio rerio*) through deadenylation and subsequent destabilisation of mRNA.

30. While RNA turnover and post-transcriptional gene silencing (PTGS) are functionally linked (Martínez de Alba et al, 2015), note that deadenylation and decapping pathways are not RNAi gene silencing pathways *per se* but are part of RNA turnover. In this document, they are considered in relation to the interplay between RNAi and RNA decay pathways; 'aberrant' RNA without a polyA tail may be subject to RNA-dependent RNA polymerase 6 (RdRP6), thereby generating dsRNA which can then enter the RNAi pathway (Baeg et al, 2017). Meanwhile decapped and deadenylated mRNAs, and siRNAs and miRNA-cleaved mRNAs are substrates for exonucleolytic decay.

31. In prokaryotes, the initial mRNA transcripts naturally possess a 5'-triphosphate group after transcription; the bacterial enzyme 5' pyrophosphohydrolase (RppH) removes a pyrophosphate molecule from the 5' end, converting the 5'-triphosphate to a 5'-monophosphate and triggering mRNA degradation by ribonucleases (Deana et al, 2008; Hsieh et al, 2013).

2.2. Cell-autonomous, Non-cell-autonomous, Systemic, & Environmental RNAi

32. In responsive organisms, RNAi silencing can act in a cell-autonomous or non-cell-autonomous manner (see Figure 2).

2.2.1. Cell-autonomous RNAi

33. In the case of cell-autonomous RNAi, the silencing process is limited to the cells in which the dsRNA is introduced (or expressed) and encompasses the RNAi process within those exposed cells. The varied biology of dsRNA-induced silencing is exemplified by the apparently cell-autonomous, non-heritable silencing in *Drosophila* and mammals compared with the systemic nature of silencing in *C. elegans* (see the following sub-sections).

2.2.2. Non-cell-autonomous RNAi

34. In the case of non-cell-autonomous RNAi, the interfering effect can propagate across cell boundaries and takes place in tissues or cells separate from the location of application of the dsRNA. Non-cell-autonomous RNAi was observed in the first RNAi experiments, conducted in *C. elegans* (Fire et al, 1998); when these nematodes are microinjected with dsRNA into head, tail, intestine or gonad arm, soaked in dsRNA solution, or fed with bacteria expressing dsRNA, RNAi is induced and the effect is transmitted across cellular boundaries (Tabara et al, 1998; Timmons & Fire, 1998). Non-cell autonomous RNAi has been described in parasitic nematodes[6] (Geldhof et al, 2007), hydra (Chera et al, 2006), planaria (Newmark et al, 2003; Orii et al, 2003), insects (Tomoyasu et al, 2008; Xu

[6] *C. elegans* is not a parasitic nematode but a free-living one, feeding on dead and decaying organic matter viz. it is a saprobic nematode.

& Han, 2008) and plants (Himber et al, 2003). In *C. elegans* and in a number of arthropods RNAi was found to be not only systemic, but also heritable (parental RNAi) due to RNA signal transmission to progeny (Zotti et al, 2018; Bucher et al, 2002; Abdellatef et al., 2015).

35. There are two different kinds of non-cell-autonomous RNAi, 'environmental RNAi' and 'systemic RNAi' (Hunter, 2006; Whangbo & Hunter, 2008; Huvenne & Smagghe, 2010).

2.2.3. Environmental and Systemic RNAi

36. Environmental RNAi refers to sequence-specific gene silencing in response to environmentally-encountered dsRNA. Certain organisms can take up dsRNA from their environment, with subsequent triggering of RNA silencing. At least two pathways for dsRNA uptake have been described: (1) a specific trans-membrane channel-mediated uptake and (2) an endocytosis-mediated uptake (Whangbo & Hunter, 2008; Huvenne & Smagghe, 2010).

37. 'Systemic RNAi' is the term used when the silencing phenomenon is locally initiated in the organism but then spreads from cell to cell throughout the whole organism. Systemic RNAi was first observed in *C. elegans* when ingested or injected dsRNAs (microinjections into the head, tail, intestine or gonad arm) led to the systemic spread of RNAi throughout the organism and transmission to its progeny.

38. For more detail, refer to the OECD publication titled *Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides* (ENV/JM/MONO(2020)26).

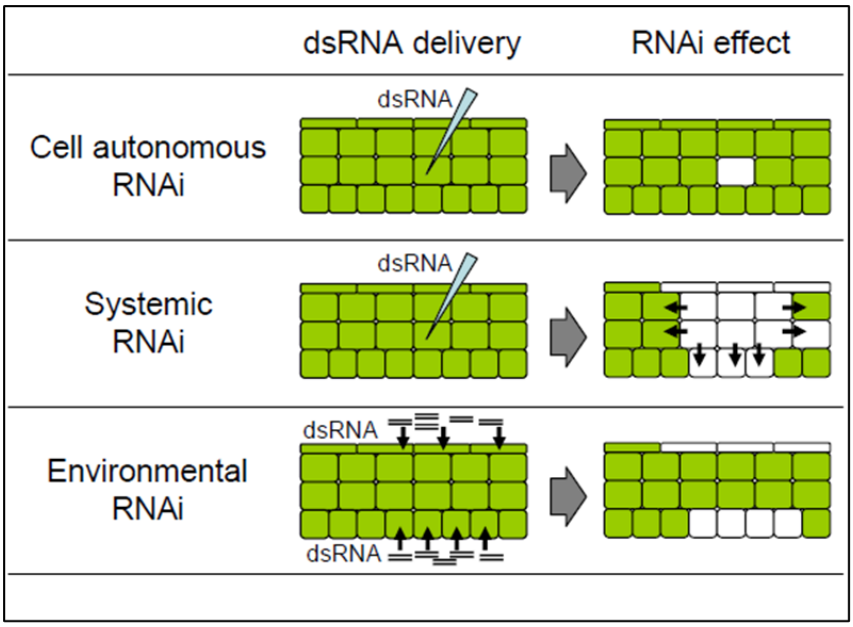

Figure 2: Cell autonomous, systemic and environmental RNAi: RNAi can act in a cell-autonomous manner, affecting only cells directly exposed to dsRNA e.g., by injection (top row of figure). Cell non-autonomous RNAi includes systemic RNAi, in which the RNAi effects propagate across cellular boundaries, mediated by transport of small RNAs (middle row). Environmental RNAi refers to the take-up of dsRNA from the environment (bottom row). [This figure is modified from Figure 5 in Paces et al, 2017. A similar illustrative diagram is found in Huvenne & Smagghe, 2010.]

Text Box: Brief summary of transcription, translation and RNA interference

1. RNA interference — or RNAi — is a cellular process that can silence the expression of specific genes: that is, it can stop proteins being produced from specific genes. RNAi can involve different types of small RNA molecules. The best-known types of RNAi act by targeting messenger RNA (mRNA) in the cytoplasm.
2. In eukaryotic cells, protein-coding genes are transcribed by RNA polymerase II (RNAPII). During transcription, an RNA molecule — the primary transcript — is synthesised using the DNA sequence as a template. The primary transcript is processed to form mRNA; regions of sequence that do not code for the protein (introns) are removed by splicing and a 'cap' is added to the 5′ end of the RNA.
3. The mRNA travels from the nucleus into the cytoplasm through the nuclear pore complex. The nuclear pore complex is one of the largest protein complexes in the cell. Filaments on the cytoplasmic side of the pore help to channel the mRNA towards the protein synthesis machinery.
4. In the cytoplasm, ribosomes carry out translation of the mRNA to form a polypeptide chain, which folds to form a protein. Ribosomes are made up of proteins and RNA molecules called ribosomal RNAs (rRNAs). Some protein folding happens during translation, but the endoplasmic reticulum is an important site of protein folding and many ribosomes are associated with it.
5. The RNAi mechanism targets the mRNAs to limit synthesis of proteins. Because siRNAs can trigger degradation of specific mRNAs, they limit production of specific proteins.
6. RNAi is now widely used in the laboratory to explore the functions of genes by experimentally silencing them. Specific siRNAs (commonly 'hairpin' RNAs, molecules that fold back on themselves so that they become double stranded) are now available to silence almost any gene in human cells or model organisms.
7. Researchers hope to use siRNAs to correct faulty gene expression in humans and to control economically- and environmentally-important pest species. Delivery mechanisms are a key consideration in the development of RNAi-based therapies and RNAi-based pest-control products; the siRNA trigger needs to be delivered efficiently and in a targeted manner.

39. The potential applications of exogenous RNAi[7], also termed environmental RNAi or eRNAi, were quickly recognised by researchers interested in human therapeutics (Lares et al, 2011; Witwer & Hirschi, 2014; Hirschi et al, 2015) and in plant protection (Baum et al, 2007; Mao et al, 2007; Mao et al, 2011; Burand & Hunter, 2013; Koch & Kogel, 2014). However, an important consideration in the use of RNAi in the human therapeutic space is the existence of multiple barriers to systemic absorption, as discussed later in this working paper.

40. The utility of RNAi for pest control was suggested by two studies published in 2006 which demonstrated that PTGS can be elicited in insects by oral administration of double-stranded RNA (dsRNA) (Araujo et al, 2006; Turner et al, 2006). Subsequently, investigations in *Aedes aegypti* mosquitoes provided the first demonstration that RNAi could be induced in insects by topical application of dsRNA (Pridgeon et al, 2014).

41. Using technology based on RNAi as a mode of action, plant protection against pests may be achieved by:

- genetic modification of the crop plant to express a precursor long dsRNA specifically directed against a pest species feeding on the crop (referred to as a plant-incorporated protectant (PIP) or *in-planta* RNAi)

[7] so-called 'spray-induced gene silencing', or SIGS (Liu et al, 2020).

- application of double-stranded RNA (dsRNA) molecules (with a nucleotide sequence specifically developed to target a gene of interest in a pest species) via spraying, stem injection, root dip, seed treatment, soil drench or hydroponics; when applied via spraying, the dsRNA on the leaf surface may be directly ingested by feeding pests or, if the uptake of the dsRNA is facilitated in some way to aid penetration of the plant cuticle, the absorbed dsRNA molecules could possibly be processed to shorter RNA molecules and distributed throughout the plant.

42. The use of dsRNA-based active ingredients could provide a valuable additional mode of action to mitigate pest pressures in a number of agricultural and horticultural crops world-wide, in a manner that is specifically directed at pest targets without adversely impacting non-target species, including beneficial insects. In this regard, the deployment of dsRNA-based active ingredients holds significant promise as compared with conventional[8] chemical insecticides, acaricides, fungicides and herbicides.

43. In a 2020 review, Dalakouras and coworkers highlighted the advances to date in the field of RNAi application to plants. The paper provides a useful overview in that it discusses the various methods of RNA delivery in plants against diverse targets including plant genes, viruses, viroids, fungi, insects, mites, nematodes, and bacterial pathogens; examines the possible shortcomings of these methods; considers the critical parameters that have to be met for a desired outcome; and explores feasible possibilities for increasing the efficiency and applicability of such treatments. It noted that, at the time of publication of the review, RNAi use in agriculture was based on the use of transgenic plants expressing double-stranded RNAs (dsRNAs) against some key crop pests. However, GMO-free RNA interference technology which does not alter the genome of the crop plant to be protected promises to be a more generally-acceptable approach (Dalakouras et al, 2020).

44. It is possible that the adoption of technology using RNAi as a mode of action will result in a reduction in human health risks as compared with the use of conventional chemical pesticides (Joga et al, 2016; Kamthan et al, 2015). However, as for conventional pesticides, regulators will have to adequately assess both environmental and human health and safety issues relating to novel technologies based on RNAi as a mode of action, before any marketing approval.

[8] The term ‘conventional’ is used in this document to refer to existing types and forms of pesticides which have been, or are in use, and for which regulatory requirements have been well established in all OECD jurisdictions.

3. Problem Formulation

The current approach used by regulatory agencies in OECD countries to assess the health risks of 'conventional' chemical and biological pesticides provides the basic framework for human health risk assessment (HHRA) of dsRNA-containing end-use products. However, due to the specific mode of action of dsRNA active ingredients (viz, Watson-Crick base pairing of complementary or near-complementary RNA sequences), the assessment approach currently used for traditional pesticides will need to be adapted, in order to investigate any potential adverse effects triggered by the dsRNA-based active ingredient under consideration.

The focus of this document is to highlight specific issues that will need to be considered in HHRAs of dsRNA-containing end-use products, thus serving as a guide during problem formulation and during the hazard and risk assessment steps.

45. In most, if not all OECD member countries, dsRNA molecules which are developed for topical application to crops for pest control will be assessed by relevant government agencies using a similar set of considerations to those applied to chemical and biological pesticides. However, as outlined in subsequent sections of this document, it is expected that certain additional issues will need to be taken in to account in considering applications for regulatory approval of pesticides with a dsRNA technical-grade active constituent(TGAC)/technical grade of the active ingredient (TGAI).

46. In order to create clarity about terminology, a distinction is made in the document between the terms active substance, technical-grade active constituent (TGAC)/ technical-grade active ingredient (TGAI) and the TGAC/TGAI as it appears after the formulation process:

47. **Active Substance**: dsRNA molecule (including any chemical modifications) with a nucleotide sequence specifically developed to target a pest species without manufacturing impurities.

48. **Technical-grade active constituent (TGAC)/ Technical-grade active ingredient (TGAI):** outcome of the dsRNA manufacturing process intended to be used in plant protection products and includes the active substance and manufacturing impurities. For plant protection products/pesticides certain tests typically need to be performed with the TGAC/TGAI rather than using the active substance. For exogenous dsRNA products, these tests may need to be performed on the end-use product which includes the TGAC/TGAI and formulation ingredients that have the potential to impact stability and uptake of the RNA.

49. **Formulation (process):** combining the TGAC/TGAI with formulants and sometimes other TGACs/TGAIs. In the formulated end-use product, the TGAC/TGAI can be modified (conjugation of dsRNA, formation of dsRNA complexes and nanoparticles etc.) to ensure e. g. stability.

50. It is hoped that this paper will provide a guide to:

- problem formulation, the first step in conducting a human health risk assessment (i.e., what are the specific issues with dsRNA-based products that will need to be considered that are not already being considered by regulators in assessing the human safety of chemical and biological pesticides and of introducing GMOs into the environment?)
- the component hazard, exposure, and risk assessment steps of the human health risk assessment.

51. As is the case with conventional pesticides, an understanding of the underlying mode-of-action (MOA) of dsRNA-based active ingredients will help to inform the risk assessment. However, a complete understanding of all the steps of a biochemical pathway by which a pesticide active ingredient acts is not required to complete a risk assessment. The key components of risk assessment viz., an estimation of exposure and identification of any hazards (e.g. effects on mortality, growth, and/or reproduction), have been, and will continue to be carried out without necessarily having detailed MOA information to hand.

52. Special factors to consider during problem formulation for human health risk assessment of dsRNA products may be broadly categorised under hazard (the intrinsic properties of the agent being assessed, and its potential adverse effect on the human body) and the extent of exposure to that agent (where the extent of exposure is determined by a number of factors including the use pattern of the product in question, and the physicochemical properties and environmental stability of its active constituent). These factors are considered in the following sections.

53. It should be noted that, at the time this document was being drafted, the OECD was not aware that any regulatory agency had published a risk assessment (or summary risk assessment) of a dsRNA applied directly to the environment as a constituent of an end-use product intended for pest control. Thus, there was no direct regulatory experience with externally applied dsRNA product to provide an example for this document and help guide regulatory considerations related to these novel pesticides. However, the Exposure and Hazard sections of this document contain several examples of regulatory considerations for specific dsRNAs produced in GM crops as plant-incorporated protectants (PIPs) which act *via* RNAi.

4. Exposure

Since exogenously-applied dsRNA products are likely to be applied using the same methods as traditional chemical pesticides, various routes of exposure are possible. These routes include oral, respiratory, ocular and dermal exposure during mixing, loading, and application via spray drift, or from subsequent formation of aerosols of applied product. All these routes are potentially relevant for agricultural/horticultural workers, operators (mixer/loaders and applicators), residents and bystanders in the vicinity of crops being treated, while the oral route is relevant for consumers of treated produce. Modifications to the dsRNA nucleotides or certain types of formulations may increase the persistence or potential for uptake, which in turn could increase the potential for exposure to the dsRNA.

54. A key step in problem formulation is the identification of plausible exposure scenarios; these will depend on the product and its proposed use and application methodology. It is expected that the currently-used exposure scenarios in the risk assessment of exogenously-applied chemical pesticides will also be applicable to exogenously-applied pesticides containing dsRNA. For the operators, possible exposure scenarios may occur during mixing and loading and during application of the product. During the task of mixing and loading, the operator may have direct contact with the formulation via the dermal, oral, or inhalation pathway. These contact scenarios may be considered together or independently of the environmental fate and stability of dsRNA molecules. For the general population, dietary exposure via residues of dsRNA on food is expected to be the main route of exposure.

55. Since the availability of generalizable data on the environmental fate (stability, persistence, mobility, etc.) of unmodified, modified, and formulated dsRNA molecules is limited at this time for dsRNA-based pesticides, these aspects will need a particular risk assessment focus. While the available evidence suggests limited stability and persistence

of unmodified dsRNA molecules, empirical data using appropriate experimental protocols may need to be provided by applicants, particularly on those products containing dsRNAs that are chemically modified and/or formulated to improve environmental stability and efficacy (i.e., enhanced uptake by the target organism). Such information will also allow better estimation of the extent and duration of possible human exposures during and following product application.

4.1. Environmental stability and persistence of unmodified dsRNAs

56. As noted in an early publication on predictive ecological risk assessments of RNAi in agriculture (Auer & Frederick, 2009) and in Section 6 of ENV/JM/MONO(2020)26 ('Fate and Distribution of dsRNA from RNAi-based Pesticides in the Environment'), a determination of the potential risks of exogenously-applied dsRNA to non-target species in the environment requires an understanding of the likely stability and persistence (or lack thereof) of dsRNA molecules in the environment. Similarly, an understanding of the environmental stability and persistence of dsRNA has direct bearing on estimating human exposure following the application of dsRNA-based pesticides.

57. In ENV/JM/MONO(2020)26 (paragraph 282), the OECD *Ad Hoc* Expert Group on RNAi-based Pesticides concluded that the available evidence suggested limited stability and persistence of unmodified dsRNA in the environment. The following studies, published since that document was drafted, provide some further data on this subject.

58. Parker et al (2019) reported that ^{32}P-dsRNA both degraded in soils (into ^{32}P-containing products) and adsorbed to particle surfaces i.e., decreasing concentrations of dsRNA in solution resulted from both dsRNA adsorption and degradation. Microorganisms in soil utilised ^{32}P-radiolabelled dsRNA, as evidenced by the observation that dsRNA degradation and the formation of (tentative) high-molecular weight products were suppressed in soil samples with reduced microbial activity (achieved by either solution filtration or Xray pre-irradiation of the soil). Therefore, decreasing levels of dsRNA in soil after application results from both adsorption to soil and degradation, both chemical and microbiological. Bachman et al., (2020) reported on results of a pilot study of topically applied dsRNA on soybean plants demonstrating similar rapid degradation under field conditions. Using quantitative reverse transcription-polymerase chain reaction (RT-qPCR) to detect dsRNA in soils, a method that is several hundred times more sensitive compared to the 32P radioactive label, Zhang and colleagues reported that dsRNA spiked into two agricultural soils (silty clay loam and fine sandy loam) dissipated within hours (Zhang et al., 2020).

59. Thus, these later publications support the conclusions previously made in the OECD's environmental risk assessment document, namely, that there is very limited stability and persistence of unmodified dsRNA in the environment.

4.2. Distribution and off-site movement of exogenously-applied dsRNA

60. In the same way that knowledge of the likely environmental stability and persistence of dsRNA pesticides is relevant to the determination of possible human exposure following application to crops, so too is an understanding of offsite movement and distribution in environmental compartments.

61. The off-site movement of applied dsRNA is considered in Section 6.3 of ENV/JM/MONO(2020)26 while the foliar uptake of dsRNA and its possible amplification in plants is discussed in some detail in Section 6.4 of that document.

62. The conclusion reached in that document was that, to the best of our current understanding, the available studies indicate that dsRNA can be taken up by foliar

application, but only if specialised formulations and/or delivery methods are used. However, given that relatively limited data exist on this topic, knowledge of more effective ways to breach plant barriers may be extended as research in this area progresses.

63. Offsite movement of dsRNA from a treatment site may occur *via* the following pathways (USEPA, 2013):

- Spray in drift
- Surface runoff from foliage and soil, following rain or crop irrigation
- Infiltration into the soil and movement into groundwater
- Physical movement of pollen from treated crops and dust from treated fields
- Physical movement on insect pollinators and other animals moving through treated crops
- Physical movement with plant debris (including dried residues)
- Uptake by plants through the roots.

64. As noted in Section 6.3 of ENV/JM/MONO(2020)26 (paragraph 252), dsRNA may be distributed throughout the environment *via* some or all of these pathways, regardless of whether it is stabilised or unstabilised. The first three pathways listed are the most likely routes for measurable migration of dsRNA from the application site to offsite terrestrial or aquatic environments. Volatilisation of dsRNA into the atmosphere is quite unlikely.

4.3. Exposure Routes for dsRNA-based products

65. Since exogenously-applied dsRNA products are likely to be applied using the same methods as conventional chemical and biological pesticides, the same routes of human exposure are possible, viz., oral, dermal and inhalation exposure. As for existing risk assessments of 'conventional' pesticides, the exposure of pesticide handlers/operators (mixers, loaders, applicators), workers, residents and bystanders to agricultural and horticultural activities, and of consumers of treated agricultural/horticultural produce need to be considered.

66. For each of these exposure routes, physiological and biochemical barriers, at the gastrointestinal, systemic, and cellular level to dsRNA accessing intracellular target sites are discussed in some detail. Furthermore, a great deal of clinical research is being undertaken in order to try to treat human diseases by silencing specific cellular functions using RNAi. The findings from this work are relevant to human health risk assessment of possible pest-control products using dsRNA; in particular, this clinical research has provided significant amounts of information about the pharmacokinetics of dsRNA, that is, its absorption, distribution, metabolism, and elimination in the human body, through the oral, dermal, respiratory, and ocular routes of exposure.

4.3.1. Oral exposure

67. Possible human exposure scenarios during and following the application of dsRNA-based products in agriculture or horticulture include incidental ingestion *via* water, soil, pesticide spray, dust or granules and direct ingestion *via* consumption of treated agricultural/horticultural commodities.

68. Extensive physical and biochemical barriers present a significant challenge to oral delivery of nucleic acids (reviewed by Petrick et al, 2013). These biological barriers to absorption include nucleases in the saliva and gastrointestinal tract, acidic conditions in the stomach, and multiple membrane barriers, including in gastro-intestinal (GI) cells, from GI cells to endothelium, across endothelium to blood and tissues, and across tissue

membranes. Together, they limit the delivery of ingested RNA into blood and organs. While there may be some individuals with a higher intestinal pH or more permeable GI epithelium, the other barriers would preclude functional uptake of ingested RNAs.

69. In the digestive tract, RNA is subject to both non-enzymatic and enzymatic degradation. The breakdown begins with mastication and exposure to degradative RNases in saliva (Park et al, 2006), followed by further digestion in the stomach and gut (USEPA, 2014); the harsh acidic conditions of the stomach denature and depurinate nucleic acids (Loretz et al., 2006; O'Neill et al, 2011). Pancreatic and intestinal nucleases and degradative enzymes (and possibly bile salts) eventually catabolise RNA to nutritionally-available mono-nucleotides and subsequent nucleosides and bases (O'Neill et al, 2011; Carver & Walker, 1995; Hoerter et al, 2011; Rehman et al, 2011; Sorrentino et al, 2003). In addition to the RNases encoded within the human genome, there are likely to be numerous others RNases provided by the collection of micro-organisms that colonise the gastrointestinal tract (USEPA, 2014).

70. The human gut also provides a physical barrier to uptake of hydrophilic compounds like siRNAs. Due to molecular size and charge, diffusion across cell membranes is difficult for RNA[9]. The low percentage of ingested nucleic acids that might be systemically absorbed from the GI tract would then encounter nucleases in the blood (Houck & Berman, 1958) and renal clearance (Molitoris et al, 2009).

71. To affect gene expression in cells, any remaining RNAs in circulation must: (1) cross cellular membranes; (2) escape from early endosomes to enter the cytoplasm[10]; and (3) avoid degradation by nucleases found within lysosomes (Gilmore et al, 2004; Manjunath & Dykxhoorn, 2010; Sioud, 2005). It is likely that the cumulative impact of these barriers would result in an insufficient amount of intact intracellular siRNA to impact cellular function, assuming the RNA is not modified or formulated to bypass these barriers in any way [11].

72. Thus, when metabolism and barriers to RNA absorption are considered, it is extremely unlikely that oral ingestion of naked/unformulated dsRNAs would reach mammalian cells in sufficient quantities to mediate any RNAi effects, or indeed, any other unwanted or adverse effects.

73. In considering human health risks from the application of PIP and non-PIP RNAi technology in agriculture, the USEPA's Science Advisory Panel (SAP) concluded that "the combination of RNases and acids found in the human digestive system are likely to ensure that all forms of RNA structure are degraded throughout the digestive process" (USEPA, 2014). Furthermore, in considering the susceptibility of plant and animal RNAs to RNases, the panel concluded that the available evidence "supports the likelihood that PIP and non-PIP RNAs expressed in plant material consumed by humans are likely to be degraded, no matter the type of RNA or its structural status when entering the human digestive system".

Dietary RNAs

74. Despite the biochemical and physiological considerations outlined above, several studies conducted in recent years have investigated the possibility that miRNAs from a variety of plant-based dietary sources can be taken up into the tissues of animal and human consumers and modify gene expression and metabolism. Several of the key studies and reviews (previously considered in ENV/JM/MONO(2020)26) are summarised below, since

[9] Carver & Walker (1995) suggested that if RNA avoided all the degradation processes, any uptake of short RNA sequences in humans would be limited to the upper small intestine.

[10] The route for complexed and naked nucleic acid uptake by cells is *via* endosomes. The mRNA must then escape from the endosome and reach a translationally-competent region of the cytoplasm (e.g., Juliano et al, 2012; Weissman, 2015).

[11] This is consistent with the clinical experience in endeavouring to deliver therapeutic RNAs.

the notion of uptake of active diet-derived small RNAs in recipient organisms could have significant implications for the safe use of RNA interference technology in agriculture.

75. In 2012, the publication of a paper by Zhang *et al* suggested that ingested plant miRNAs could act to regulate animal metabolism; this report described the uptake of plant-derived miRNAs into the serum, liver and a few other tissues in mice following consumption of rice, as well as apparent gene regulatory activity in the liver. The paper was met with considerable scientific scepticism but served to stimulate a significant amount of research. Indeed, it would have very significant implications for our understanding of human nutrition and therapeutics. While a few laboratories have reported supportive findings regarding the bioactive potential of dietary RNAs in mammals, most research cannot replicate and validate such findings. Some key papers in this area are summarised and discussed below.

76. In studies designed to evaluate the reproducibility of the reported findings of the Zhang et al (2012) publication, Dickinson and co-workers found little evidence for the presence of miRNAs in blood prior to or following dietary intake of miRNA-rich plant material and observed that the reported physiological changes in mice ascribed to RNAi effects were due to dietary imbalance (100% raw rice) and variability in detection methods (Dickinson et al, 2013). More recently Chan and Snow (2017), whose own research has been unable to validate the bioavailability of dietary plant miRNAs, outlined the type of experimental evidence that would be needed to support the theory of biologically-meaningful dietary uptake of dsRNA. They reviewed the literature and suggested that studies in this field have suffered from "technical artifact and a lack of reproducibility"; they noted that data reported as supportive "typically reveals descriptive phenomenology where multiple interpretations, including technical artefact, could explain the results". Thus, in a 2016 paper by Pastrello and co-workers, it was reported that, in a large nutrigenomics study cohort and in a randomised dose-controlled trial, there was a significant positive correlation between the daily amount of broccoli (*Brassica oleracea*) consumed and the amount of miRNA in the blood. They also suggested that these Brassica miRNAs regulate expression of human genes and proteins *in vitro*, and that miRNAs cooperate with other Brassica-specific compounds in a possible cancer-preventive mechanism. However, this paper was retracted by the authors on 22 May 2017 as they "no longer [had] confidence in the data to support [their] central conclusion – that is, the detection of *Brassica oleracea* microRNAs in the bloodstream of humans who consumed broccoli". This retraction was based on the incorrect design of the miRNA primers, with anti-sense design of all the forward primers for broccoli miRNA detection (Pastrello et al, 2017).

77. This negligible level of plant miRNA uptake from the diet was also demonstrated in primates by Witwer et al (2013) and in mice by several independent groups using different dietary sources (Dickinson et al, 2013; Liang et al, 2014; Snow et al, 2013).

78. Chan and Snow (2017) concluded that, unless some unknown mechanisms are involved, insufficient levels of plant miRNAs are present in mammalian plasma or serum to be active *via* canonical RNA interference mechanisms. This conclusion was supported by a 2017 report (Kang et al, 2017) which is probably the most extensive assessment of diet-derived miRNAs to date; examination of small RNA (sRNAs) in >800 datasets from human tissues and body fluids revealed that, although dietary sRNAs could be detected, they were present at ca. 5 copies per cell or fewer, far below the levels shown for their endogenous counterparts which may reach 50,000 copies per cell for some miRNA entities. Furthermore, feeding experiments using different plant diets in rats and different milk diets in pigs did not find any evidence of substantial uptake of dietary sRNA.

79. The reviews by Chan and Snow (2017) and Kang et al (2017) concluded that, based on the weight of evidence, the uptake and canonical RNA interference activity of dietary miRNAs is not a "prevalent" mechanism in mammals. While these conclusions were conservatively worded, the USEPA's 2016 Science Advisory Panel extensively reviewed a large number of studies and clearly concluded that there was no reliable evidence that exogenous dsRNA could be taken up from the gut into mammalian circulation to exert its functions in the ingesting organism (USEPA, 2016a).

80. However, the question of whether such transfer might occur in specialised contexts, including those which could occur under various conditions of dsRNA concentration, modification, and application, continues to be a subject of debate and, since the contents of the previous OECD document on this subject (viz. *Considerations for the Environmental Risk Assessment of the Application of Externally or Applied or Externally Applied ds-RNA-Based Pesticides*) were drafted, several more relevant papers have been published. These are reviewed here.

81. Link et al (2019) studied the presence of plant-derived miRNAs in a variety of foods, their stability to processing, and their detectability in GI mucosa and faeces of healthy subjects consuming the foods. They concluded that both plant and animal miRNAs are detected in foods, irrespective of processing. Plant-derived miR168 was detected in human faeces, normal gastric and colon mucosa, and peritoneal ascites samples from patients with liver cirrhosis; it was not detectable in blood or serum. Accepting that some of their detected concentrations may have been artefactual (e.g., the concentration of miR168 in raw milk), they suggested that their use of a TaqMan-based assay provided a greater specificity for miRNAs than methods used in some previous studies. Noting that their dietary study included only six healthy volunteers and, taking into account assay uncertainties, they concluded that whether food-derived xenomiRs may be associated with changes in cellular function or behaviours remains to be answered. The authors concluded that "our understanding of the cross-kingdom talk of miRNAs is still in infancy, and it is still too early to make a final statement on potential use of xenomiRs in the prevention and treatment of malignant, inflammatory, or metabolic diseases."

82. Using an improved miRNA processing and detection method Huang, Davis & Wang (2018) sought to resolve the discrepancy in the literature regarding the cross-kingdom transfer of plant miRNAs (miRNAs) into mammals. Their study in mice, using corn or corn miRNA extract as the source of dietary plant miRNAs (including miR156a, miR164a, and miR167a), tested the hypothesis that dietary plant-derived miRNA can survive the GI tract and be systemically absorbed; the presence or absence of the ingested miRNAs were analysed in the diet, caecum, faeces, liver, and whole blood. Methodology was aimed at overcoming the detection reliability issue by combining the sequence specificity of PCR and the resistance of plant miRNAs (with 3' end methylation) to periodate oxidation. This study utilised periodate oxidation followed by alkaline beta-elimination to more efficiently degrade those miRNAs without 3' end methylation (i.e., endogenous mammalian miRNAs) than in some previous studies; this ensured that the detected miRNA(s) in the biological samples were of plant origin. To eliminate the matrix effect as a variable, a control treatment utilised an autoclave method to degrade miRNAs in the corn or corn extract before administration to control groups.

83. Less than 1% of corn miRNAs was found in the GI tract after oral and gastric processing, and none was detected in the caecum, faeces, liver, or in the blood of mice following supplementation of corn miRNAs in the diet or given by gavage. In conjunction with an *in vitro* digestion study with an oral and gastric digestion phase, the study authors concluded that corn miRNAs were extensively degraded during the digestive process and were not taken up into circulation or tissues in mice. A substantial fraction of the corn miRNA was degraded in the oral phase in the *in vitro* digestion study (2 minutes at 37°C in the oral phase digestion buffer with human saliva α-amylase); it was noted that other *in vitro* digestion studies only employed a simulated gastric phase. Therefore, at least for miRNAs derived from corn, the conclusion of the Huang, Davis & Wang (2018) study was that they are not likely to be available for systemic absorption.

84. With respect to conflicting results of different studies regarding the detection of exogenous miRNAs in human or animal models, Huang, Davis & Wang (2018) commented that key issues appeared to be (1) the reliability of miRNA detection and (2) the biological significance of miRNA at the detected levels. The issue of detection reliability relates to false detections and the authenticity of a plant origin of detected miRNAs. Oversampling in database analyses, potential contamination in sequencing studies, and false positive detections in PCR assays [false positive detections can occur in PCR-based assays,

especially at high (>30) cycles] were cited as possible causes of false detections in previous reports.

85. To investigate whether development of the intestine in mammals is regulated by dietary miRNAs, Li et al (2019) fed weanling C57BL/6 male mice with diets containing miR156 miRNA; miR156 is one of the most conserved miRNAs in plants and is highly expressed in at least 42 plants studied, including the widely-consumed plants, soybean, wheat, and maize. miR156 was reported at significantly higher levels in gut tissue of mice fed with the maize diet, and both body weight and intestinal tract weight were decreased significantly, suggesting that intestine development was greater in weanling mice with lower plant MIR156 intake. The study authors found that miR156 regulates proliferation of intestinal cells both in *in vitro* and *in* vivo by inhibiting the Wnt/β-catenin signalling pathway in mouse intestine. However, because the claim for nutritional balance was not substantiated by data (e.g., a nutritional profile of all administered diets), the level of confidence in the conclusions from this paper should reflect this lack of critical information. Furthermore, *in situ* hybridization (ISH) and fluorescence ISH results revealed greater miR156 expression in intestinal crypt cells than at the mucosal surface. These results appear to provide direct evidence that maize miR156 can be absorbed, rather than simply adhering to the surface of mucosal cells and the absorption is affected by the dosage of plant miRNAs ingested. However, there did not appear to be evidence of the detection of miR156 in blood, plasma or serum in the mouse studies reported by Li et al (2019). The authors argued that there possibly may be selective absorption of different dietary miRNAs. However, since only a single animal was analysed and these methods provide only qualitative information, and no histopathological assessment or other more in-depth tissue analysis was completed the level of confidence in the conclusions from this paper should reflect these facts.

86. In a 2014 paper, Lusk raised concerns about the fact that trace quantities of contaminating nucleic acids are widespread in laboratory environments, but their presence has received little attention in the context of high throughput sequencing. Contamination must be considered a potential source of signals of exogenous species in sequencing data, even if these signals are replicated in independent experiments, vary across conditions, or indicate the presence of nucleic acid from species which seems, *a priori*, unlikely to contaminate a sample; that is, in some cases, contaminants can effectively mimic behaviour intuitively expected from true signals. His analyses suggested that, despite replication of experiments and the inclusion of 'plausible' controls, contamination could explain all of the observations used to support a published claim (Spisák et al, 2013) that complete genes pass from food to human blood. Lusk also called into question several publications that based their conclusions on observations of rare matches to exogenous species in RNA sequencing data e.g., Wang et al (2012) and Zhang et al (2012); this latter paper is discussed in some detail above.

87. Several reports of cross-kingdom transfer and biological activity were reviewed during a USEPA Scientific Advisory Panel (SAP) meeting to consider issues affecting human health and ecological risk assessments of plant-incorporated protectants (PIPs) using RNAi as a mode of action (USEPA, 2016a). The Panel concluded that studies describing cross-kingdom transfer of endogenous RNA contained a number of experimental concerns, including: (1) a lack of appropriate controls; (2) absence of sufficient data to support health-based conclusions; (3) *in vitro* studies purporting uptake into cells or across model membrane barriers have used non-physiological quantities of nucleic acid carriers such as extracellular vesicles (Witwer & Halushka, 2016); and (4) failure to consider stoichiometric estimates of target abundance and strength of siRNA-target interactions in the facilitation of canonical regulatory functions (e.g., Snow et al, 2013). The Panel also concluded that improvements in analytical methods were required to address identified concerns with sensitivity and specificity related to the potential for environmental contamination in biological samples (Lusk, 2014; Witwer, 2015; Witwer & Halushka, 2016) and that there were questions about the validation of analytical methods

(sensitivity, specificity and reproducibility) for reliable detection and quantification of sRNAs (Chan & Snow, 2017; Witwer & Halushka, 2016).

88. In a 2020 publication, McNeill & Hirschi provided an overview of the controversy around the bioavailability and bioactivity of dietary RNAs within consumers. They noted that, in recent years, several studies have suggested that miRNAs from a variety of plant-based diets can be transferred to tissues of animal or human consumers and modify gene expression. However, their view was that this conclusion remains controversial because many other research groups have either failed to detect the presence of dietary miRNAs in the circulation or any biological functionality of negligible amounts.

89. McNeill & Hirschi (2020) concluded that, although it is an intriguing concept, any role of dietary sRNAs in influencing gene expression and metabolism is far from being proven. Their overarching conclusion was that "experimental conditions and assays need to be standardised and widespread successful replication of fundamental studies needs to be firmly established before moving forward".

4.3.2. Dermal exposure

90. Workers and bystanders in or around the treatment area during product mixing and loading and during application, or moving through the treated area following product application, could be dermally exposed. Furthermore, persons walking near by the application field (bystanders), or persons live close by the field of application (residents) may also be exposed to this kind of pesticides. As discussed below [also Section 7.1.5, paragraphs 293-299, in ENV/JM/MONO(2020)26], physical and physicochemical barriers are likely to limit dermal absorption.

91. Information on systemic absorption following dermal exposure to unmodified dsRNA in vertebrates is limited[12], and generally comes from studies developing delivery methods for siRNA therapeutics for treatment of skin diseases. siRNA has poor dermal permeability due to its size, charge, and susceptibility to degradation. Therapeutics using siRNA must employ strategies to cross the outer layer of epidermis; some strategies used to date have included conjugation to cholesterol, cell-penetrating peptides, liposomes, lipid nanoparticles, and transfection reagents (Chen et al, 2014; Ibaraki et al, 2019; Colombo et al, 2019). In these studies, specialised formulations and modifications such as these were employed to facilitate cellular uptake, while short siRNAs (21-23nt) were used to bypass the innate immune response. Therefore, the unmodified siRNA controls in these studies have provided preliminary data to help understand possible effects from dermal exposure to RNA.

92. The data suggest that, in its unmodified form, siRNA does not readily cross dermal barriers and is not capable of uptake at a level that causes silencing of the target gene. Both *in vitro* (e.g., Chen et al, 2014; Ibaraki et al, 2019) and *in vivo* studies (e.g., Hsu & Mitragotri, 2011; Ibaraki et al, 2019; Kasiewicz & Whitehead, 2019) indicate that modification of the siRNA and/or specialised delivery methods are required for siRNA to cause significant effects following dermal exposure.

93. Evidence that dermal exposure to siRNA alone does not result in cellular uptake also comes from Ibaraki et al (2019); fluorescently-labelled siRNA was nearly undetectable by fluorescence microscopy on a skin biopsy after 10 hours of siRNA exposure (5 µg), even when the stratum corneum (outer layer of epidermis) was removed before siRNA application. In contrast, siRNA encapsulated in liposomes permeated up to 100 µm into the skin and significantly reduced target protein production (Ibaraki et al, 2019). The impact of formulation on ncRNA persistence in skin has also been demonstrated by Ma et al., 2014,

[12] For this working paper, only studies using topical application of RNA were examined for information on dermal exposure. Studies in which RNA was administered through more invasive routes such as subcutaneous injection were not considered, since they are of limited relevance to human dermal exposure.

where siRNA at the administration site following subcutaneous injection was observed for up to 7 days when formulated with chitosan, while siRNA alone persisted for less than one day. Intradermal (Srivastava et al., 2017) or subcutaneous (Urgard et al., 2016) injection of formulated miRNAs has also demonstrated the ability to modify inflammatory responses at the target site. Regarding non-injection routes of administration, Desmet et al. (2016) used a liposomal encapsulation method which allowed delivery of siRNA to the epidermis in human skin samples.

94. Overall, dermal application of unformulated siRNAs does not appear to result in dermal permeation, cellular uptake, or target silencing. Given that dsRNA differs from siRNA only in length of the nucleotide chain (and hence, significantly higher molecular weight), it may be reasonably concluded that dermal exposure to unformulated dsRNA would not result in any significant dermal absorption, or adverse effects[13]. However, further studies of dermal exposure to dsRNA-based pesticides could be informative since existing studies have primarily focused on siRNAs in specialised formulations.

4.3.3. Respiratory exposure

95. It is likely that dsRNA-based biopesticide products will be applied using similar methods to traditional chemical pesticides, including application of sprays, granule, or powder formulations, some of which may result in dust formation. Therefore, respiratory exposure *via* spray drift or from subsequent formation of aerosols of applied product is possible. Inhalation of dust from dried spray or granule/powder formulation could be another possible source of exposure, as could inhalation of pollens from some treated agricultural/horticultural crops; however, barriers to respiration of particles are discussed below.

96. The deposition of particles in the lung is dependent on the aerodynamic diameter of the inhaled droplets; most agricultural particles (e.g., pollen) are large enough that they are not deposited in the lung, but rather, are cleared from the upper respiratory tract, leading to secondary oral exposure rather than pulmonary exposure (Sherman et al, 2015). Therefore, it might be helpful to provide the aerodynamic diameter of the RNAi-based agents for evaluation purposes.

97. For example, inhalation of pollen containing residues of crop-applied dsRNA (if exogenously-applied dsRNA happened to be taken up and translocated to the pollen or if pollen is sprayed directly), could be a possible source of exposure. However, this route of exposure is unlikely to be of significance as pollens are limited in number and both pollen and agricultural dust/aerosols tend to be large particles that do not migrate to the small capillaries of the lungs (EFSA, 2014). Such particles will be deposited in and cleared from the upper respiratory tract, resulting in secondary oral exposure rather than pulmonary exposure[14]. Supporting this conclusion, Sherman et al (2015) also noted that there was a history of safe inhalation exposure of dsRNA contained in plant pollen and dust; since the particle size of plant dust and pollen is generally larger than inhalable particles (generally considered to be <10 µm), inhaled pollen and plant dust particles would be cleared from the upper airways and result in secondary oral exposures, not inhalation exposures. Goldstein et al (2004) also considered the non-respirability of pollens (including corn pollens which are spherical grains ranging in size from 90 to 100 µm), and of agricultural dusts which are generally of non-respirable sizes. In addition, there is a general assumption that dsRNA contained within the pollen tissue, such as is the case for dsRNA-producing plants or dsRNA that was applied exogenously and subsequently taken up into the plant

[13] In an RNAi-responsive organism, access of a dsRNA to an intracellular target site is not necessarily sufficient for an RNAi response to be induced - the dsRNA concentration needs to be high enough to induce a response.

[14] On this basis, oral toxicity studies would be sufficient to cover investigations of this exposure route.

cells, results in negligible respiratory exposure due to the confinement of the dsRNA within the pollen tissue.

98. The available literature on respiratory exposure to RNA indicates that, under certain conditions, an RNAi response can be induced by both unmodified and formulated siRNA. Until respiratory toxicity studies are conducted using putative dsRNA-based pesticides, most of the data informing our understanding of the effects of respiratory exposure in vertebrates to dsRNA will be derived from the clinical literature. However, limitations of the current data should be kept in mind when assessing respiratory exposure to dsRNA-based pesticides; existing studies have mainly used shorter sequences of RNA designed to bypass potential immune responses such as binding to Toll-like receptors and activation of the interferon response, whereas dsRNA-based pesticides are likely to employ longer dsRNA, which could potentially activate these pathways. To date, clinical data on inhalation of longer dsRNAs are lacking. Furthermore, it is likely that direct respiratory tract exposure *via* intratracheal or intranasal administration of a TGAC/TGAI or formulated RNA, as used in animal studies to mimic inhalation exposure for RNAi-based therapeutics may not be wholly applicable to the inhalation exposure scenarios expected with dsRNA-based pesticides.

99. Recent reviews (Thanki et al, 2018; Youngren-Ortiz et al, 2017) also noted that the major barriers to the delivery of naked siRNA molecules to the lung include the presence of mucus, alveolar fluid, alveolar macrophages, and mucociliary clearance, as well as the unfavourable physicochemical properties of siRNAs (negative charge, large molecular weight) and their instability in plasma (half-life ~10 min). Furthermore, after being transported intracellularly into the lysosomes *via* endocytosis, unmodified siRNAs are degraded in lysosomes, thus reducing the activity of siRNA therapeutics (Tseng et al, 2009). Because of these physicochemical barriers, chemical modifications of siRNA have been, and are being developed in the field of lung therapeutics in an effort to increase stability, specificity and potency, and to reduce the likelihood of immune responses and off-target effects (Rodrigues & Petrick, 2020). For example, lipid nanoparticles are currently being used in clinical trials to deliver mRNA for treatment of cystic fibrosis. siRNAs are being developed as an inhalable dry powder using polyethylenimine (PEI) or by using chitosan derivatives (Okuda 2018, Li 2018). Additionally, therapeutic use of naked RNA molecules such as siRNA and mRNA, can be delivered to the lung but delivery must occur intratracheally or through the use of a nebulizer (Chow 2020, Ng 2019, Tiwari 2018).

100. Under certain conditions respiratory exposure (e.g. in a therapeutic setting) appears to induce RNAi responses more readily than following oral exposure. For example, in a study using unmodified siRNA targeting plasminogen activator inhibitor-1 (PAI-1), a gene involved in disease progression of pulmonary fibrosis, intranasal administration in mice reduced target protein levels in bronchoalveolar fluid after 14 days and improved survival rate (Senoo et al, 2010).

4.3.4. Ocular exposure

101. In exposure assessments for pesticide products based on chemical or biological active constituents, potential exposure to pesticides in sprays, mists and dusts *via* the eyes is always considered, particularly for workers who may be exposed to the product during mixing, loading and application. Thus, it is appropriate to consider the feasibility of this route as a potential route of systemic absorption for dsRNA-based pesticide products as well.

102. Since members of the family of RNases are found in a range of mucosal secretions, including tears (Gupta et al, 2012), the absorption of an unmodified, unformulated dsRNA from spray mist depositing on the eyes may be reduced by RNase activity. However, studies of RNA therapeutics targeting the eye indicate that even unmodified siRNA applied via eye drops is more stable in ocular fluids than in plasma, and intravitreal exposure to unmodified siRNA resulted in significant activation of the innate immune system via Toll-Like Receptor 3 (Appendix 1). Overall, these clinical results

demonstrate that effects from ocular exposure to even unmodified siRNA need to be considered in the human health risk assessment of a dsRNA-based pesticide.

4.4. Bioavailability in humans

103. Several factors will affect the bioavailability of a dsRNA active ingredient in an exogenously-applied pesticide product, including pharmacokinetics of dsRNA and several barriers to systemic uptake in humans.

4.4.1. Pharmacokinetics of dsRNA – preclinical research

104. Pharmacokinetics is defined by the International Union of Pure and Applied Chemistry as the "process of the uptake of drugs by the body, the biotransformation they undergo, the distribution of the drugs and their metabolites in the tissues, and the elimination of the drugs and their metabolites from the body over a period of time" (IUPAC, 1997). Several pharmacokinetic studies have been conducted using unmodified dsRNA to elucidate the potential efficacy of gene silencing through dsRNA in the clinical context. Many of these studies have been conducted with intravenously-administered dsRNA on test animals, as many dsRNA-based therapeutics are expected to be administered in this manner to achieve maximum efficacy. While this particular exposure route is not directly relevant to the exposure considerations for exogenously-applied dsRNA-based pesticides, information derived from these studies is still relevant for the risk assessment of dsRNA-based pesticides as they provide insight into the behaviour of dsRNA in the mammalian system and thus the bioavailability in humans.

105. For a comprehensive review of the pharmacokinetics of exogenous non-coding RNA (ncRNA), including absorption, distribution, metabolism, and excretion, refer to Section 3.1.4 of Davalos et al., (2019). Relevant excerpts are provided below:

"In general, RNA-based experimental therapeutics (as exemplified by siRNAs or miRNAs) have shown dose-dependent increases in both the siRNA or miRNA maximum serum concentration (Cmax) and AUC (i.e. the integral of the concentration-time curve) (Zuckerman and Davis, 2015; Beg et al., 2016). Most experimental siRNA drugs did not demonstrate dose accumulation over time, except in cases where the mononuclear phagocyte system is saturated (Zuckerman and Davis, 2015). Scaling dosing from preclinical animal studies to humans (i.e. based on body weight or surface area) is challenging and apparently depends on the mechanism of clearance. For example, the pharmacokinetics of a naked siRNA formulated into polymer-based nanoparticles in mice, rats, monkeys and humans exhibited blood Cmax shortly after intravenous administration and rapid elimination across all species in correlation with the body weight (Zuckerman et al., 2014). The pharmacokinetic profile investigated during the development of exogenous RNA therapeutics has shown to be influenced by the route of administration"

Distribution:

"[…] naked oligonucleotides, including ncRNAs, rapidly distribute to the liver, lung and kidney after iv injection. Within the first minutes they are found in the bladder, suggesting rapid renal elimination. Chemical modifications or formulations increase their distribution to other tissues or delay clearance. Exosome-loaded RNAs have recently been shown to be an alternative tool for distribution to different tissues. ncRNAs by oral administration seem to distribute to the GI tract or, if formulated, systemically."

Metabolism:

"There are few studies which have evaluated the metabolism of exogenous RNAs administered to animals or humans, most of which only investigate their degradation. Naked siRNAs seem to be rapidly degraded when administered iv (Viel et al., 2008), decreasing by ≈50% within the first 3 min, although certain chemical modifications (i.e. 2'F, 2'OME) can slightly increase this time. Stability of naked siRNA was

reduced (<5 min) compared to that of other chemically modified or formulated siRNA when administered by iv injection in mice (Gao et al., 2009). Indeed, incubation of naked siRNA with plasma or foetal bovine serum causes a very rapidly degradation of the genetic material (Jiang et al., 2012; Liu et al., 2011; Braasch et al., 2004; Urban-Klein et al., 2005). Unmodified aptamers in general are also very rapidly degraded (≈10 min) (Zhou and Rossi, 2016). When naked siRNA is administered as formulated (i.e. nanoparticles) it was shown that the renal filtration barrier can separate the siRNA from its carrier (Naeye et al., 2013; Zuckerman et al., 2012). In the eye, siRNAs are degraded by endonucleases without preferences for one side of the duplex (as observed also for chemically modified siRNAs) (Beverly et al., 2006). Early in vitro studies also showed that exogenous RNAs taken up by cells are rapidly degraded (Shanmugam and Bhargava, 1966), and exogenous RNA injected into the blood is rapidly degraded to nucleosides, ribose phosphate and free bases (Sved, 1965)."

Excretion:

"The renal filtration barrier owns an effective size cut-off of ≈40 Å(Batsford et al., 1987; Bohrer et al., 1978), but is also influenced by net molecular charge discrimination (Brenner et al., 1978). This is supposed to facilitate rapid renal clearance of small molecules and free siRNA (Zuckerman et al., 2012; Naeye et al., 2013) since ASO, siRNAs and aptamers are commonly smaller than this cut-off (Huang et al., 2010). By comparing naked siRNAs and ribose-modified siRNAs (2'-O-Methyl or 2'F) using PET scans in rats and mice, the kidney was identified as the main organ for siRNA elimination (Viel et al., 2008). However, it is worth noting that also the liver participates in siRNA elimination. In other studies, siRNA appeared to accumulate in the bladder shortly after administration (Hatanaka et al., 2010). This was also the case for modified siRNAs (LNA) compared to their versions conjugated with PEG administered in mice, in which their levels peaked in the bladder 5 min post injection and they were found in the urine as soon as 1 min after injection (Iversen et al., 2013)."

106. Pharmacokinetic studies in male CD-1 mice indicated a very short plasma half-life for two different siRNAs given by single intravenous injection (Christensen et al, 2013). This absorption, distribution, metabolism, and elimination (ADME) study used two modified but unformulated siRNAs, both internally labelled with tritium, at a dose of 5 mg/kg bw; they were a 21mer MRP4 (multidrug resistance protein isoform 4) siRNA which targets the transporter protein MRP4 (expressed in the brush-border membrane of rat kidneys) and a 21mer SSB (Sjögren Syndrome antigen B) siRNA which targets the ubiquitous gene, SSB. The MRP4 siRNA was modified by dTdT[15] overhangs, while the SSB siRNA was stabilised by selective incorporation of 2´-O-methyl pyrimidines throughout the nucleotide sequence.

107. Overall, distribution in total radiolabelled components was similar following intravenous dosing with the two tritiated siRNAs. Radioactivity was quickly distributed throughout the body. MRP4 siRNA and SSB siRNA remained detectable in all tissues at 24 h and 48 h, respectively. Renal excretion was the main route of elimination; the kidneys, followed by the liver, were the primary organs for distribution of radioactive components (consistent with other unformulated siRNA biodistribution studies reported in mice). The metabolism of the two siRNAs was rapid and extensive; 5 mins after administration, neither parent nor metabolite nucleotides could be detected in plasma, but radiolabelled nucleosides were observed (Christensen et al, 2013).

108. In the metabolism profiles obtained from various organs and tissues, only radiolabelled nucleosides were found, suggesting rapid metabolism of siRNAs and that small molecular weight metabolites were responsible for the distribution pattern of total radiolabelled components. No parent compound was observed in the urine; the recovered radioactivity was associated with both oligonucleotide and nucleoside metabolites.

[15] dT = 2´-deoxy thymidine

4.4.2. Lessons from research on medical applications

109. In clinical research, the effective delivery of dsRNA-based pharmaceuticals to target organs by the oral, dermal and inhalation routes has had several challenges. dsRNA therapies based on native forms of dsRNA have not shown efficacy by the oral route of administration without some protection from exposure to stomach acids and RNases. For dermal and inhalation routes of drug delivery, some form of conjugation, encapsulation or chemical modification is necessary to facilitate trans-membrane movement and to reduce rapid renal clearance.

110. Several clinical research studies have been conducted to support efforts to try to treat various human diseases by exploiting the human RNAi mechanism to silence specific cellular functions. The findings from this work are relevant to human health risk assessment of possible pest-control products using dsRNA; in particular, this clinical research has provided significant amount of information about the pharmacokinetics of dsRNA, that is, its absorption, distribution, metabolism, and elimination in the human body.

111. To date, three RNAi-based injectable therapeutics have been approved by the Food and Drug Administration (FDA) in the United States, namely ONPATTRO (for treatment of TTR-mediated amyloidosis), GIVLAARI (for treatment of acute hepatic porphyrias), and Lumasiran (for treatment of primary hyperoxaluria type 1; Hu et al., 2020 and FDA-CDER, 2020). In addition, several other therapeutics, targeting various conditions are currently in clinical trials (Hu et al., 2020).

112. Several overviews of the application of RNAi in the treatment of human diseases have been published to date, including Sullenger & Nair (2016), Chi et al (2017), Stein & Castanotto, (2017), Levanova & Poranen (2018), Chalbatani et al (2019), and Smith & Zain (2019).

113. In comparison to antisense oligonucleotide (ASO) and siRNA-based therapeutics, Chi et al (2017) noted that the systemic delivery of double-stranded siRNAs (12 kDa average) to the cytoplasm of cells is more challenging due to the have a larger molecular mass and high negative charge of siRNAs due to the position of aromatic nucleobases in the interior of the molecule, with hydrated phosphates on the exterior. This results in generally poor interactions with cell membranes, resulting in rapid renal excretion.

114. In reviews of the use of siRNAs in cancer therapy (Chalbatani et al, 2019) and in the treatment of human viral infections (Levanova & Poranen, 2018) the authors noted that rapid degradation in serum and low cellular uptake of unmodified siRNAs were major limitations to the therapeutic use of these molecules. They noted that virtually all the siRNA molecules developed for therapeutic use are modified in some way, although only to the extent that the modifications do not hinder or prevent binding to the RISC – thus not leading to the required RNA interference. A key research focus in the area of RNAi therapeutics is therefore the development of chemical modifications to siRNAs which (1) avoid exonuclease degradation; (2) increase plasma circulation time; (3) enhance transmembrane transport and cellular uptake; (4) increase affinity to RISC; (5) prevent loading of the passenger strand; (6) lower the innate immune-stimulation response; and (7) avoid miRNA-like off-target effects (see Section 5).

115. In a review of FDA-approved oligonucleotide therapies, Stein & Castanotto (2017) noted that oligonucleotide (ON) therapies been under clinical development for approximately 30 years, during which time numerous clinical trials had been performed. Of all the molecules evaluated as of January 2017, the FDA had assessed that six provided clear clinical benefit in rigorously- controlled trials. They were as follows: Vitravene (*aka* Fomivirsen) – an antisense ON; Macugen (formerly Pegaptanib) - an aptamer; Kynamro (*aka* Mipomersen) – an antisense gapmer; Exondys 51 (*aka* Eteplirsen) – a splice-switching ON; Defitelio (*aka* Defibrotide) – a non-specific MOA based on charge-charge interactions of its phosphodiester constituents with proteins; Spinraza (*aka* Nusinersen) – an antisense oligonucleotide. (No siRNAs were included in this list.)

116. In a review of ONs in human therapies, Smith & Zain (2019) considered the wide range of ONs undergoing drug development research, including "antisense gapmers, steric blockers, splice-switching ONs, siRNAs, antagomirs (anti-miRNAs), miRNA mimics, aptamers, DNA decoys, DNAzymes, synthetic guide strands for CRISPR/Cas, and innate immunity-stimulating ONs". For siRNAs, these reviewers noted that delivery remains a major obstacle; while systemic administration and intrathecal infusion have been used to access the liver and brain, respectively, the development of methods to deliver siRNAs into other organs is key to their expanded use in human therapeutics.

117. The Table in Appendix I ('dsRNA-Based Therapeutic Products – Selected Examples') illustrates a range of dsRNA-based human therapies which have been developed over the past decade. Some of these examples were undergoing clinical trials when this document was drafted while others failed to meet safety or therapeutic targets and have been discontinued. The Table provides summary information about the mode of action of the therapeutic RNA, its structure and form, the dose route used in trials, and adverse effects seen. The therapeutic products are listed by target organ – liver, tumours, and the eye. Of the extensive list of treatments targeting diseases of the liver, only two RNAs were not covalently modified, and both of those were packaged in proprietary lipid nanoparticles (LNPs)[16]. Of the two examples of therapeutics targeting tumours, both RNAs were chemically modified, while the two therapeutics targeting diseases of the eye were naked siRNAs, for delivery intravitreally or as eye drops.

118. However, experimental data collected related to different possible exposure pathways for mammals have been obtained on a reduced number of specimen and the outcomes sometimes diverge, also because of dissimilarities in experimental protocols and laboratory setups. These uncertainties need to be specifically considered when data for risk assessment are being generated.

4.4.3. Lessons from research on medical applications: oral exposure

119. Little is known about the pharmacokinetic properties of exogenous RNA under conditions which could alter intestinal permeability, such as consumption of non-steroidal anti-inflammatory drugs, Crohn's disease, etc. (Davalos et al., 2019). One study (Frede et al., 2016) indicated that ncRNA absorption may be increased under inflammatory conditions: uptake of nanoparticle-formulated siRNA was increased in "epithelial cells, lamina propria lymphocytes, and cells from the mesenteric lymph nodes including dendritic cells and T cells" from mice models of induced colonic inflammation, compared to the same cell types from healthy mice. However, similar studies using unmodified and unformulated ncRNA have not been conducted, so the impact of disease states on gastrointestinal uptake of naked siRNA remains unknown.

120. With respect to the oral absorption of RNA-based therapies, O'Driscoll et al (2019) reviewed the progress and feasibility of the oral delivery of RNA-based drugs; they noted the lack of clinical trial data and concluded that "while progress has been made through innovative formulation strategies, to date clinical translation of oral products has not been realized".

121. A 2016 review (Sullenger & Nair, 2016) of the status of clinical research on the application of RNA-based therapeutics in treating a range of human diseases concluded that two major pharmacokinetic challenges existed to the development of synthetic oligonucleotide therapeutics, namely:

- their limited oral bioavailability; and
- the rapid rate at which short RNAs are cleared from the circulation.

[16] Liver cells are reached by many types of liposomal and polymer delivery systems (Weissman, 2015).

122. This rapid clearance from circulation was also observed with RNAs designed to be nuclease-resistant. All the 5 Phase-3 clinical trials of synthetic siRNAs underway up to the time of the Sullenger & Nair (2016) review either used local delivery of the siRNA (e.g., intraocularly to treat age-related macular degeneration or retinal ganglion apoptosis) or involved modifications to the siRNA, in order to target delivery to the liver or kidneys after intravenous administration (e.g., formulating the siRNA with N-acetylgalactosamine to facilitate uptake by, and release into the cytoplasm of hepatocytes).

123. Another 2016 review on the clinical application of therapeutic oligonucleotides concluded that "the effective delivery of oligonucleotides to their intracellular sites of action remains a major issue" (Juliano, 2016). This review did not discuss barriers to uptake following oral ingestion of nucleotides but considered those in place following intravenous administration of oligonucleotides, including those packaged in nanoparticle delivery systems.

124. For a more comprehensive review of lessons from therapeutic applications involving ncRNA delivery via the oral route, the reader is referred to Section 3.2.2 of Davalos et al. (2019).

4.4.4. Lessons from research on medical applications: dermal exposure

125. In a diabetic mouse model, application of 100–500 nM therapeutic siRNA to wounded skin resulted in significant target silencing, localisation within wound tissues, and accelerated wound healing when the siRNA was encapsulated in fluorescently-labelled lipid nanoparticles (Kasiewicz & Whitehead, 2019). Localisation of nanoparticles was visualised in and around wound bed cells, suggesting that siRNA can enter tissue and elicit silencing effects when appropriately formulated (Kasiewicz & Whitehead, 2019).

126. The dsRNA may be formulated in a mixture containing further ingredients (co-formulants), which may influence the dermal permeation of dsRNA. Therefore, for formulation containing dsRNA, the extend of dermal absorption may be tested in the same way as it is already evaluated for formulations containing chemicals or micro-organisms as active substance.

127. Cell culture studies provide some insight into results of exposure to siRNA when certain barriers to dermal uptake, such as the outer epidermal layer, are not present. When treated with 25 μM siRNA alone, human keratinocytes displayed target gene silencing of ~18%; however, when the siRNA was conjugated to a cell-penetrating peptide and formulated within a phospholipid nanovesicle, target gene product silencing increased to 80% (Chen et al, 2014). Likewise, Ibaraki et al (2019) observed 10% uptake of siRNA alone in murine macrophage cell culture, but nearly 100% uptake with a liposome formulation and transfection agent.

128. In a study on mice by Hsu & Mitragotri (2011), 10 μM of siRNA (2-O-methyl modified) complexed with a dermal penetrating peptide (or appropriate controls) were applied topically to a 3 cm^2 area of skin on the back of the animal for 24-72 hours. The siRNA or peptide administered alone did not result in significant knockdown of target protein production, but in complex with the dermal penetrating peptide, significant target silencing was observed (Hsu & Mitragotri, 2011). Similar results were obtained by Colombo et al (2019) in experiments in which 40 - 50 mg of a 21bp siRNA formulation (containing up to 10 μM RNA) did not elicit a silencing effect in mice after application to undamaged skin, while siRNA possessing several modifications (such as including several dNTPs in the siRNA and conjugating the siRNA to cholesterol) resulted in significant suppression of the target gene product.

4.4.5. Lessons from research on medical applications: respiratory exposure

129. The therapeutic potential of RNAi-based agents has been demonstrated preclinically for many lung diseases, e.g., lung cancer, respiratory infections, inflammatory diseases and pulmonary fibrosis. Hence, extensive clinical research into the development of inhaled RNAi therapeutics is being conducted (e.g., Thanki et al, 2018). Clinical studies to date have demonstrated that the use of the pulmonary route to effectively deliver unmodified RNA molecules in humans is not straightforward.

130. Some recent therapeutic studies on inhaled RNA have shown advances in the delivery of RNAi-based drugs to the lungs (reviewed by Youngren-Ortiz et al, 2017; Thanki et al, 2018). However, as discussed below, a common conclusion across these studies is the requirement for specifically engineered formulations to overcome biological and physicochemical barriers to effective delivery of RNAi-based therapies. In some instances this requires the need to develop suitable devices for pulmonary administration of these inhalable formulations was pointed out (Thanki et al, 2018).

a) These barriers include:

- The vascular endothelial barrier: The capillary lumen is surrounded by a tightly-abutted layer of endothelial cells, forming a barrier between blood and the parenchymal space. The vascular endothelium allows passage of molecules the size of individual oligonucleotides but limits the passage of those in nanoparticle packaging.
- The reticuloendothelial system: The administration of oligonucleotides in nanoparticles will usually result in a large fraction of the material being taken up by the RES, particularly Kupffer cells.
- Renal excretion: Renal clearance plays an important role in the pharmacokinetics of oligonucleotides. siRNA and uncharged oligonucleotides do not bind extensively to plasma proteins and are readily cleared by the kidneys. Many oligonucleotides fall in the size range of 3–6 nm or less and are ultra-filtered by the kidneys (see review by Juliano, 2016). For siRNAs, the liver and kidneys are the key sites of siRNA uptake. Phosphorothioate-modified oligonucleotides bind to plasma proteins, slowing their renal clearance and permitting greater distribution to tissues, with most found in liver and kidneys; their renal excretion mainly involves nuclease degradation products. Uncharged morpholino oligonucleotides are rapidly cleared by the kidneys, largely as intact molecules, and display lower levels of tissue accumulation than phosphorothioates (Juliano, 2016). Molitoris et al (2009) showed that an siRNA targeted to p53, a pivotal protein in the apoptotic pathway, was cleared within minutes of intravenous administration to Wistar and Sprague-Dawley rats. The kidneys, and in particular, the proximal tubule cells, were overwhelmingly the primary site of tissue distribution. The authors noted that the rapid clearance of the oligonucleotide from the body minimised exposure of other organs/cells.

b) Thus, renal clearance plays an important role in the pharmacokinetics and bio-distribution of all types of 'free' oligonucleotides.

131. Davalos et al. (2019) summarized additional literature on ncRNA effects on the respiratory tract:

" The literature describes other naked siRNAs tested by intratracheal (Perl et al., 2005) or intrapulmonary administration for local therapy (Rosas-Taraco et al., 2009), although with minor chemical modifications. Non chemically modified miRNA mimicking hairpins have also been administered intranasally in mice models (Das et al., 2014). Intranasal administration of siRNAs in rats for brain targeting has resulted in very low levels of radioactive siRNAs in the brain or plasma when naked siRNAs were administered compared to a formulated version (Perez et al., 2012). miRNAs administered by intranasal injection using transfection reagents reached the dorsal root ganglia and the olfactory bulb (Cheng et al., 2015) and, using another nanovector delivery system through intranasal administration (as drops), it reached the brain, exerting a local biological effect (Zhuang et al., 2016)."

132. Lam et al (2012) reviewed respiratory exposure studies relating to RNAi in vertebrates, mainly in a therapeutic context, using siRNAs of usually 21–23 base pairs which are designed to bypass the interferon response elicited by longer dsRNA. Similar to the existence of physicochemical barriers to oral uptake of dsRNA there are a number of barriers to uptake of dsRNA along the airways of mammals. These include the presence of mucus, phagocytosis by macrophages, alveolar fluid, and the highly branched structure of the lungs limiting particle sizes which can be deposited in the lower airways (Lam et al, 2012). These barriers may limit respiratory exposure to dsRNA.

133. Intranasal administration of slightly modified anti-viral siRNAs (containing deoxythymidine dinucleotide [dTdT]) reduced viral symptoms in both Rhesus macaques (Li et al, 2005) and humans (DeVincenzo et al, 2010). When intratracheal administration of naked unmodified siRNA in mice was compared to either locked nucleic acid (Moschos et al, 2011) or 2-O-methylated siRNA (Gutbier et al, 2010), rapid renal filtration was observed for unmodified siRNA within minutes, while the chemically-modified / formulated siRNA was still detected in peripheral blood and pulmonary tissue hours after administration (Gutbier et al, 2010).

134. In summary, while the potential clinical effectiveness of dsRNA-based pharmaceuticals might be apparent in cell culture studies, *in vivo* delivery to target organs by the oral, dermal and inhalation routes has proved to be problematic. dsRNA therapies based on native forms of dsRNA have not shown efficacy by the oral route of administration without some protection from exposure to stomach acids and RNases (Petrick et al, 2013). For dermal and inhalation routes of drug delivery, some form of conjugation, encapsulation or chemical modification is necessary to facilitate trans-membrane movement and to reduce rapid renal clearance. Thus, available evidence from human clinical research suggests that the likelihood of any systemic exposure of mammals to unmodified, unformulated RNA molecules applied in the field as pesticides is quite low (see also Section 7.1.5, paragraphs 303-306 in ENV/JM/MONO(2020)26). However, if the dsRNA in a pesticide product is modified or formulated in any way, this could increase the potential for systemic exposure, as the clinical experience described in the section above has shown that these modifications allow dsRNA to potentially bypass some of the existing barriers to uptake.

Lessons from research on medical applications: ocular exposure

135. Several examples of the distribution of ncRNA in the eyes are available in the scientific literature, since the eye was the target for the first RNA-based therapy, fomirisen (Vitravene) approved by the US FDA[10] and the EMA[11]. These are summarized by Davalos et al. (2019):

"Intravitreally injected naked siRNA (slightly modified, dTdT) distributed throughout the eye (vitreous, iris, retina, retinal pigment epithelium and sclera) of rabbits when administered at 2 mg/eye, and the pattern of ocular distribution was similar in male and female rabbits (Dejneka et al., 2008). The radioactive half-life (radiolabelled siRNA) in the vitreous fluid exceeded 48 h. No systemic treatment-related clinical signs were observed in the rabbits following a single intravitreal dose of the siRNA (Dejneka et al., 2008). Intravitreal injection of naked siRNAs in rats elicited a long-lasting ocular biological effect (Roy et al., 2011). Two hours post-injection the siRNA was localized in the retina vascular cells and pericytes, and its effect peaked one week post-injection at a ≥1 μM concentration (Roy et al., 2011). Other research groups have not observed accumulation of naked siRNAs (5 μg injected in the vitreous) 24 h post-injection, compared to its formulated version, suggesting rapid degradation of unprotected siRNA in the vitreous (Turchinovich et al., 2010). Conjugated or formulated siRNAs have also been tested in animal models (Zhang et al., 2014; Janout et al., 2014). Intravitreal injection of miRNAs has been described in the literature using either naked miRNAs in mice (Liu et al., 2015), rats (Qin et al., 2016) or using transfection reagent in rats (McArthur et al., 2011) or exosomes in rats (Mead and Tomarev, 2017).."

4.4.6. Modified dsRNA and pesticide formulation

Plants

136. Product formulations may have a significant effect on absorption and the bioavailability of dsRNA (e.g., Brown & Ingianni, 2013). For example, different types of dsRNA carriers have been demonstrated to affect stability and uptake of dsRNA into plant cells. Jiang et al. (2014) used water-soluble cationic fluorescent nanoparticles with peripheral cationic groups for electrostatic binding to dsRNA for efficient delivery of dsRNA into plant cells. Application of mixtures of 3.6 nm nanoparticles and specific dsRNAs to root tips of *Arabidopsis* seedlings resulted in uptake of dsRNA nanoparticle complexes into the root system and into plant cells, followed by systemic silencing of target genes after 3 days continuous treatment.

137. Not only cellular uptake, but also dsRNA stability can be affected by dsRNA carriers. Numata et al. (2014) complexed a fusion peptide of a polycation and a cell-penetrating peptide with siRNA via ionic interactions. dsRNA-peptide complexes which are in the size range of 100 to 300 nm were much more resistant to RNase treatment than naked dsRNA. Moreover, there was evidence for cellular uptake of siRNA-peptide complexes and transient local gene silencing in leaf epidermal cells after infiltration of Arabidopsis thaliana and poplar leaves. As a more sophisticated delivery vehicle, Zhang et al. (2019) designed DNA nanostructures with attachment loci for conjugation to biological molecules. For DNA nanostructures of 2.4 to 16 nm conjugated to siRNA and applied on the abaxial side of *Nicotiana benthamiana* leaves via infiltration, a high degree of colocalization with the plant cytosol was observed. Loading on these DNA nanostructures protected siRNA from degradation inside the cell and enabled efficient transient siRNA mediated silencing of a marker transgene. The conformation and compactness of the DNA nanostructure did not only affect the degree of cellular uptake, but also seemed to affect the type of gene silencing mechanism (mRNA degradation vs. translational inhibition) triggered. Although these reports demonstrate the effects of carrier molecules on dsRNA stability and uptake into plant cells, it has to be noted that leaf infiltration is highly unlikely to be used in agricultural field applications.

138. In contrast to the above mentioned methods which relied on carrier uptake, Mitter et al. (2017a, 2017b) used positively charged layered double hydroxide (LDH) clay nanosheets (size range 15 – 120 nm) as dsRNA carrier in order to protect dsRNA from RNase degradation and wash-off from the leaf surface and to facilitate steady release of the dsRNA through slow LDH breakdown under environmental conditions. By permitting prolonged dsRNA persistence on plant leaves as compared to naked dsRNA, LDH nanosheets have been demonstrated to enable sustained protection against plant viruses infected via mechanical inoculation. With regard to mammalian cells, LDH nanoparticles that were in the range of 30 – 150 nm have been demonstrated to deliver siRNA into HEK293T and NIH3T3 cells in vitro (Ladewig et al., 2009).

Mammals

139. dsRNA modifications (e.g. chemical stabilization, use of enhancers) is being developed to try to overcome barriers in mammals for therapeutic use of gene silencing. siRNAs which are formulated and/or encapsulated are able to bypass these barriers and alter gene expression in target cells. For example, oral administration to mice (at a dose of 20 µg/kg, once daily for 8 days) of β1,3-d-glucan-encapsulated *Map4k4* siRNA particles resulted in a significant reduction of Map4k4 mRNA levels in macrophages from spleen, liver, and lung tissue (Aouadi et al., 2009).

140. Similarly, Ballarín-Gonzalez et al. (2013) reported high levels of intact siRNA detectable in the stomach, small intestine, and colon of mice 1 and 5 hours after oral gavage of 78 µg siRNA, when the siRNA (non-chemically modified) was formulated with chitosan nanoparticles. Higher levels of siRNA was isolated from organs of animals treated with the chitosan/siRNA nanoparticles compared to those treated only with naked siRNA.

Compared to naked siRNA, the chitosan nanoparticle-formulated siRNA was found at levels up to 11-fold higher in the distal small intestine at 5 hours.

141. In a paper from Christensen et al. (2014), the pharmacokinetics of a single intravenous dose of 2.5 mg/kg bw of [^{3}H]-SSB siRNA, formulated in a liposome nanoparticle (LNP) vehicle, was studied in male CD-1 mice. Tissue distribution of radioactivity and biostability were determined using quantitative whole-body autoradiography and y LC-MS with radio-detection and RT-qPCR, respectively. The pharmacokinetics of a cationic lipid, one of the main excipients of the LNP vehicle, was also investigated using LC-MS, and its distribution was characterized using MALDI-MS imaging. The parent guide strand of the siRNA when administered with the LNP carrier could be detected up to 168 h; in contrast, no intact parent guide strand could be observed at 5 minutes post dosing of unformulated SSB siRNA alone. Therefore, the pharmacokinetics of the formulated LNP vehicle was used to determine the *in vivo* disposition of the siRNA. The *in vivo* distribution of radioactivity was broad, and the highest concentrations were found in spleen, liver, oesophagus, stomach, adrenal glands and seminal vesicle wall. In summary, the LNP carrier itself was determined to drive the kinetics and distribution of the SSB siRNA. Compared to the unformulated SSB siRNA, the plasma AUC of the siRNA in an LNP vehicle was very significantly increased, with metabolism and renal clearance significantly reduced (Christensen et al, 2014).

142. Another example of the impact of RNA modifications comes from the therapeutic Cemdisiran (ALN-CC5), which was tested for treatment of complement-mediated diseases by suppressing liver production of Complement 5 (C5) protein (Badri et al, 2020; Hill et al, 2016). Delivered subcutaneously, the RNA in Cemdisiran is present as a GalNac-siRNA conjugate and possesses phosphorothioate, 2′-OMe, 2′-F modifications to the RNA itself. With these modifications, potent gene silencing was achieved for more than a year following a single dose (Badri et al, 2020; Hill et al, 2016). This illustrates the importance of considering modifications to RNA in impacting the duration of exposure.

143. Evidence suggests that, in the absence of encapsulation to prevent degradation, or the inclusion of chemical stabilisation and penetration enhancers, the absorption of RNA across the GI tract is unlikely (Akhtar, 2009; Jain, 2008).

144. Chemical modification of dsRNA to improve delivery is being exploited in the pharmaceutical research, to find effective drug formulations. In a study on triple negative breast cancer-bearing mice (Liu et al, 2019), a dsRNA was encapsulated into aldehyde sodium alginate–polyethyleneimine (mPEI) nanoparticles and administered by intra-tumoral injection, in an endeavour to silence the aggressive genes of TNBC. The cationic polymer nanoparticles increased dsRNA uptake into the breast cancer cells, provided lysosomal escape ability, and enhanced the knockdown effect on the target gene products (Fra-1 gene and down-stream MMP-1 and MMP-9 genes), in vitro and in vivo, thereby inhibiting the invasion and migration of cancer cells.

4.5 Considerations on data requirements– exposure

145. Though laboratory and clinical testing are being conducted at a steady pace, available scientific literature on the exposure pathways of dsRNA to mammals is not completely understood. And the evidence about possible transfer of environmental dsRNA to mammals is inconclusive. Therefore, in conducting risk assessment on newly developed pesticides based on RNAi mechanism, particular care should be given to addressing existing uncertainties for plausible relevant scenarios.

146. As with all pesticide products, the impact of the use-pattern and application method for a dsRNA-based pesticide on the route and extent of human exposure requires consideration. In many cases, specific product formulations may be designed to overcome problems of environmental stability of the active constituent and/or overcome barriers to uptake in the target organism. Special attention will need to be paid by registrants to how the changes to the naked form of the dsRNA (e.g., chemical modifications to component nucleosides, covalent additions of carrier molecules, or the addition of penetration-

enhancing co-formulants) could affect (1) environmental persistence, mobility and distribution, and hence, potential human exposure; and (2) the systemic uptake of the dsRNA active constituent in exposed humans. Thus, data and/or studies on specific dsRNA formulation containing these or similar modifications and their impact on environmental persistence and on systemic uptake are necessary to characterise the risks that could possibly arise from exposure[17].

147. Thus, if a dsRNA active constituent in a proposed RNAi-based pesticide product was covalently modified or formulated (e.g. with carrier to aid plant penetration and/or protect against degradation by RNases), then the conduct of the following studies using appropriate experimental methods should be considered:

- studies on environmental persistence
- environmental distribution and off-site movement
- pre-clinical absorption, distribution, metabolism and elimination (ADME) studies, looking at the potential for the modifications/formulations to significantly increase systemic exposure (relative to the naked dsRNA). Alternatives to radiolabeling could be used if needed, such as bioluminescence or fluorescence. However, it is noted that ADME studies using radiolabeled material have been performed successfully for nucleic acid-based therapeutics; see e.g. Shadid et al., 2021.

148. Any additional testing in relation to estimating the extent of possible human exposure may depend on specific regulatory requirements in the different OECD member countries or jurisdictions.

[17] See also paragraph 304 of Section 7.1.5 in ENV/JM/MONO(2020)26

5. Hazard

This section summarises information about the potential hazards (or intrinsic toxicity) of double-stranded RNAs (dsRNAs) and related compounds. As for any pesticide, the hazards of a putative dsRNA active ingredient (and the associated end-use product) must be characterized via different routes of administration. Additionally, dsRNA presents some unique considerations of possible immune system stimulation and RNAi machinery saturation.
It reviews lessons which may be learned from research on the clinical development of RNAi therapies. Importantly, the impact of formulation ingredients and/or modification of the dsRNA nucleotides are considered, as they may have a unique impact on the hazard of dsRNA. The section concludes with a brief consideration of data requirements for hazard assessment.

5.1. Toxicity

5.1.1. Oral/dietary toxicity

149. Please see https://www.regulations.gov/docket/EPA-HQ-OPP-2014-0293/document for access to the US EPA's review of mammalian toxicology studies and its human health risk assessments for both the limited use seed increase and commercial use of DvSnf7 corn.

150. In a 28-day oral toxicity study, DvSnf7_968 dsRNA was administered to CD-1 mice (10/sex per dose) by gavage at doses of 0 (vehicle control - nuclease free water), 1.06, 11.0, or 105 mg/kg bw per day. Negative controls received 104 mg/kg bw per day of yeast

RNA. The animals were examined for clinical signs, mortality, body weight, food consumption, clinical pathology, organ weights, and gross and histopathology examination. There were no treatment-related effects on clinical signs, mortality, body weight parameters, food consumption, organ weights, or gross and histopathological findings at any dose (Petrick et al, 2016). The doses tested were many orders of magnitude higher than likely human dietary exposures arising from the expression of this dsRNA as a plant-incorporated protectant; exposures were calculated from measurements of dsRNA residues in crop commodities and expected dietary consumption.

151. A lack of toxicity in mice was noted for orally-administered siRNAs and a long dsRNA with 100% sequence identity to mouse vacuolar ATPase at doses of up to 48 mg/kg bw and 64 mg/kg bw, respectively (Petrick et al, 2015).

152. A 90-day dietary toxicology study investigated the effects on Sprague-Dawley rats of consuming high-amylose transgenic rice generated using antisense RNA inhibition. Rats (10 per sex per group) were fed diets containing either 70% of the transgenic rice line flour or its near-isogenic rice flour, or the control diet. There were no differences observed in body weight, food consumption, haematological parameters, organ weight, or urine analysis (Zhou et al., 2011). Some changes were observed in serum chemistry parameters; however, they were determined not to be biologically significant or attributable to the transgenic rice diet.

153. The same transgenic rice was used in a three-generation reproduction feeding study in Sprague-Dawley rats (Zhou et al., 2014), using the same experimental groups described above (20 female and 10 male rats per group in F0). No major differences were observed in animal survival, health status, behaviour, body weight, food consumption, or reproductive capacity. Although statistically significant differences in clinical chemistry parameters were observed in animals given the transgenic rice diets compared to those receiving the standard diet, they were not considered adverse or biologically significant because they were in the historical control range (Zhou et al., 2014). Overall, the authors concluded that consumption of the transgenic rice over three generations was unlikely to cause any risk to rat health in reproduction or development.

154. To evaluate the potential dietary hazard from the use of MON 87411 corn products containing the DvSnf7 dsRNA plant-incorporated protectant (PIP), the USEPA assessed the relevant toxicological database for DvSnf7 dsRNA which was submitted by Monsanto[18]. In several reports (including USEPA, 2016b; 2017) the USEPA concluded that the database supporting the safety of DvSnf7 dsRNA was adequate to assess human health risk under the US Food Quality Protection Act (FQPA). The available toxicity studies are described below.

155. In a 28-day oral toxicity study (Wahle, 2014), DvSnf7_968 RNA was administered to 10 CD-1 mice/sex/dose by gavage at doses of 0 (vehicle control - nuclease free water), 1.06, 11.0 and 105 mg/kg bw per day. A group of 10 CD-1 mice/sex served as negative controls and received 104 mg/kg bw per day of Torula yeast RNA. The animals were examined for clinical signs, mortality, body weight, food consumption, clinical pathology, organ weights, and gross and histopathology examination. There were no treatment-related effects on clinical signs, mortality, body weight parameters, food consumption or gross and histologic pathology. The NOAEL was 105 mg/kg bw per day (maximum dose tested).

156. In a 90-day oral toxicity study (Mertens, 2013), MON 87411, a biotechnology-derived corn conferring resistance to corn rootworm and containing a modified *Bacillus thuringiensis* Cry3Bb1 protein, was administered to 16 Sprague Dawley rats/sex in the diet at 33% (w/w) (equivalent to 1899 and 2303 mg/kg cage body weight/day for males, and females, respectively). The mean DvSnf7 expression value in grain was 0.091×10^{-3} µg/g fresh weight (SD= 0.028×10^{-3} µg/g). Control animals (16/sex) received conventional

[18] In 2018 Monsanto became a part of the crop science program of Bayer AG.

ground corn grain in the diet at 33% (w/w) (equivalent to 1924 and 2168 mg/kg cage body weight per day for males, and females, respectively). Evaluated parameters included clinical signs, mortality, functional observation battery (FOB), body weight, food consumption, clinical pathology, organ weights, and gross and histopathology examination. There were no treatment-related effects on mortality, clinical signs, FOB, body weight, body weight gain, food consumption, food efficiency, clinical pathology, organ weights, gross pathology, or microscopic pathology. The NOAEL was 1899 and 2303 mg/kg (grain) total cage body weight per day for males and females, respectively (maximum dose tested).

157. The USEPA concluded that the results from these two subchronic studies indicated the absence of any dietary hazard associated with DvSnf7 dsRNA at very high doses i.e., DvSnf7 dsRNA is of low toxicity.

158. In the US, a tolerance exemption exists for plant-incorporated protectant nucleic acids (40 CFR 174.507). Given this, and the lack of mammalian toxicity reported for DvSnf7 dsRNA, a dietary risk assessment would not normally be conducted. However, in this case the USEPA did carry out a very conservative dietary risk assessment for DvSnf7 RNA. Risk estimates were below levels of concern for the general US population and all population subgroups (USEPA, 2016b). It concluded that there were no concerns regarding the safety of the DvSnf7 dsRNA in MON87411. The data provided did not indicate this dsRNA possesses different characteristics, or is likely to pose a greater risk, than other RNAi mediators naturally present in corn. A history of safe human consumption of RNAi mediators exists, including those with homology to human genes.

159. Food Standards Australia New Zealand (FSANZ) has evaluated MON87411 corn (FSANZ, 2014); this assessment of DvSnf7 dsRNA in MON87411 corn was restricted to the safety of human consumption of the dsRNA and did not address any risks to human health more broadly, i.e., exposures by other routes (such as might occur when applying a dsRNA as a sprayable pesticide). FSANZ indicated that the evidence published to date does not indicate that dietary uptake of these RNAs from plant food is a widespread phenomenon in vertebrates (including humans) or, if it occurs, that sufficient quantities are taken up to exert a biologically-relevant effect; the level of DvSnf7 dsRNA present in grain from MON87411 is extremely low, and the anti-DvSnf7 effect observed in corn rootworm is also highly specific to only a very small number of closely-related beetles. The regulatory conclusion of FSANZ was that grain containing the DvSnf7 dsRNA is as safe for human consumption as is grain derived from conventional corn varieties.

160. With respect to DvSnf7 dsRNA expressed in a four-event stack maize, the EFSA Panel on Genetically Modified Organisms (GMO) (2019) concluded that “the molecular characterisation, the comparative analysis and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins and dsRNA in the four-event stack maize” does not give rise to food safety concerns.

5.1.2. Dermal toxicity

161. At the time of drafting of this document, the authors were not aware of any publicly-available studies which specifically examined relevant toxicological endpoints of dsRNA administered via the dermal route. However, there have been animal studies looking at therapeutic applications of siRNA administered to the skin, from which some insights into dermal toxicity can be gained.

162. Several studies have demonstrated the applicability of RNA-based therapeutics for dermal disease states (Desmet et al., 2016; Hsu and Mitragotri, 2011; Srivastava et al., 2017; Ibaraki et al., 2019). However, many of these studies used either skin cells in vitro, reconstructed epidermis, or skin samples, so toxicological conclusions cannot be derived from their outcomes.

163. The study by Srivastava et al (2017) included in vivo investigations with mouse model of imiquimod-induced psoriasis-like inflammation, where miRNA or a scrambled control were injected intradermally in a transfection agent. However, the effects of the scrambled control were not compared to the vehicle control in terms of clinical outcomes, so the toxicological effects of the non-specific miRNA could not be determined.

164. In a mouse model of atopic dermatitis (Ibaraki et al., 2019), clinical observations of the various experimental groups can be compared to provide a putative indication of the effects of siRNA dermally. siRNA was designed to target the NF-κB (RelA) gene, which is associated with allergy induction. The siRNA was encapsulated in a liposome, and delivered along with a cell-penetrating peptide (AT1002). The in vivo results showed improved clinical observations in the siRNA-containing liposome with AT1002 treatment, where hyperplasia was suppressed. The treatment significantly hindered any rise in the clinical score compared to the untreated group. In contrast, hyperplasia and the clinical scores of the untreated, naked siRNA, and liposome control groups continued to increase and symptoms worsened. However, since the naked siRNA did not worsen clinical score compared to the untreated group, this suggests that this siRNA alone did not contribute to the clinical observations over 7 days.

5.1.3. Respiratory toxicity

165. At the time of drafting of this document, the authors were not aware of any publicly-available studies which specifically examined relevant toxicological endpoints of dsRNA administered via the pulmonary or inhalation routes. However, there have been therapeutic applications of siRNA administered to the lungs, from which some insights into respiratory toxicity can be gained (refer to Section 5.3.2).

5.1.4. Ocular toxicity

166. At the time of drafting of this document, the authors were not aware of any publicly-available studies which specifically examined relevant toxicological endpoints of dsRNA administered via ocular routes. However, there have been therapeutic applications of siRNA administered to the eye, from which some insights into ocular toxicity can be gained (refer to Section 5.3.3).

5.1.5. Toxicity upon Injection

167. Several studies in mammalian test species have given preliminary data on the toxicity of large doses of dsRNAs via the intravenous route:

168. Intravenous injection of therapeutic siRNAs with 100% identity to the mouse ApoE gene did not produce gene silencing in the expected target site (mouse liver) at doses of 50 mg/kg bw, unless the apoB-1-siRNA was conjugated with a cholesterol tag to facilitate distribution of the siRNA to organs (liver and jejunum); these results show that presence of a specific siRNA in blood does not necessarily indicate that gene silencing will result (Soutschek et al, 2004). This was a mechanistic study, and no toxicology endpoints were reported.

169. At intravenous doses of up to 200 mg/kg bw in rats, a stabilised siRNA matching rat p53, a key transcript in many pathways including cell cycle regulation, was readily degraded and was without evidence of toxicity (Thompson et al, 2012).

170. In non-human primates, kidney and liver toxicity was observed at the highest dose tested (27 mg siRNA/kg) following repeated intravenous administration of a non-chemically modified siRNA targeting the M2 subunit of ribonucleotide reductase. Escalating doses also led to increased IL-6 and INF-gamma levels (Heidel et al., 2007).

171. Vaishnaw and colleagues noted that unformulated siRNAs have generally been well tolerated in preclinical safety studies at intravenous doses >100 mg/kg bw, suggesting that potential changes in transcriptional profiles (e.g., target or off-target gene product suppression) do not appear to occur with unformulated RNAs nor impact their safety *in vivo* (Vaishnaw et al, 2010).

172. The analysis by Vaishnaw et al (2010) of the use of RNAi in human therapeutics generally indicated that, despite high degrees of off-target complementarity for a number of lead molecules, *in vitro* potency for on- and off-target effects was clearly distinguishable, with IC50s separated by several orders of magnitude. These researchers concluded that hybridization-dependent off-target gene product suppression was unlikely to be a significant concern for *in vivo* safety in preclinical studies; when this was considered together with low exposures to small RNAs, they concluded that the likelihood for any toxicologically relevant off-target gene product suppression effects in humans (or animals) due to applications of RNAi was likely to be very low.

173. Supporting these conclusions, pharmaceutical studies indicate a very short half-life for injected RNAs that have been chemically stabilised (Christensen et al, 2013), suggesting that absorption from the GI tract and digestive barriers are not the only impediments to potential activity of ingested RNAs. In pre-clinical studies, intravenous injection of a stabilised RNA targeting a mouse gene product did not demonstrate distribution to, or gene product suppression in the liver or jejunum, despite a very high dose of 50 mg/kg bw (Soutschek et al, 2004), while in studies by Thompson et al (2012), intravenous doses of up to 200 mg/kg bw of a stabilised RNA[19] targeting an endogenous gene in rats were without any adverse effects; this also suggests that barriers outside the GI tract are sufficient to preclude activity of exogenous nucleic acids.

5.2. Other Potential Hazards

174. While clinical research has provided significant amounts of information about the human pharmacokinetics of dsRNA, it has also provided some information on possible off-target effects. Off-target effects of RNAi therapy can be broadly classified as sequence specific and non-specific. The former can arise from limited siRNA complementarity to non-target mRNAs and has been characterised in highly-exposed *in vitro* systems and following systemic RNA administration, in experiments employing agents to facilitate systemic delivery. The latter, resulting in immune- and toxicity-related responses, arise due to the construction of the RNA sequence, its modification, or the delivery vehicle.

175. With respect to toxicity, both ASOs and siRNAs can induce off-target effects *via* sequence homology to closely related target sequences or by nonspecific interactions with crucial proteins (as outlined in section 5.5 of this document). However, there is also the possibility that the passenger strand of siRNAs may suppress the translation of non-target mRNAs, resulting in a safety concern unique to therapeutic siRNAs (Chi et al, 2017).

176. Off-target effects associated with siRNA delivery can be divided into three broad categories:

- miRNA-like off-target effects, referring to siRNA-induced sequence-dependent regulation of unintended mRNA transcripts through partial sequence complementarity

[19] Partially protected from nuclease degradation using a methoxy modification on the 2' position of the ribose sugar; this modification occurs naturally in mammalian cells.

- inflammatory responses through the activation of Toll-like receptors (TLRs)[20] triggered by siRNAs or delivery vehicles (e.g., cationic lipids and viruses)
- high-dose effects on miRNA processing and function through the saturation of the endogenous RNAi machinery by exogenous siRNAs (for a review, see Yang & Yang, 2016). This suppression of RNAi machinery can occur following very high doses in experimental systems.

5.2.1. Unintended immune responses

177. Innate immune systems of higher organisms rely on pattern recognition proteins and other factors to identify potentially pathogenic invaders, including foreign dsRNAs[21]. Weissman (2015) noted that "there are more innate immune receptors that recognize RNA than any other foreign or self-molecule, indicating the importance of RNA in the immune system" and that RNA, including *in vitro* transcribed, induces innate immune responses through the activation of pattern recognition receptors, whose function is to identify and respond to pathogenic RNAs". Thus, there is at least a theoretical potential for environmental dsRNAs to stimulate innate immune responses in mammals.

178. siRNAs are able to trigger mammalian endosomal immune cascades or cytoplasmic pathways (Sioud, 2015); the injection of small RNA fragments (fewer than 30 nt) stimulated an immune reaction in mammals. Some Toll-like receptors recognise and respond to the sequence, length, and structure of siRNAs; it appears that small changes in nucleotide sequence (Robbins et al, 2009) or the presence of naturally-occurring modified nucleosides[22] in RNAs (Weissman, 2015) can mitigate immune-stimulatory effects in a given organism.

179. In mice, the immune stimulation by siRNA led to reductions in lymphocytes and platelets, largely correlated with a cytokine response (Judge et al, 2005). Systemic inflammation and damage to organs including the gut were reported when 5 µg/g weight of foreign RNA was injected into mice (Zhou et al, 2007).

180. It has been suggested that, in a 28-day oral toxicity study of siRNAs and a long dsRNA targeting vacuolar ATPase in mice, Petrick et al (2015) had observed inflammation in 1/8 male mice (in the lung, grade minimal) at an oral dose of 64 mg/kg bw 218bp dsRNA per day and 1/8 female mice (in the stomach, grade minimal) at 48 mg/kg bw siRNAs per day. However, according to the pathologist in this study, no treatment-related gross lesions or microscopic findings were observed after dosing with a 21-mer siRNA pool or 218 bp dsRNA test materials. This inflammation was cited in the study report as not being related to treatment as it was not seen in both sexes or in both RNA treatment groups, nor in a separate oral RNA study. Further, according to the study authors, this is a common background finding and does not provide evidence of inflammation following oral exposure (Petrick et al, 2015).

181. Please see https://www.regulations.gov/docket/EPA-HQ-OPP-2014-0293/document for access to the US EPA's review of mammalian toxicology studies and

[20] A class of proteins that play a key role in the innate immune system. The ability of the immune system to recognise molecules that are broadly shared by pathogens is, in part, due to the presence of so-called Toll-like receptors (TLRs) that are expressed on the membranes of leukocytes, cells of the immune system that are involved in protecting the body against infectious disease and other foreign invaders.

[21] There are some similarities in the innate immune response of insects and mammals (Lundgren & Jurat-Fuentes, 2012). It has been hypothesised that the risk of immune-stimulation by dsRNAs may be one reason why RNA-dependent RNA polymerase (RdRP), responsible for amplifying the abundance of siRNAs in some organisms, has not been found in mammals or insects (Agrawal et al, 2003; Dillin, 2003).

[22] Such as pseudouridine, 5-methyluridine, 2-thiouridine, 5-methylcytidine, and N6-methyladenosine

its human health risk assessments for both the limited use seed increase and commercial use of DvSnf7 corn.

182. In a 28-day repeat-dose oral toxicity study (Petrick et al., 2016), mice were administered DvSnf7 dsRNA at 1, 10, or 100 mg/kg bw by oral gavage. According to the study authors, there were no treatment-related effects in "body weights, food consumption, clinical observations, clinical chemistry, hematology, gross pathology, or histopathology endpoints". The NOAEL was therefore determined to be 100 mg/kg bw. The pathologist in the repeat-dose gavage study reported in Petrick et al (2016) concluded that, at high doses of dsRNA (100 mg/kg bw), there was no inflammation in the stomach or elsewhere in the GI tract after direct exposure by gavage. Furthermore, the study pathologist and the reviewing pathologist considered that any gross or histological changes occasionally seen in individual animals were incidental findings and there was no correlation with the diet regime, leading to the conclusion that there was no dsRNA-related inflammation in this oral dosing study.

183. The conclusion reached above, viz. that it is unlikely that novel dsRNAs applied as PIPs would cause an immune response in non-target mammals, has been reached by regulatory authorities in Australia, Canada, and the USA and Europe (FSANZ, 2014; CFIA, 2015; USEPA, 2014; EFSA, 2014).

184. An example of siRNA activation of TLRs was reported by Cho et al (2009); 21nt or longer non-targeted siRNAs were able to suppress hemangiogenesis and lymphangiogenesis in mouse models of neovascularisation, independently of RNA interference, by directly activating Toll-like Receptor 3 (TLR3), a double-stranded RNA immune receptor, on the surface of blood endothelial cells. This action was as efficient as a 21nt siRNA specifically targeting vascular endothelial growth factor-A. In contrast, a 7nt non-targeted siRNA, which was too short to activate TLR3, did not affect angiogenesis in these models.

185. Another example of TLR3 activation comes from the therapeutic Bevasiranib, designed to target VEGF for treatment of age-related macular degeneration. In testing the effects of Bevasiranib, even naked siRNA delivered intra-vitreally triggered significant activation of Toll-like receptor 3 (TLR3) and its adapter molecule TRIF, inducing the secretion of interleukin-12 and interferon-Ɣ (Kleinman et al, 2008; Hu et al, 2020b). Indeed, further clinical investigations were terminated in 2009.

186. Whitehead et al (2011) commented that "one of the significant challenges facing the field today is the differentiation between therapeutic effects caused by target-specific, RNAi-mediated gene silencing and those caused by non-specific stimulation of the innate immune system". Their review highlighted ways in which siRNA could be engineered either to avoid or provoke an innate immune system response.

187. In its white paper on the use of technology employing RNAi as a pesticidal mode of action, the USEPA noted that, since no new protein is being expressed by RNAi, the use of this technology should not present any allergenicity issues (USEPA, 2013). Despite the lack of allergenicity concerns for RNA, allergenicity or sensitization issues may still occur in RNAi-based exogenous pesticides if other biologically-derived molecules are present as contaminants [see also Section 3.5 ('Formulation chemistry – effects on dsRNA stability and environmental persistence') in ENV/JM/MONO(2020)26].

188. The literature from pharmaceutical research on undesired effects arising from RNAi-based therapies (e.g., the potential interaction with the innate immune system of humans and other mammals) needs to be put in context. The route of exposure for most of this literature is not directly relevant for human health risk assessment of topically-applied dsRNA-based products. While the majority of studies utilised intravenous injection, the primary route of exposure for humans to topical dsRNA-based pesticide products would most likely be *via* the diet. Thus, an innate immune response via oral exposure to a topically-applied dsRNA would be unlikely since dsRNA would not be absorbed in any

meaningful amounts from the gastrointestinal tract; these barriers are discussed in detail above (Section 4.3.1).

5.2.2. Saturation of RNAi machinery

189. High levels of siRNAs occurring in a cell as a result of the introduction of exogenous dsRNA can saturate a cell's RNAi machinery and thereby alter endogenous gene expression (Agrawal et al, 2003; Dillin, 2003; Katoch et al, 2013). Essentially, there are a limited number of RISC complexes present within a cell, and the excess exogenous siRNAs may saturate these, thereby preventing them from carrying out typical homeostatic functions in regulating endogenous gene expression (Kahn et al, 2009). Jackson and Linsley (2010) suggested that small RNAs could have "effects on the expression of genes predicted to be under the control of endogenous microRNAs". Saturation of RNAi machinery could also lead to reduced defences against viral infection (Dillin, 2003) and disrupt other pathways in which RNAi machinery is involved, such as RNA-induced transcriptional silencing (RITS) (see Appendix 4 in ENV/JM/MONO(2020)26), possibly resulting in heritable changes in gene expression due to histone modifications.

190. The process of saturation has been better documented with expression of small hairpin RNA, although it is also known from high-dose *in vitro* studies with transfected siRNAs as well (Jackson & Linsley, 2010). Grimm et al (2006) hypothesised that the toxicity and mortality observed in mice infused with a high dose of short hairpin RNAs (shRNAs), a form of stably transfected and over-expressed RNAs, was due to competition with miRNA components. Kahn et al (2009) found that siRNA concentrations from 4 nM were able to saturate RISC components, while saturation of Exportin-5 and Argonaute proteins (especially AGO2) was observed in mice when 5×10^{11} to 2×10^{12} copies of exogenous siRNAs were introduced (Grimm, 2011). High copies of viral-associated RNA (10^8 copies/cell) were also able to saturate the RNAi pathway (Andersson et al, 2005). However, it is noted that these data come from experiments with transformed cells in culture; the relevance to *in vivo* systems is yet to be determined.

191. One regulatory agency suggested that on the basis of most of the available data, it seems unlikely that, following the application of a dsRNA-based pesticide, the extent of systemic exposure of organisms, including humans, in the surrounding environment would be sufficient to saturate their RNAi machinery (USEPA, 2014).

192. The conclusion that saturation is an unlikely hazard is supported by the ubiquitous presence of RNA in the environment, leading to the need for eukaryotic organisms to develop barriers and other mechanisms in order to avoid saturation of the RNAi machinery that is critical to maintaining their cell processes. For example, long endogenous dsRNAs are prevalent in plants, and sequence analysis of predicted long dsRNA transcriptomes of major crops reveals complementarity with human genes (Jensen et al, 2013); *in silico* evaluations also note the potential for sequence alignments. Species responsive to RNAi are being exposed to many thousands of endogenous and exogenous RNAs - see Ivashuta et al (2015) for a description of exogenous RNA sources in the diet of species responsive to environmental RNA.

193. Given this, it seems reasonable to assume that organisms have developed mechanisms to limit the potential for saturation of their RNAi biochemical pathways (in the same way that they have developed mechanisms to limit amplification of sRNAs). Finally, given the stoichiometric requirements for effective RNAi (100-1,000 copies per cell required) and most non-target organisms are made up of billions to trillions of cells, saturation of RNAi machinery appears unlikely to create a potential concern for non-target organisms.

5.2.3. Effects on the gut microbiome

194. Bacteria and archaea have RNA-based regulatory systems, but the machinery differs from those in eukaryotic systems (see Section 5.2.1 of ENV/JM/MONO(2020)26 for a detailed discussion of RNAi machinery in micro-organisms). Dietary composition (e.g., fibre, fat content) has been reported to alter the gut microbiome of humans and other mammals. There is no evidence that specific dietary RNA sequences have any such effects (USEPA, 2014); RNA uptake by microbes is generally followed by catabolism to provide nutrition. No evidence has been reported to suggest that eukaryotic dsRNA is amplified by bacteria.

5.3. Lessons from research on medical applications

195. Successful delivery of dsRNA therapeutics has been achieved using both naked as well as modified siRNA. Select examples are described below.

196. In several clinical trials on RNA therapeutics, dexamethasone premedication was required to reduce infusion-related adverse effects or reactions (i.e. hypersensitivity, flushing, oedema, etc.) (Beg et al., 2016). For some siRNAs used in the clinic, a predosing hydration protocol has been used to protect the kidneys (Zuckerman et al., 2014), thus reducing in humans the toxicities observed in animals.

197. In human clinical trials, PRO-040201 (TKM-ApoB), an unmodified siRNA targeting ApoB expression, was formulated in lipid nanoparticles for the intravenous treatment of hypercholesterolemia. It was generally well tolerated in Phase I trials, with no signs of liver toxicity. However, one of 2 subjects at the highest dose reported flu-like symptoms, considered to be consistent with immune system stimulation. Because of this, and the observation that cholesterol reductions were only transient, further development of PRO-040201 was terminated (Burnett et al, 2011; Burnett & Rossi, 2012; Chen et al, 2018; Hu et al, 2019a; Ozcan et al, 2015).

5.3.1. Oral applications

198. In a mouse model of dextran sodium sulfate (DSS)-induced colitis, Laroui et al. (2014) targeted CD98 (which shows increased expression in inflammatory gut immune responses) for downregulation using siRNA, as a proposed treatment for inflammatory bowel disease. siRNA/polyethyleneimine (PEI) targeting CD98 was loaded within nanoparticles composed of biodegradable polylactic acid (PLA). Nanoparticles were delivered in a chitosan/alginate hydrogel by oral gavage at a concentration of 5 mg/mL (although the dose of siRNA itself was not described). Weight loss was the primary physiological endpoint investigated: over an 8-day period, the mice dosed with CD98 siRNA/PEI–loaded NPs lost only 3% of their initial body weight, which was a statistically significant improvement over the DSS-treated group (10% weight loss) and the DSS-treated group dose with empty NPs (10% weight loss). On the protein level, myeloperoxidase levels (a measure of inflammation) were 4-fold lower in mice treated with CD98 siRNA/PEI-loaded NPs compared to mice which did not receive the nanoparticles. On the mRNA level, qPCR showed that CD98 mRNA expression was reduced 3-fold in mice treated with CD98 siRNA/PEI-loaded NPs, compared with mice treated with DSS alone or DSS and empty NPs.

199. From this study, the toxicological conclusions that can be derived are limited since the dose of siRNA received per animal was not quantified (only the concentration of the siRNA/nanoparticle solution was given). Also, no scrambled/non-specific siRNA controls were used, limiting the conclusions that can be drawn on the specificity of the siRNA itself, and the model system was a disease state. Therefore, this study does not indicate the possible oral toxicological effects of a non-specific siRNA in a healthy animal. Nevertheless, the CD98 siRNA/PEI-loaded NPs appeared to improve weight loss and myeloperoxidase

levels, indicating that the delivery of siRNA itself did not result in adverse effects in this experimental system. This study also shows that oral delivery of siRNA to a target site in the digestive tract can be successful, if the siRNA is complexed/formulated.

200. A second study from the same research group (Xiao et al., 2014) used nanoparticles with both siRNA and antibody targeting CD98. Histological examination of colon tissues from the DSS control group or treatment control group (DSS + hydrogel with scrambled siRNA-loaded NPs) showed clear signs of inflammation, including epithelial disruption, goblet cell depletion, and significant infiltration of inflammatory cells into the mucosa. In contrast, tissues from the treatment group showed less inflammation, and other markers of colitis severity were also reduced (body weight loss, myeloperoxidase activity, etc.). In this study, the effects of the scrambled siRNA treatment group were not more severe than the effects observed with no siRNA treatment, suggesting no obvious non-specific effects of siRNA via oral administration (even with a formulation optimised to enter cells) in this experimental system.

201. In a rat model of hepatic injury (He et al., 2014), a single dose of 50 µg/kg of 2'-*O*-methyl modified siRNA targeting TNF-a encapsulated in chitosan nanoparticles (NP) was delivered by oral gavage. Controls included a scrambled siRNA+NP group and a vehicle (PBS) group. No clinical signs such as body weight were reported. However, when examining liver histology, the TNF-a siRNA-NP treatment improved observations of inflammation more than the scrambled siRNA+NP or PBS controls. TNF-a levels were reduced by 65% in serum, but were also significantly lower in macrophages isolated form the spleen, liver, and lung. This indicates that the siRNA nanoparticles were successfully absorbed through the intestine but, notably, were also able to infiltrate and exert systemic RNAi within other tissues.

202. Another study (Kriegel and Amigi, 2011) tested the ability of orally delivered encapsulated siRNA (1.2 mg/kg bw) targeting TNF-a to reduce inflammation in a mouse model of DSS-induced colitis. Endpoints examined were markers of inflammation (TNF-a mRNA and interferon levels), intestinal histology (cell infiltration, goblet cell depletion, irregular mucosal structure) and clinical symptoms (weight loss and colon length). Some non-specific effects were observed in the scrambled/inactive siRNA and delivery vehicle groups, underscoring the importance of including these types of controls in RNAi studies. However, while non-specific, the effects observed in the scrambled siRNA group were not more adverse than the DSS-induced colitis alone, suggesting that in this biological system, oral administration of scrambled siRNA did not result in observable toxicological effects.

5.3.2. Respiratory applications

203. The use of RNA as an inhalable therapeutic is currently being explored as a novel method to treat various respiratory conditions such as lung cancer, respiratory infections, and chronic inflammatory respiratory illnesses (Chow 2020, Qui 2016). Inhalable RNA has the potential to be a non-invasive treatment method to target and treat tissues locally, and therefore minimize systemic side effects (Chow 2020, Qiu 2016, Roh 2022). However, the challenge in further developing this therapy for clinical use lies in the delivery of the RNA molecule, as the human respiratory system contains various physical and biological barriers which impede its uptake.

204. Extracellularly, pulmonary delivery of RNA is challenging due to the structure of the human lung, which is highly branched with variations in length and diameter. Particle deposition in the lung is favorable for those with an aerodynamic diameter between 1 and 5 µm (Lam 2012, Labiris 2003). Smaller particles (between 0.1 and 1 µm) are easily exhaled while larger particles are unlikely to move past the bifurcations early on in the respiratory tract or will be trapped by the network-like structure of mucus, which is continuously shed, inhibiting RNA interaction at the epithelium (Lai 2009, Salathe 2007). At the alveolar level, RNA molecules are likely to be removed by alveolar macrophages before reaching the target site.

205. Intracellularly, the barriers for RNA delivery are dependent on the type of RNA molecule used and cellular compartment target site. For example, siRNA or mRNA must access the cytoplasm to undergo RISC activation. For cytoplasmic access, RNA molecules would likely enter the cell via endocytosis. However, this route of entry is prone to lysosomal degradation. For shRNA or miRNA vectors, nuclear entry is further complicated due to the high selectivity of the nuclear membrane (Dean 2005).

Formulated RNA

206. Because of these impediments to delivery, developers have explored the use of RNA delivery systems to maintain the integrity of the RNA molecule and increase the probability of reaching the target site. The most commonly used delivery systems include viral vectors, polymer-based materials, and lipid-based materials.

207. Viral vectors for packaging and delivering RNA have been explored using adenoviruses, adeno-associated viruses, and retroviruses to express DNA (which encodes shRNA or miRNA to induce RNAi). The use of viral vectors allows for nuclear access of the RNA, however limitations to this delivery method include toxicity, insertional mutagenesis associated with retroviruses, and immunogenicity associated with adenoviruses (Couto 2010).

208. Non-viral vectors, such as lipid, polymer, and peptide-based delivery methods, exhibit an improved safety profile when compared to viral vectors (Chow 2020, Qui 2016). Of the three, the most commonly used is the lipid-based delivery system, which includes cationic liposomes, solid lipid nanoparticles, solid nanostructured lipid carriers, lipidoids, and pH-response lipids. While lipid-based delivery has demonstrated high efficacy at delivering RNA, challenges of this technique are their poor structural stability and early RNA release due to fusion with pulmonary surfactants (Qui 2016, Garcia 2009). Despite this, lipid nanoparticles are currently in an ongoing clinical trial to deliver mRNA to the lung through nebulization for the treatment of cystic fibrosis (CF). MRT5005 delivers mRNA encoding fully functional CFTR protein and in Phase I/II clinical trials, interim results indicated that MRT5005 was well tolerated among patients that received low and mid-dose levels (8-16 mg) and no adverse events were reported in patients which received up to 24 mg. After a single mid-dose of MRT5005, patients exhibited marked improvement of lung function.

209. Polymer-based methods include the use of both synthetic and natural materials, including poly lactic-co-glycolic acid (PLGA), polyethylenimine (PEI), chitosan, and dextran (Chow 2020, Qui 2016). Polymer-based materials exhibit lower toxicity in relation to lipid-based methods and are easier to chemically modify. For synthetic polymers, PEI is a favorable candidate for formulation due to its high cationic charge density, aqueous solubility, and wide pH-buffering capacity. The development of siRNA/PEI inhalable dry powder has been used to exert gene silencing in mice with lung metastasis (Okuda 2018). Limitations for using this nonbiodegradable polymer include relatively high toxicity. Studies using biodegradable polymers to deliver mRNA through nebulization have been shown to circumvent both local and systemic toxicity after repeated dosing in mouse models (Patel 2019). Natural polymers, like chitosan, have been pursued for their biocompatibility and biodegradability, as chitosan can effectively cross the mucus layer in the airway due to its mucoadhesive and mucopermeable properties (Li 2018). In their study, Capel et al., 2018, used water-soluble chitosan derivatives in the development of powder formulation of inhalable siRNA to silence EGFR in a mouse model of lung cancer. Mice were dosed intratracheally with the aerosolized siRNA/chitosan formulation and their results indicated no adverse effects due to repeated administration of the formulation and a gene silencing effect resulting in tumor reduction.

210. The use of peptide delivery systems which can effectively transfect cells while maintaining low toxicity are currently being developed. Because peptides can mediate transfection through mechanisms which differ from lipids or polymers, they can be utilized to increase cellular uptake (Qui 2016). The limitation in using peptide delivery systems is the lack of cell specificity and few in vivo studies have shown successful delivery to the

lung. Qui et al., 2017, was able to develop a surfactant protein B mimic, KL4 peptide, for pulmonary delivery of both siRNA and miRNA. KL4 demonstrated effective transfection efficiency and, upon PEGylation, KL4 was formulated as an inhalable powder through spray-drying. After a single intratracheal administration in mice, no inflammatory response or toxicity was observed and the biological activity of the mRNA was preserved (Qui 2017, 2019).

Innate RNA

211. In addition to using delivery vectors, another approach taken by developers involves the use of naked RNA, either modified or unmodified. This approach exploits the absence of serum (and therefore nucleases) in the lung for the successful pulmonary delivery of RNA. Several in vivo studies have demonstrated that both siRNA and mRNA can be transfected in the lung following pulmonary delivery (Chow 2020). To further develop this technology, researchers have aimed their focus on identifying lung cell types which are most vulnerable to unmodified RNA transfection.

212. Ng et al., 2019, examined the distribution and activity of naked siRNA in the mouse lung following intratracheal administration and found that silencing activity of the siRNA was most prominent in lung epithelial cells, dendritic cells, and alveolar macrophages. Other studies have corroborated these results, finding that siRNA was mostly in the lung epithelial cells but not the endothelial cells (Xu 2020).

213. In the development of their synthetic modified mRNA, Tiwari et al., 2018, aimed to express neutralizing antibodies in the lung via intratracheal delivery to prevent RSV infection in mice. In this study, the authors compared the delivery of naked mRNA against mRNA used with a polymer-based delivery vector. Their results suggested that the transfection efficiency of naked mRNA was either better than, or comparable to, these polymers.

214. Overall, these studies suggest that the future of inhalable RNA as a therapeutic option is promising. However, there is indication that in order for successful pulmonary delivery, the RNA molecule itself may need to be formulated or administered through methods which include nebulization or intratracheal delivery

5.3.3. .Ocular applications

215. The human eye is composed of various compartments that differ in form and function. The eye thereby comprises several anatomical and physiological barriers that challenge the delivery of drugs (Gupta et al., 2021; Gote et al., 2019). These barriers include the tight corneal and conjunctival epithelium or the blood-ocular barrier and topical drug delivery is further impeded by dilution through lacrimation and the corneal tear film with its rapid turnover, the blinking reflex or the naso-lacrimal duct drainage (Baran-Rachwalska et al., 2020).

216. Direct delivery of RNA-based drugs into eyes can be achieved by either using topical applications or intravitreal injections. These ways of application will allow higher bioavailability at the site of desired action compared to systemic delivery and could reduce systemic side effects (Gupta et al., 2021; Guzman-Aranguez et al., 2013).

217. As RNA in its native form is unstable after application and prone to rapid degradation, chemical modifications as encapsulation within nanoparticles or exosomes might protect the active substance. The combination of RNA with peptides or lipids and the use of transfection agents enables to overcome eye associated barriers and facilitates the cellular uptake and the biodistribution of the active substance (Schiroli et al., 2019; Turchinovich et al., 2010; Baran-Rachwalska et al., 2020; Mead and Tomarev, 2017; Liu et al., 2011). Successful and precise application of RNA has been shown through intravitreal application (Roy et al., 2019; Turchinovich et al., 2010; Zhang et al., 2014; Janout et al., 2014; Liu et al., 2015; Qin et al., 2016; McArthur et al., 2011; Dejneka et al., 2008). The majority of side effects were considered to be unrelated to the use of RNA and the absence

of systemic treatment-related clinical signs proved the clinical safety and tolerability of this mode of action. However, direct injection into the vitreous seems to be rather an unrealistic exposure scenario concerning RNAi use as a pesticide.

218. Benitez-Del-Castillo et al. (2016) included 156 healthy subjects and patients with dry eye disease in their study that were topically treated with a synthetic, 19-base pair double-stranded RNA oligonucleotide duplex formulated in phosphate buffered saline. Significant improvement after instillation was reported with a low proportion of patients with medication-related adverse effects. According to pharmacokinetic analysis the RNA oligonucleotide duplex could not be detected in plasma sample as rapid degradation is expected when the active substance reaches the systemic circulation. Even though RNAi technology seems to cause hardly any adverse effects in clinical trials, sequence- and target independent effects after RNA treatment cannot fully be excluded as siRNAs might induce unanticipated vascular or immune effects via the activation cell-surface toll-like receptor 3 (TLR3) so that this potential hazard of dsRNA should be taken into account for human health risk assessment (Kleinmann et al., 2008; Reich et al., 2003).

5.4. Impact of Formulation and Modification of RNA

219. The oligonucleotides utilised in clinical studies are usually covalently modified and extensively formulated to improve persistence and bypass cellular barriers to RNA uptake.

5.4.1. Impact of formulation ingredients

220. A number of formulation strategies have been developed in the past decade to address issues related to the delivery, bioavailability, or potential toxicity of RNAi in human therapeutic products. It is expected that exogenously applied dsRNA products in agriculture will incorporate specific carriers to facilitate cell uptake in target organisms and penetration through plant surface. (Christiaens et al., 2020). However, it is likely that the economic realities of agricultural and horticultural production will have an impact on the development of complex formulation technologies. In general, agricultural chemical companies need to balance multiple factors in the development of end-use product formulations, including: product stability during shipment and storage; product performance (i.e., loading, mixing, efficacy); the environmental load of formulation components; and the costs of development, formulation components and production). In formulating dsRNA-based products, companies will endeavour to find formulation technologies compatible with a biological active constituent in order to: ensure product stability in commercial channels; not negatively impact product efficacy in the field; find formulation types that do not require significant changes in handling or application as cf. conventional pesticide products. Successful delivery of dsRNA-based therapeutics in humans have used a variety of formulation strategies, including chitosan polymer derivative nanoparticles, liposome delivery and lipid encapsulation, cationic lipids, polyethyleneglycol (PEG)-lipid conjugates, and N-acetylgalactosamine (GalNAc), and various nanoparticle formulations.

221. For agricultural applications several strategies have been pursued, including chemical modifications of the dsRNA molecule and the use of a variety of delivery vehicles. Chelating substances, such as EDTA can increase the environmental stability of naked dsRNA and chemical modifications of dsRNA molecules is a strategy currently pursued by some chemical companies (Christiaens et al., 2020). Different types of nanocarriers (e.g. liposomes, polymers, and peptides) could increase the stability of dsRNA in the insect body or increase cellular uptake rate. Cationic polymers are another class of carriers which proved effective in increasing efficacy of RNAi against insect pests such as mosquitoes (Zhang et al., 2010; Lopez et al., 2019), and Lepidoptera (He et al., 2013; Christiaens et al., 2018; Parsons et al., 2018). Peptides (CPP) were used recently as a carrier for dsRNA in insects and increased sensibly the interfering capacities (Gillet et al. 2017). Nano-sized polyelectrolyte complexes with dsRNA were produced using chitosan cross-linked to

sodium tripolyphosphate. When administered to the larvae of *Aedes aegypti* a significant increase of larval mortality and gene silencing was obtained (Dhandapani et al., 2019).

222. A novel approach was followed by Mitter et al. (2017) who developed a non-toxic and biodegradable layered-double hydroxide nanoparticle (Bioclay or LDH) that maintained the biological activity of dsRNA up to 20 days after spray on plants. Another interesting feature of this product is the fact that this protective layer is degraded under acidic conditions and therefore does not prevent RNA degradation by gastric fluids. While, at this time, there is limited information about the efficiency of delivery of siRNA through LDH in mammals *in vivo*, siRNA-loaded LDH has been shown *in vitro* to deliver RNA with very high efficiency (99%) into mammalian HEK293T cells and subsequent downregulation of a target transcript (Ladewig et al., 2009; Ladewig et al., 2010).

223. It is clear then, that for the risk assessment of dsRNA-based pesticides, careful attention should be paid to how the proposed formulation of the product may impact the toxicological properties, the process of cellular uptake and the environmental persistence of the dsRNA itself.

5.4.2. Impact of modifications of dsRNA nucleotides

224. *Phosphorothioate:* Replacement of one of the non-bridging oxygens of a phosphodiester linkage with a sulphur (phosphorothioate) is a common backbone modification used in RNA-based therapeutics. This modification increases RNA stability by reducing susceptibility to nuclease cleavage. However, some adverse effects including nonspecific protein binding (Brown et al., 1994), unspecific target genes (Winkler et al., 2010) or platelet activation (Flierl et al., 2015) can be attributed to phosphorothioate modifications, when compared to non-modified (phosphodiester) counterparts.

225. *2'-O-methylation:* Plant miRNAs possess 2'-O-methylation at the 3' end of the molecule. This modification makes the miRNA more resistant to RNases and oxidation. While this modification does not appear to have adverse toxicological effects, it does increase the stability of the molecule and therefore may impact its toxicokinetic properties. In vitro stability assays suggest that that methylation has a protective effect on the stability of plant miRNA (Zhang L et al., 2012), when compared to a synthetic form (without 2'-O-methylated 3' ends). Supporting this observation in vivo, oral delivery of 50µg/kg 2'-O-methyl-modified siRNA duplex targeting TNFα-specific reduced serum TNFα levels at a systemic level; in contrast, no effects were observed with liposome-formulated, control or naked RNA (He et al., 2015).

226. *Combined phosphorothioate and 2'-O-methylation:* Using imaging techniques in baboons (positron emission tomography - PET -and [18F]-labelling), Tavitian et al. (1998) showed that naked oligonucleotides have limited distribution into organs and tissues and rapid elimination. In 5 minutes after intravenous injection, naked oligonucleotides were already found in the bladder. One hour after injection, almost all naked oligonuleotides were found in the bladder or residually in the kidney, while the phosphorothioate oligonucleotides were found largely in the liver and kidney, and the 2'-O-methyl ones were in an intermediate distribution (Tavitian et al., 1998). Kinetic studies with radiolabelled oligonucleotides also showed a dramatic reduction of naked oligonucleotides in the plasma within the first 10 min, while 2'-O-methyl and phosphorothioate-modified ones showed a much slower reduction during the first 60 min. Overall, this suggests that 2'-O-methyl or phosphorothioate modifications cause increased retention in various tissues such as liver and kidney, and take longer to be metabolised compared to unmodified oligonucleotides.

5.5. Bioinformatics as a guide to hazard assessment

227. The use of bioinformatics, or sequence matching information, is discussed in detail in Sections 7.1.7 (paragraphs 312 – 313) and 7.5.4 (paragraphs 363 - 376) of

ENV/JM/MONO(2020)26 and the reader is referred to these sections for a detailed consideration of this topic. Critical to the use of sequence analysis in assessing the likelihood of off-target effects in humans of dsRNA products is an understanding of the degree of sequence similarity necessary for effective RNA-mediated gene product suppression, as well as an understanding of a number of structural and biological factors which can affect binding of small RNAs to mRNA. As pointed out in paragraph 179 of Section 5.2.4 in ENV/JM/MONO(2020)26, there is also a broad general distinction between plants and animals in the targeting of endogenously-produced miRNAs; plant miRNAs are usually perfectly or nearly perfectly complementary to their target gene products and induce direct mRNA cleavage of the target mRNA transcript by RISC, while animal miRNAs tend to be more divergent in their sequence recognition,[23] especially when compared to siRNAs. Thus, miRNA-like off-target effects (e.g. translational inhibition) could potentially arise from imperfect pairing of dsRNA-derived siRNAs with mRNA in mammals. There are some dsRNA sequence design considerations to minimize this potential, such as the use of siRNA pools to limit off-target effects (Neumeier and Meister, 2021). Sequence-dependent off-target effects require a stoichiometry between small RNAs and their targets, which may not necessarily be met with long dsRNA with only partial sequence similarity (Hannus et al, 2014). Thus, the type of RNA that is used in the pesticide formulation should also be considered. A key conclusion of these considerations is that bioinformatic prediction of a potential binding site in the human transcriptome for a dsRNA does not necessarily mean that a hazard will result i.e., a bioinformatics assessment is not a hazard assessment. However, bioinformatics analysis of the sequence information relating to a dsRNA designed to target a particular pest species can indicate the likelihood that the dsRNA may also regulate gene expression of less complementary targets in humans. In general, the possibility of silencing occurring in the presence of mismatches between a dsRNA sequence and a human transcript and the resulting possibility of sequence-unrelated off-target effects also indicate the continued importance of bioassays in assessing the actual spectrum of activity of dsRNA (Christiaens et al, 2018, Arpaia et al, 2020).

228. Based on the considerations outlined above, a bioinformatics-based search for potential targets in the human transcriptome that shows less than perfect complementarity to a dsRNA sequence should be included in the HHRA for pesticidal RNAs. Generally, the more thermodynamically favorable the interaction between the pesticide RNA and the potential human off-target, the more likely it is to result in binding and degradation of that off-target and therefore the more likely it is to present a potential hazard. Several factors influence whether off-target effects may ultimately occur. The most apparent one is the number of mismatches between the pesticidal RNA and the potential off-target, i.e., the more mismatches, the less thermodynamically favorable the interaction, and the less likely it is for off-targets to occur. The general approach to this assessment involves individual alignment of all possible 21 nt (of the longer pesticide RNA sequence) to the sequences of the human transcriptome. While there is no defined threshold of tolerated mismatches that reliably predicts that degradation will also occur *in vivo*, the following examples may provide a starting point in determining a mismatch threshold. In their risk assessment of Onpattro™ (patisiran), an siRNA-based injectable drug for the treatment of hereditary transthyretin-mediated amyloidosis, the USFDA used a range of 3 to 5 mismatches of the therapeutic siRNA to potential off-targets in the human transcriptome to determine whether subsequent *in vitro* studies for any of these hits would be required. For those human targets that met

[23] This point is recognised by EFSA in its regulatory requirements (EFSA, 2017); according to Regulation (EU) No. 503/2013, when an RNAi mechanism is used in a GM plant, a bioinformatic analysis to identify potential 'off target' genes is required to be included in the application for approval. However, the EFSA GMO Panel acknowledged the limitations of bioinformatic searches for potential off-targets of siRNAs produced by GM plants because "in plants a set of parameters allows for a reasonable prediction of RNAi off-target gene products while in human and animals the extent of complementarity between the small RNA and the target is more limited and therefore these prediction tools do not allow for sufficiently reliable predictions."

that threshold, the relative affinity of the siRNA to the putative off-targets was compared to the affinity to its intended target, the transthyretin (*TTR*) mRNA in transfected HepB3 cells. In this case, because the siRNA showed less than 1000-fold affinity to all potential off-targets when compared to *TTR*, those initially identified off-targets were not further considered (USFDA, 2018). While the number of off-targets in the human transcriptome was not disclosed in this study, the fact that none of them showed significant affinity in the subsequent *in vitro* assays may indicate that 3-5 mismatches is a conservative approach to identifying biologically relevant hits. A similar threshold (i.e., < 5 mismatches) was employed in the evaluation of Oxlumo® (lumasiran) two years later (USFDA, 2020). Oxlumo® is an injectable GalNAc-siRNA for the treatment of primary hyperoxaluria type 1. Again, none of the initially identified off-targets subsequently showed significant affinity in an *in vitro* assay.

229. Other factors that influence the likelihood for off-target effects to occur involve the type of RNA that is used (e.g., siRNA, miRNA), whether the RNA was chemically modified, where in the RNA/RNA duplex the mismatches occur, and the nucleic acid composition of the predicted RNA/RNA duplex (Ui-Tei, 2013; Pandey and Verma, 2021; Kobayashi et al., 2022). For example, it has been shown that high complementarity in the so-called seed region of an siRNA, which encompasses nucleotides at position 2 - 8 of the siRNA guide strand can result in miRNA-like off-target effects (Ui-Tei et al., 2008). Interestingly, a recent computational analysis of the seed region of 2'-OMe-modified siRNA suggests that for these chemically modified siRNAs in particular nucleotide position 2-5 is essential for avoiding off-target effects, while nucleotide position 6-8 is involved in off-target activities (Kobayashi et al., 2022). Additionally, the processing of dsRNA into "pools" of siRNAs may limit potential off-target effects due to stoichiometric requirements of RNAi (Hannus et al, 2014). While there is no universally accepted set of rules that must be followed in the evaluation of off-target effects through bioinformatics, there are examples in the published literature that provide helpful guidance (e.g., Ui-Tei, 2013, Pandey and Verma, 2021). While many of these are written from the perspective of developers of siRNA therapeutics who seek to design an siRNA that is specific to a human target, the same set of rules can be applied to determining how likely it is that an off-target effect will occur if a potential off-target is identified for a pesticidal RNA.

230. In addition to the sequence specific effects outlined above, sequence-unrelated effects could occur if exposure to unspecific dsRNA affects the human RNAi machinery, rather than a specific transcript sequence. These effects could include saturation of the RNAi machinery or immunostimulation. While there is no evidence to date for RNAi machinery saturation in insects, there is literature showing that this can occur and can lead to fatality in mice, possibly due to competition with essential miRNAs (Grimm et al. 2006). However, it should be noted that these effects were only seen when the shRNA was expressed constitutively in the liver of these mice, which presents a worst-case exposure scenario.

231. In invertebrates, the immune system can recognize dsRNA as a virus associated molecular pattern, and therefore activate an antiviral response (McMenamin et al., 2018). However, it is unclear whether such stimulation of the immune response could lead to adverse effects on the fitness of invertebrate species. Robbins et al. (2009) report an in vitro study on mammalian in which the injection in the blood stream of small fragments of nanocarrier-formulated siRNA (to facilitate cellular uptake) could activate the mammalian innate immune system via a toll-like receptor (TLR) pathway which recognized and responded to the RNAi. Induction of the immune system by siRNA mainly increases the probability of an antibody reaction. Evading the recognition of siRNA by Toll-like receptors may be achieved in engineered RNA by introducing chemically modified nucleotides while designing dsRNA molecules (Mansoori et al., 2016)

232. It may be concluded that sequence complementarity alone is not sufficient for hazard identification or risk assessment, since *in silico* identification of matches to a putative target is not sufficient to determine the potential for gene product suppression or potential for downstream effects, even assuming that a given small RNA was able to reach

a target in sufficient quantities to mediate an RNAi effect. An examination by regulatory agencies of the use of sequence information by the product inventor/developer/applicant in designing a pesticidal dsRNA to be as selective as possible for a target pest should be an important aspect of a regulatory risk assessment contributing to a weight of evidence approach for safety assessment. That is, the scientific rationale for the design of the dsRNA should provide a guide to regulators as to the likely selectivity of the resulting siRNA for the target pest and its specificity for the target gene product.

233. In other words, while bioinformatics can provide important information for the design of possibly selective target genes in the target organism and reduce uncertainties in the HHRA, sequence data cannot be used as a stand-alone predictor of off-target effects of a dsRNA in humans. Other effects also need to be considered in a weight-of-evidence approach, such as the extent of human exposure to the dsRNA through the application of the pesticide, existing barriers to systemic exposure (which may limit target-site access), formulation and modification of the RNA (which may increase target site access) and differences in RNAi machinery between the pest organism being targeted by a particular dsRNA and humans.

234. With respect to the views of regulatory agencies as to the value of bioinformatics, it is noted that:

- According to EU Regulation (EU) No. 503/2013, when an RNAi mechanism is used in a GM plant, a bioinformatic analysis to identify potential 'off target' genes is required to be included in the application for approval (EFSA, 2017). However, EFSA's GMO panel acknowledged the limitations of bioinformatic searches for potential off-targets of siRNAs produced by GM plants because "in plants a set of parameters allows for a reasonable prediction of RNAi off-target genes while in human and animals the extent of complementarity between the small RNA and the target is more limited and therefore these prediction tools do not allow for sufficiently reliable predictions" (EFSA, 2017).
- The Biopesticides and Pollution Prevention Division (BPPD) of the USEPA considered and utilised the bioinformatics analysis that was submitted by Monsanto for approval of DvSnf7 dsRNA expressed in GM corn (USEPA, 2016a; 2016b; 2017).
- Regulatory or regulatory advisory bodies in other jurisdictions (including in Australia, Canada and the EU) also reviewed the same bioinformatics analysis submitted by Monsanto for approval of DvSnf7 dsRNA expressed in GM corn, noting that the anti-DvSnf7 effect observed in corn rootworm was also highly specific to only a very small number of closely-related species.

5.6. Considerations on data requirements – hazard

235. *Toxicity:* The available literature to date on toxicological effects of dsRNA in mammals comes primarily from studies conducted to support the commercialization of genetically modified plants expressing dsRNA (relevant to the oral route of exposure), and from studies on RNAi-based therapeutics (relevant to the inhalation, dermal, ocular, and injection administration routes). For oral and dermal administration, there is insubstantial evidence to date for adverse effects of dsRNA. For inhalation, ocular, and injection administration, a range of effects are observed; however, much of the available evidence comes from human therapeutic administration scenarios which often result in significantly more direct and greater exposures than would be expected for workers and others exposed to dsRNA-based pesticides.

236. Given that effects are dependent on the method of application, formulation, and/or modification of the RNA itself, empirical toxicity testing on dsRNA-based pesticides may be needed to characterise their hazards. As mentioned in the Environmental Considerations

document conclusions (ENV/JM/MONO(2020)26, paragraph 414), dsRNA-based pesticides may take longer to be effective compared to conventional chemicals; for this reason, the exposure and observation period of toxicity studies should be extended if appropriate. Since modifications to the dsRNA nucleotides and/or specific end-use product formulations may impact the toxicological characteristics of dsRNA-based pesticides, special consideration should be given to the appropriate test material for toxicity studies.

237. *Immune system stimulation*: In animals, induction of the innate immune response appears to occur after systemic exposure to RNAs administered by injection. There does not appear to be any evidence in the literature to date to suggest that this occurs following oral exposure, the most relevant exposure route for the general population in considering the dietary risks to human health from agricultural use of externally-applied dsRNAs. Considering the non-dietary exposure, the dermal and inhalation may be the most relevant exposure pathways. Additionally, as described in Appendix I, immune system stimulation via TLR3 receptors has been observed following ocular exposure of unmodified siRNAs. Therefore, immune system stimulation should be considered a potential hazard of dsRNA, which should be taken in the context of route of exposure during the human health risk assessment.

238. *Saturation of RNAi machinery*: Toxicological studies in mammals exhibited no adverse findings at oral doses many times greater than anticipated human exposure to externally-applied dsRNAs, suggesting that saturation of RNAi machinery is unlikely to be a cause of adverse effects from exposure to exogenous dsRNA.

239. *Effects on the gut microbiome*: Dietary composition (e.g., fibre, fat content) has been reported to alter the microbiome, but there does not appear to be any published evidence to date to suggest that specific dietary RNA sequences have any such effects. Thus, it appears unlikely that human health would be affected by any indirect effect arising from exposure of the gut microbiome to dsRNA.

240. *Lessons from clinical development of RNAi therapies*: The route of exposure for most of the clinical therapeutic literature is not directly relevant for dietary risk assessment of topically-applied dsRNA-based products. While the majority of clinical studies to date have utilised intravenous injection, the main routes of exposure of humans to topical dsRNA-based pesticide products would be *via* oral (especially the diet) and dermal routes, with inhalation exposure possible. However, results from studies of siRNA-based therapeutics yield useful information for inhalation, dermal, and ocular hazard, which are relevant to the risk assessments for workers and bystanders which will form part of the HHRA of any new dsRNA-based pesticide.

241. *General:* A useful summary reference covering issues related to human health hazards of dsRNA is a review published in 2020 by Rodrigues and Petrick, titled *Safety considerations for humans and other vertebrates regarding agricultural uses of externally applied RNA molecules*; it summarises information presented at an OECD Conference on RNA interference (RNAi)-based pesticides, held in Paris, France, from 10 – 12 April 2019.

242. As for all pesticide products, the toxicological hazard via oral, dermal, inhalation, and other routes of administration will need to be characterised. Furthermore, specific product formulations or modifications to the dsRNA (e.g., chemical modifications to component nucleosides, covalent additions of carrier molecules, or the addition of cell-penetration-enhancing formulants) may be used to overcome problems of environmental stability or barriers to uptake in the target organism. These dsRNA modifications and formulations have the potential to affect the systemic uptake and toxicological properties of the dsRNA active constituent in exposed humans. Therefore, alterations to toxicological testing protocols may be needed if such dsRNA modifications or formulations are used in a proposed dsRNA-based pesticide.

243. In order to inform the hazard assessment of dsRNA-based pesticides which have been modified as outlined above, product-specific empirical testing may be required to more fully characterise the potential hazards of the product. The necessity and extent of

such testing will depend on what is known about the product's active constituent and its co-formulants, and possibly on the regulatory requirements in the different OECD member countries or jurisdictions. As noted in Section 4.5 ('Considerations on data requirements – exposure'), data and/or studies on the possible impact on environmental mobility and persistence and, hence, on human exposure, that a modified dsRNA active ingredient and/or formulation presents are necessary, in order to help characterise the risks that could possibly arise from exposure to that formulation.

244. As noted in Section 10 ('Conclusions') of ENV/JM/MONO(2020)26, protocols for studies addressing the possible toxicological hazards of dsRNA-based products may require some revisions regarding how they are carried out for conventional pesticides; because dsRNA-based pesticides may take longer to display efficacy. Evaluations may need to account for this time lag by extending the study observation period. Sub-lethal endpoints could be considered depending on the product under evaluation.

245. Additionally, when altered or formulated to increase persistence and/or uptake, dsRNA-based pesticides have an unknown potential for lifecycle and reproductive effects. Life cycle studies (development and reproduction) and studies on other non-lethal effects may need to be considered for these types of dsRNA-based pesticides. For example, OECD Test Guideline 422, *The Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*, could be used.

246. Although there is currently no evidence suggesting transgenerational effects in humans, it is known that in some insect species the effect of dsRNA might be transmitted to the next generation. In one of such cases Abdellatef et al (2015) reported that when the aphid *Sitobion avenae* is fed transgenic barley expressing dsRNA, target gene expression levels were downregulated in the first-generation adults and persisted for several subsequent generations. Reduced target gene expression correlated with a decline in growth, reproduction, and survival rates. Morphological and physiological aberrations such as winged adults and delayed maturation were maintained over seven aphid generations. Relatedly, Khajuria *et al*. (2015) and Coleman *et al.* (2015) reported similar effects in western corn rootworm (*Diabrotica virgifera virgifera*) and the green peach aphid (*Myzus persicae*), respectively, in which oral administration of dsRNA to the parental generation resulted in the second-generation exhibiting effects induced by downregulation of the target gene.

247. How these findings in insects would translate to adverse in effects mammals is unclear and, specifically in the case of unmodified dsRNA, are unlikely. There are clear biological differences between the uptake of dsRNA in insects and mammals as well as their reproductive processes. For example, in the case of the aphid, they are a parthenogenetic species. In some cases an adult female even can carry both daughter and granddaughter embryos simultaneously, potentially facilitating the exposure of subsequent generations to the parental dsRNA.

248. In discussing mRNA-based therapeutics, Weissman (2015) noted that, "since mRNA, unlike DNA and viral vectors, does not contain promoters or antibiotic resistance genes, and cannot integrate into the genome and disruption of genes does not occur, at least for protein replacement, … testing for genome integration, germline transmission, carcinogenicity and genotoxicity should not be required". However, the mode of action of mRNA is different than that of dsRNA (including miRNAs and siRNAs). Given that miRNAs (which result in gene silencing similar to siRNAs) play roles in cell-cycle regulation (e.g. Li et al 2019), implications for carcinogenicity and genotoxicity of dsRNA-based products may be different than those of mRNA-based products discussed by Weissman (2015). The data requirements to characterise these hazards of dsRNA-based pesticides will likely be considered on a case-by-case basis as regulatory approaches are developed.

5.6.1. US EPA Approach

249. While data requirements specific to sprayed or externally applied dsRNA-based pesticides have not been enacted in the U.S., EPA bases its requirements for these pesticides on the biochemical pesticide requirements, outlined in Subpart U—Biochemical Pesticides of 40 CFR Part 158. For toxicity, these include the following.

- Acute oral toxicity – rat
- Acute dermal toxicity
- Acute inhalation toxicity – rat
- Primary eye irritation - rabbit
- Primary dermal irritation
- Dermal sensitization
- 90-day oral (one species)
- 90-day dermal – rat
- 90-day inhalation – rat
- Prenatal developmental - rat preferably
- Bacterial reverse mutation test
- *In vitro* mammalian cell assay

250. Often, the technical grade material of the pesticide is tested in mammalian toxicity studies along with product formulations for acute mammalian toxicity and irritation studies. When the formulation is expected to impact persistence and/or uptake, the formulated product may need to be tested for all studies. Some of this data may be appropriate to waive according to 40 CFR Part 158.45. Further, data not listed in Subpart U for Biochemical Pesticides that is more specific to sprayed or externally applied dsRNA-based pesticides (e.g., bioinformatics, information on potential for stability/uptake) may be required according to 40 CFR Part 158.75.

6. Risk Management

251. Pesticides have been, and continue to be, an indispensable tool in primary production of crops and livestock; they are used in most sectors of agriculture and horticulture to manage populations of economically-important pest species. However, many pesticides can present hazards to human health and there are potential risks associated with their use, if farmers and/or applicators are exposed when mixing and applying the product or working in treated fields, bystanders and residents are exposed to spray/dust drift in the vicinity of agricultural/horticultural operations, or the general public is exposed to residues in food and drinking water.

252. Sprayable dsRNA technology – also-called 'spray-induced gene silencing,' (SIGS)[24] - does not involve genetic manipulation, that is, there is no permanent and heritable change to the genome of crops (or livestock) being protected. Therefore, their authorization process will not follow the specific requirements for genetically modified organisms but will need regulation as new pesticides.

253. The risk of a pesticide to humans is assessed by estimating its potential to cause harm (due to the inherent toxicological properties of the pesticide) and the extent of exposure to the pesticide; the extent of exposure depends on both the exposure amount (dose) and the duration of exposure to that dose. If exposure to a pesticide can be significantly reduced – or even eliminated – during its use or following its application, then even hazardous substances can be used with relatively low risk.

254. For a new generation of pesticides based on dsRNA technology, the same regulatory process for risk management is relevant. That is, the inclusion of information on the label about the active constituent and the end-use-product, information about crop use and application rates, and recommendations about what PPE should be worn, if required, based on the outcome of the risk assessment.

255. With respect to exposure to dsRNA from dsRNA-based pesticides, the literature suggests that the likelihood of any significant systemic absorption of naked, unformulated dsRNAs following oral (Section 4.3.1), dermal (Section 4.3.2), exposure may be minimal. However, there is some published evidence that dsRNAs could possibly cause an inflammatory response if there were significant inhalation exposure (*via* a non-sequence-specific immunostimulatory effect; Rodrigues & Petrick, 2020). Overall, as with other pesticides, the risk mitigation measures for dsRNA-based pesticides will be a function of the hazards and exposure characterised on a product-specific basis.

[24] The expression of RNAi silencing constructs in plants (targeting pests and pathogens) is likewise referred to as host-induced gene silencing, or HIGS (see e.g., Liu et al, 2020).

7. Conclusions

256. The following conclusions can be drawn:

257. Available evidence suggests that dsRNAs have a long record of safe consumption by humans and other mammals. These dsRNAs include both long and short dsRNAs with sequence identity to genes/exons in humans and other organisms consuming them. Nucleic acids are naturally-occurring components of plant- and animal-derived foods and feed and are routinely consumed by humans and animals.

258. The potential for exposure as well as human responsiveness to dsRNAs targeting specific agricultural/horticultural pests are the key parameters to consider in the risk assessment of external dsRNA applications. While bioinformatics will provide important information, sequence data alone should not and cannot be used as a stand-alone predictor of off-target effects in humans.

259. Significant physiological and biochemical barriers exist in humans and other vertebrates to limit the uptake and distribution of exogenous RNAs arising from oral and dermal. These barriers include nucleases in saliva, denaturation and depurination in the acid pH of the stomach, nucleases in the digestive tract, pancreatic secretions of bile salts and degradative enzymes, cellular membrane barriers, the polysaccharide coating of the intestinal epithelium, and intracellular degradation in endosomes and lysosomes. The widespread distribution of such barriers is likely to be a consequence of the widespread presence of RNAs in the environment, molecules which could be biologically active if they were easily able to access cells.

260. As with all pesticide products, the impact of the use-pattern and application method for a dsRNA-based pesticide on the route and extent of human exposure requires consideration. In many cases, specific product formulations may be designed to overcome problems of environmental stability of the active constituent and/or overcome barriers to uptake in the target organism (e.g. Mitter et al., 2017). Special attention will need to be paid by registrants to how the changes to the naked form of the dsRNA (e.g., chemical modifications to component nucleosides, nanocarriers, covalent additions of carrier molecules, or the addition of penetration-enhancing co-formulants) could affect (1) environmental persistence, mobility and distribution, and hence, potential human exposure; and (2) the systemic uptake of the dsRNA active constituent in exposed humans. Thus, data and/or studies on the possible impact on environmental persistence and on systemic uptake and toxicity that a specific dsRNA formulation presents are necessary to characterise the risks that could possibly arise from exposure to that formulation.

261. This suggests that, to inform the risk assessment of dsRNA-based pesticides, product-specific empirical testing will be required to fully characterise hazards and exposure. The necessity and extent of such testing will depend what is known about the product's active constituent and its co-formulants, and possibly on the regulatory requirements in the different OECD member countries or jurisdictions.

262. As noted in Section 10 ('Conclusions') of ENV/JM/MONO(2020)26, non-target organisms vary in their responsiveness to RNAi, and any protocols for studies addressing the possible toxicological hazards of dsRNA-based products may require some revisions cf. how they are carried out for conventional pesticides. Because dsRNA-based pesticides may take longer to display efficacy, any evaluation may need to account for this time lag

by extending the study observation period. Life cycle studies (development and reproduction) and studies on other non-lethal effects may also need to be considered.

8. References

Note: All references cited in this document are listed below; the list also includes relevant references which were previously cited in the OECD document, Considerations for the Environmental Risk Assessment of the Application of Externally or Applied or Externally Applied ds-RNA-Based Pesticides.

Abdellatef E, Will T, Koch A, Imani J, Vilcinskas A, Kogel KH. Silencing the expression of the salivary sheath protein causes transgenerational feeding suppression in the aphid Sitobion avenae. Plant Biotechnol J. 2015 Aug;13(6):849-57. doi: 10.1111/pbi.12322. Epub 2015 Jan 14. PMID: 25586210.

Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV et al (2018) Patisiran, an RNAi therapeutic, for hereditary Transthyretin Amyloidosis. N Engl J Med 379(1): 11-21. DOI: 10.1056/NEJMoa1716153

Agrawal N, Dasaradhi PVN, Mohmmed A, Malhotra P, Bhatnagar RK & Mukherjee SK (2003) RNA interference: Biology, mechanism, and applications. Microbiol Mol Biol Rev 67: 657-685. DOI: 10.1128/MMBR.67.4.657-685.2003

Akhtar S (2009) Oral delivery of siRNA and antisense oligonucleotides. J Drug Target 17: 491- 495. DOI: 10.1080/10611860903057674

Andersson MG, Haasnoot PJ, Xu N, Berenjian S, Berkhout B & Akusjärvi G (2005) Suppression of RNA interference by adenovirus virus-associated RNA. J Virol 79(15): 9556–9565. DOI: 10.1128/JVI.79.15.9556-9565.2005

Aouadi M, Tesz GJ, Nicoloro SM, Wang M, Chouinard M, Soto E, Ostroff GR, Czech MP. 2009. Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation. Nature 458(7242):1180-4. doi: 10.1038/nature07774.

Araujo RN, Santos A, Pinto FS, Gontijo N F, Lehane MJ & Pereira MH (2006) RNA interference of the salivary gland nitrophorin 2 in the triatomine bug Rhodnius prolixus (Hemiptera: Reduviidae) by dsRNA ingestion or injection. Insect Biochem Mol Biol 36: 683–693. DOI: 10.1016/j.ibmb.2006.05.012

Arpaia S, Christiaens O, Giddings K, Jones H, Mezzetti B, Moronta-Barrios F, Perry JN, Sweet J, Taning CN, Smagghe G & Dietz-Pfeilstetter A (2020) Biosafety of GM crop plants expressing dsRNA: Data requirements and EU regulatory considerations. Frontiers in Plant Science 11: Article 940 (13 pp). DOI: 10.3389/fpls.2020.00940

Auer C & Frederick R (2009) Crop improvement using small RNAs: Applications and predictive ecological risk assessments. Trends Biotechnol 27(11): 644-651 DOI: 10.1016/j.tibtech.2009.08.005

Badri P, Jiang X, Borodovsky A, Najafian N, Kim J, Clausen VA, Goel V, Habtemariam B & Robbie GJ (2020) Pharmacokinetic and pharmacodynamic properties of Cemdisiran, an RNAi therapeutic targeting Complement Component 5, in healthy subjects and patients with Paroxysmal Nocturnal Hemoglobinuria. Clin Pharmacokinet (13 Oct 2020). DOI: 10.1007/s40262-020-00940-9.

Ballarín-González B, Dagnaes-Hansen F, Fenton RA, et al. 2013. Protection and Systemic Translocation of siRNA Following Oral Administration of Chitosan/siRNA Nanoparticles. Mol Ther Nucleic Acids. 2013;2(3):e76. doi:10.1038/mtna.2013.2

Balwani M, Sardh E, Ventura P, Peiró PA, Rees DC, Stölzel U et al (2020) Phase 3 trial of RNAi therapeutic Givosiran for Acute Intermittent Porphyria. N Engl J Med 382: 2289-2301. DOI: 10.1056/NEJMoa1913147

Baran-Rachwalska P,Torabi-Pour N, Sutera F. M, Ahmeda M, Thomas K, Nesbit MA, Welsh M, Moore CBT, Saffie-Siebert SR. 2020. Topical siRNA delivery to the cornea and anterior eye by hybrid silicon-lipid nanoparticles. J Control Release 10;326:192-202

Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson S, Plaetinck G, Munyikwa T, Pleau M, Vaughn T & Roberts J (2007) Control of coleopteran insect pests through RNA interference. Nat Biotechnol 25: 1322–1326. DOI: 10.1038/nbt1359

Beg MS, Brenner AJ, Sachdev J, Borad M, Kang YK, Stoudemire J, et al. 2016. Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. Invest New Drugs.

Benitez-Del-Castillo JM, Moreno-Montanes J, Jimenez-Alfaro I, Munoz-Negrete FJ, Turman K, Palumaa K, Sadaba B, Gonzalez MV, Ruz V, Vargas B, Paneda C, Martinez T, Bleau A-M, Jimenez AI. 2016. Safety and Efficacy Clinical Trials for SYL1001, a Novel Short Interfering RNA for the Treatment of Dry Eye Disease. Invest Ophthalmol Vis Sci. 57:6447–6454

Brown A & Ingianni E (2013). Pesticide usage and exposure patterns. Lecture conducted from University of Maryland Pesticide Education and Assessment Programs. http://pesticide.umd.edu/products/leaflet_series/leaflets/PIL36.pdf

Brown DA, Kang SH, Gryaznov SM, DeDionisio L, Heidenreich O, Sullivan S, et al. 1994. Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. J Biol Chem 269, 26801-26805

Burand JP & Hunter WB (2013) RNAi, future in insect management. J Invertebr Pathol 112(Suppl. 1): S68-S74. DOI: 10.1016/j.jip.2012.07.012

Burnett JC, Rossi JJ & Tiemann K (2011) Current progress of siRNA/shRNA therapeutics in clinical trials. Biotechnol J 6(9): 1130–1146. DOI: 10.1002/biot.201100054

Burnett JC & Rossi JJ (2012) RNA-Based therapeutics: Current progress and future prospects. Chem Biol 19(1): 60-71. DOI: 10.1016/j.chembiol.2011.12.008

Capel, V. et al. (2018) Water-soluble substituted chitosan derivatives as technology platform for inhalation delivery of siRNA. Drug Deliv. 25, 644–653

Carver JD & Walker WA (1995) The role of nucleotides in human nutrition. J Nutr Biochem 6: 58–72.

Cervantes A, Alsina M, Tabernero J, Infante JR, LoRusso P, Shapiro G et al (2011) Phase I dose-escalation study of ALN-VSP02, a novel RNAi therapeutic for solid tumors with liver involvement. J Clin Oncol 29 (15_suppl): 3025. DOI: 10.1200/jco.2011.29.15_suppl.3025

CFIA (2015) Decision Document DD2015-111: Determination of the safety of Monsanto Canada Inc.'s Corn (Zea mays L.) Event MON 87411. Canadian Food Inspection Agency. http://www.inspection.gc.ca/plants/plants-with-novel-traits/approved-under-review/decision-documents/dd2015-111/eng/1474581080652/1474581194137#a4-4

Chalbatani GM, Dana H, Gharagouzloo E, Grijalvo S, Eritja R, Logsdon CD, Memari F, Miri SR, Rad MR & Marmari V (2019) Small interfering RNAs (siRNAs) in cancer therapy: a nano-based approach. Int J Nanomedicine 14: 3111–3128. DOI: 10.2147/IJN.S200253

Chan SY & Snow JW (2017) Formidable challenges to the notion of biologically important roles for dietary small RNAs in ingesting mammals. Genes Nutr 12: 13. DOI: 10.1186/s12263-017-0561-7

Chassy B, Hlywka JJ, Kleter GA, Kok EJ, Kuiper HA, McGloughlin M, Munro IC, Phipps RH & Reid JE (2004) Nutritional and safety assessments of foods and feeds nutritionally improved through biotechnology. Compr Rev Food Sci Food Saf 3: 38 - 104.

Chen C, Yang Z & Tang X (2018) Chemical modifications of nucleic acid drugs and their delivery systems for gene-based therapy. Med Res Rev 38: 829-869. DOI: 10.1002/med.21479

Chen M, Zakrewsky M, Gupta V, Anselmo AC, Slee DH, Muraski JA, Mitragotri S (2014) Topical delivery of siRNA into skin using SPACE-peptide carriers. J Control Release 179:33-41. doi: 10.1016/j.jconrel.2014.01.006Chen H-M, Chen L-T, Patel K, Li Y-H, Baulcombe DC (2010) 22-nucleotide RNAs trigger secondary siRNA biogenesis in plants. Proc Natl Acad Sci USA 107: 15269-15274

Cheng C, Kobayashi M, Martinez JA, Ng H, Moser JJ, Wang X, et al. 2015. Evidence for Epigenetic Regulation of Gene Expression and Function in Chronic Experimental Diabetic Neuropathy. J Neuropathol Exp Neurol 74, 804-817.

Chi X, Gatti P & Papoian T (2017) Safety of antisense oligonucleotide and siRNA-based therapeutics. Drug Discovery Today 22(5): 823-833. DOI: 10.1016/j.drudis.2017.01.013

Cho WG, Albuquerque RJC, Kleinman ME, Tarallo V, Greco A, Nozaki M, Green MG, Baffi JZ, Ambati BK, De Falco M, Alexander JS, Brunetti A, De Falco S & Ambati J (2009) Small interfering RNA-induced TLR3 activation inhibits blood and lymphatic vessel growth. Proc Natl Acad Sci USA 106: 7137-7142. DOI: 10.1073/pnas.0812317106

Chow, M.Y.T. et al. (2020) Inhaled RNA Therapy: From Promise to Reality. Trends in Pharmacological Sciences. 41, 715-729.

Christensen J, Litherland K, Faller T, van de Kerkhof E, Natt F, Hunziker J, Krauser J & Swart P (2013) Metabolism studies of unformulated internally [3H]-labeled short interfering RNAs in mice. Drug Metab Dispos 41: 1211-1219. DOI: 10.1124/dmd.112.050666

Christensen J, Litherland K, Faller T, van de Kerkhof E, Natt F, Hunziker J et al (2014) Biodistribution and metabolism studies of lipid nanoparticle-formulated internally [3H]-labeled siRNA in mice. Drug Metab Dispos 42(3): 431-440. DOI: 10.1124/dmd.113.055434

Christiaens O, Dzhambazova T, Kostov K, Arpaia S, Joga MR, Urru I, Sweet J & Smagghe G (2018) Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants. EFSA supporting publication 2018: EN-1424. 173 pp. DOI: 10.2903/sp.efsa.2018.EN-1424

Christiaens, O., Petek, M., Smagghe, G., & Taning, C. N. T. (2020). The use of nanocarriers to improve the efficiency of RNAi-based pesticides in agriculture. In Nanopesticides (pp. 49-68). Springer, Cham.

Christiaens, O., Tardajos, M. G., Martinez Reyna, Z. L., Dash, M., Dubruel, P., and Smagghe, G. (2018). Increased RNAi efficacy in Spodoptera exigua via the formulation of dsRNA with guanylated polymers. Front. Physiol. 9:316. doi: 10.3389/fphys.2018.00316

Codex Alimentarius Commission (2008) Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants. Codex Alimentarius, CAC/GL 45-2003

Coleman, A. D., Wouters R. H. M., Mugford, S. T., Hogenhout, S. A., Persistence and transgenerational effect of plant-mediated RNAi in aphids, Journal of Experimental Botany, Volume 66, Issue 2, February 2015, Pages 541–548, https://doi.org/10.1093/jxb/eru450

Colombo S, Harmankaya N, Water JJ & Bohr A (2019) Exploring the potential for rosacea therapeutics of siRNA dispersion in topical emulsions. Experimental Dermatology 28(3): 261-269. DOI: 10.1111/exd.13881

Couto, L.B.; High, K.A. Viral vector-mediated RNA interference. Curr. Opin. Pharmacol. 2010, 10, 534–542.

Cruz C, Tayler A & Whyard S (2018) RNA interference-mediated knockdown of male fertility genes in the Queensland Fruit Fly Bactrocera tryoni (Diptera: Tephritidae). Insects 9: 96 (13 pp). DOI: 10.3390/insects9030096

Dalakouras A, Wassenegger M, Dadami E, GanopoulosI, Pappas ML & Papadopoulou K (2020) Genetically Modified Organism-free RNA interference: Exogenous application of RNA molecules in plants. Plant Physiology 182: 38–50. DOI: 10.1104/pp.19.00570

Das S, Kumar M, Negi V, Pattnaik B, Prakash YS, Agrawal A, et al. 2014. MicroRNA-326 regulates profibrotic functions of transforming growth factor-beta in pulmonary fibrosis. Am J Respir Cell Mol Biol 50, 882-892.

Davalos A, Henriques R, Latasa MJ, Laparra M, & Coca M. (2019) Literature review of baseline information on non-coding RNA (ncRNA) to support the risk assessment of ncRNA-based genetically modified plants for food and feed. EFSA supporting publication 2019:EN-1688. 220 pp. DOI: 10.2903/sp.efsa.2019.EN-1688

Dean, D.; Strong, D.; Zimmer, W. Nuclear entry of nonviral vectors. Gene Ther. 2005, 12, 881–890.

DEEM/FCID (2018) Dietary Exposure Evaluation Model – Food Commodity Intake Database, Version 4.02, Durango Software, LLC. United States Environmental Protection Agency, https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/deemfcidcalendex-software-installer

Dejneka NS, Wan S, Bond OS, Kornbrust DJ, Reich SJ. 2008. Ocular biodistribution of bevasiranib following a single intravitreal injection to rabbit eyes. Molecular Vision 14:997-1005

Desmet E, Bracke S, Forier K, Taevernier L, Stuart MC, De Spiegeleer B, et al. 2016. An elastic liposomal formulation for RNAi-based topical treatment of skin disorders: Proof-of-concept in the treatment of psoriasis. Int J Pharm 500, 268-274.

DeVincenzo J, Lambkin-Williams R, Wilkinson T, Cehelsky J, Nochur S, Walsh E, et al. (2010) A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus. Proc Natl Acad Sci USA 107: 8800-8805.

Dhandapani, R. K., Gurusamy, D., Howell, J. L., & Palli, S. R. (2019). Development of CS-TPP-dsRNA nanoparticles to enhance RNAi efficiency in the yellow fever mosquito, Aedes aegypti. Scientific reports, 9(1), 1-11.

Dickinson B, Zhang Y, Petrick JS, Heck G, Ivashuta S & Marshall WS (2013) Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nat Biotechnol 31: 965–967. DOI: 10.1038/nbt.2737

Dillin A (2003) The specifics of small interfering RNA specificity. Proc Natl Acad Sci USA 100: 6289-6291. DOI: 10.1073/pnas.1232238100

Dix DJ, Gallagher K, Benson WH, Groskinsky BL, McClintock JT, Dearfield KL & Farland WH (2006) A framework for the use of genomics data at the EPA. Nature Biotechnol 24: 1108-1111. DOI: 10.1038/nbt0906-1108

Dong K, Sun L, Liu JT Gu S-H, Zhou JJ, Yang RN et al. (2017) RNAi-induced electrophysiological and behavioral changes reveal two pheromone binding proteins of Helicoverpa armigera Involved in the perception of the main sex pheromone component Z11–16:Ald. J Chem Ecol 43: 207-214. DOI: 10.1007/s10886-016-0816-6

Dyrbuś K, Gąsior M, Penson P, Ray KK & Banach M (2020) Inclisiran—New hope in the management of lipid disorders? J Clin Lipidol 14(1): 16-27. DOI: 10.1016/j.jacl.2019.11.001

EFSA (2014) International scientific workshop on risk assessment considerations for RNAi-based GM plants, (4-5 June 2014, Brussels, Belgium). EFSA supporting publication (Parma, Italy). Available online at: http://www.efsa.europa.eu/sites/default/files/corporate_publications/files/705e.pdf

EFSA (2017) Minutes of the 118th Plenary meeting of the Scientific Panel on GMO. 25-26 Oct. 2017, Parma, Italy. European Food Safety Authority, Genetically Modified Organisms Unit, Scientific Panel on GMO. (https://www.efsa.europa.eu/sites/default/files/event/171025-m.pdf ; accessed on 20 June 2021)

EFSA Panel on Genetically Modified Organisms (GMO) (2011) Guidance for risk assessment of food and feed from genetically modified plants. EFSA J 9: 2150 (37 pp). DOI: 10.2903/j.efsa.2011.2150

EFSA Panel on Genetically Modified Organisms (GMO) (2019) Assessment of genetically modified maize MON 87427 x MON 89034 x MIR162 x MON 87411 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2017-144). EFSA Journal 17(11): 5848 33pp. DOI: 10.2903/j.efsa.2019.5848

FAO/WHO (1996) Biotechnology and food safety. Report of a Joint FAO/WHO Consultation, Rome, Italy, 30 Sept - 4 Oct 1996, Paper No. 61FAO/WHO (2000)

FAO/WHO (2000) Safety aspects of genetically modified foods of plant origin: Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, 29 May – 2 June 2000. World Health Organization, Geneva.

FAO/WHO (2001) Safety assessment of foods derived from genetically modified microorganisms. Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology. Food & Agriculture Organization of the United Nations, World Health Organization.

Flierl U, Nero TL, Lim B, Arthur JF, Yao Y, Jung SM, et al. 2015. Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators. J Exp Med 212, 129-137

Flisiak R, Jaroszewicz J & Łucejko M (2018) siRNA drug development against hepatitis B virus infection, Expert Opin Biol Ther 18(6); 609-617. DOI: 10.1080/14712598.2018.1472231

Fortunato O & Iorio MV (2020) The Therapeutic potential of microRNAs in cancer: Illusion or opportunity? Pharmaceuticals (Basel) 13(12): 438. DOI: 10.3390/ph13120438

Foss FM, Querfeld C, Zain J, Pinter-Brown LC, Joyce R, Moskowitz A et al (2019) Phase I trial of cobomarsen, a mir-155 inhibitor, in HTLV-1 associated adult T cell lymphoma/leukemia. miRagen Therapeutics, Inc. poster, downloadable at http://www.viridiantherapeutics.com/publications/2019/01/Phase-I-Trial-of-Cobomarsen-a-MiR-155-Inhibitor-in-HTLV-1-Associated-Adult-T-cell-Lymphoma-Leukemia.pdf (accessed on 22 February 2021)

Frede A, Neuhaus B, Klopfleisch R, Walker C, Buer J, Müller W, Epple M, Westendorf AM. 2016. Colonic gene silencing using siRNA-loaded calcium phosphate/PLGA nanoparticles ameliorates intestinal inflammation in vivo. J Control Release;222:86-96. doi: 10.1016/j.jconrel.2015.12.021.

FSANZ (2013) Response to Heinemann et al on the regulation of GM crops and foods developed using gene silencing. Food Standards Australia New Zealand, http://www.foodstandards.gov.au/consumer/gmfood/Documents/Heinemann%20Response%20210513.pdf (Accessed on 21 June 2021)

FSANZ (2014) Safety assessment report – Application A1097: Food derived from herbicide-tolerant & insect-protected corn line MON87411. Food Standards Australia New Zealand. Document A1097-GM-CFS-SD1 at http://www.foodstandards.gov.au/Search/pages/results.aspx?k=DvSnf7 (Accessed on 4 March 2018)

FSANZ (2020) Safety assessment – Application A1202: Food derived from herbicide-tolerant and insect-protected corn line DP23211. Food Standards Australia New Zealand, 1 October 2020 [136-20] Supporting document 1, 48 pp. A1202 SD1.pdf (foodstandards.govt.nz (Accessed on 21 June 2021)

Garcia-Mouton, C. et al. (2019) The lord of the lungs: the essential role of pulmonary surfactant upon inhalation of nanoparticles. Eur. J. Pharm. Biopharm. 144, 230–243

Gilmore IR, Fox SP, Hollins AJ, Sohail M & Akhtar S (2004) The design and exogenous delivery of siRNA for post-transcriptional gene silencing. J Drug Target 12: 315-340. DOI: 10.1080/10611860400006257

Gillet, F.-X., Garcia, R. A., Macedo, L. L., Albuquerque, E. V., Silva, M., and Grosside-Sa, M. F. (2017). Investigating engineered ribonucleoprotein particles to improve oral RNAi delivery in crop insect pests. Front. Physiol. 8:256. doi: 10.3389/fphys.2017.00256

Goldstein DA, Shelton PE, Cullen MR, Easterday PA, Eppard PJ & Cabanilla BR (2004) Responding to the challenge of novel technology: An industrial hygiene and safety program for antibody production in maize. J Occup Env Med: 46(8): 784-790. DOI: 10.1097/01.jom.0000135691.69649.f8

Gonzalez V, Sadaba B, Moreno-Montanes J, Ruz V, Paneda C & Jimenez AI (2015) Pharmacokinetics and biodistribution of bamosiran in human and animal model, a novel treatment for glaucoma. Invest Ophthalmol Vis Sci 56: 5748. [Abstract, Annual Meeting of the Association for Research in Vision & Ophthalmology (ARVO), Annual Meeting Abstract. June 2015]

Gote V, Sikder S, Sicotte J, Pal D. 2019. Ocular Drug Delivery: Present Innovations and Future Challenges. J Pharmacol Exp Ther 370:602–624

Grimm D, Street KL, Jopling CL, Storm TA, Pandey K, Davis CR et al (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441(7092): 537–541. DOI: 10.1038/nature04791

Grimm D (2011) The dose can make the poison: lessons learned from adverse in vivo toxicities caused by RNAi overexpression. Silence. 2: 8. DOI: 10.1186/1758-907X-2-8

Gupta A, Kafetzis A. N., Tagalakis A. D., Yu-Wai-Man C. 2021. RNA therapeutics in ophthalmology - translation to clinical trials. Exp Eye Res. 205:108482

Gupta SK, Haigh BJ, Griffin FJ & Wheeler TT (2012) The mammalian secreted RNases: Mechanisms of action in host defence. Innate Immun 19(1): 86–97. DOI: 10.1177/1753425912446955

Gutbier B, Kube SM, Reppe K, Santel A, Lange C, Kaufmann J, et al. (2010) RNAi-mediated suppression of constitutive pulmonary gene expression by small interfering RNA in mice. Pulm Pharmacol Ther 23, 334-344.

Guzman-Aranguez A, Loma P, Pintor J. 2013. Small-interfering RNAs (siRNAs) as a promising tool for ocular therapy. Br J Pharmacol. 170(4):730-47

Habtemariam BA, Karsten V, Attarwala H, Goel V, Melch M, Clausen VA et al (2020) Single-dose pharmacokinetics and pharmacodynamics of transthyretin targeting N-acetylgalactosamine–small interfering ribonucleic acid conjugate, vutrisiran, in healthy subjects. Clin Pharmacol Ther 109(2): 372-382. DOI: 10.1002/cpt.1974

Hannus M, Beitzinger M, Engelmann JC, Wieckert M-T, Spang R, Hannus S, Meister G. (2014) siPools: highly complex but accurately defined siRNA pools eliminate off-target effects. Nucleic Acids Research 42(12): 8049-8061.

He, B., Chu, Y., Yin, M., Müllen, K., An, C., and Shen, J. (2013). Fluorescent nanoparticle delivered dsRNA toward genetic control of insect pests. Adv. Mater. 25, 4580–4584. doi: 10.1002/adma.201301201 Heidel JD, Yu Z, Liu JY, Rele SM, Liang Y, Zeidan RK, et al. 2007. Administration in non-human primates of

escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. Proc Natl Acad Sci U S A 104, 5715-5721

He C, Yin L, Song Y, Tang C, Yin C. 2015. Optimization of multifunctional chitosan-siRNA nanoparticles for oral delivery applications, targeting TNF-alpha silencing in rats. Acta Biomater 17, 98-106.

Hill A, Valls AG, Griffin M, Munir T, Borodovsky A, Kawahata N et al. (2016) A subcutaneously administered investigational RNAi therapeutic (ALN-CC5) targeting complement C5 for treatment of PNH and complement-mediated diseases: preliminary phase 1/2 study results in patients with PNH. Blood 128: 3891. DOI: 10.1182/blood.V128.22.3891.3891

Hirschi KD, Pruss GJ & Vance V (2015) Dietary delivery: a new avenue for microRNA therapeutics? Trends Biotechnol 33: 431-432. DOI: 10.1016/j.tibtech.2015.06.003

Hoerter JA, Krishnan V, Lionberger TA. &Walter NG (2011) siRNA-like double-stranded RNAs are specifically protected against degradation in human cell extract. PLoS One 6: e20359. DOI: 10.1371/journal.pone.0020359

Houck JC & Berman LB (1958) Serum ribonuclease activity. J Appl Physiol 12(3): 473- 476.Houseley J & Tollervey D (2009) Review: The many pathways of RNA degradation. Cell 136(4): 763-776. DOI: 10.1016/j.cell.2009.01.019

Hsu T & Mitragotri S (2011) Delivery of siRNA and other macromolecules into skin and cells using a peptide enhancer. Proc Natl Acad Sci USA 108, 15816-15821 DOI: 10.1073/pnas.1016152108

Hu X, Steimel JP, Kapka-Kitzman DM, Davis-Vogel C, Richtman NM, Mathis JP, Nelson ME, Lu AL & Wu G (2019a) Molecular characterization of the insecticidal activity of double-stranded RNA targeting the smooth septate junction of western corn rootworm (Diabrotica virgifera virgifera). PLoS ONE 14(1): e0210491. DOI: 10.1371/journal.pone.0210491

Hu B, Weng Y, Xia X-H, Liang X-J & Huang Y (2019b) Clinical advances of siRNA therapeutics. J Gene Med e3097. DOI: 10.1002/jgm.3097

Hu Z, Boeckman CJ, Cong B, Steimel JP, Richtman NM, Sturtz K, Wang Y, Walker CL, Yin J, Unger A, Farris C & Lu AL (2020a) Characterization of DvSSJ1 transcripts targeting the smooth septate junction (SSJ) of western corn rootworm (Diabrotica virgifera virgifera). Sci Rep 10(11139): 1-13. DOI: 10.1038/s41598-020-68014-1

Hu B, Zhong L, Weng Y, Peng L, Huang Y, Zhao Y & Liang X-J (2020b) Therapeutic siRNA: state of the art. Signal Transduct Target Ther 5: 101. DOI: 10.1038/s41392-020-0207-x

Huang H, Davis CD & Wang TTY (2018) Extensive degradation and low bioavailability of orally consumed corn miRNAs in mice. Nutrients 10: 215-227. DOI: 10.3390/nu10020215

Ibaraki H, Kanazawa T, Kurano T, Oogi C, Takashima Y, & Seta Y (2019) Anti-RelA siRNA-Encapsulated Flexible Liposome with Tight Junction-Opening Peptide as a Non-invasive Topical Therapeutic for Atopic Dermatitis. Biol Pharm Bull 42: 1216-1225. DOI: 10.1248/bpb.b19-00259

IUPAC (1997) Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford. Online version (2019-) created by S. J. Chalk. ISBN 0-9678550-9-8. https://doi.org/10.1351/goldbook

Ivashuta S, Zhang Y, Wiggins BE, Ramaseshadri P, Segers GC, Johnson S et al (2015) Environmental RNAi in herbivorous insects. RNA 21: 840–850. DOI: 10.1261/rna.048116.114

Jackson AL & Linsley PS (2010) Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat Rev Drug Discov 9: 57–67. DOI: 10.1038/nrd3010

Jackson NAC, Kester KE, Casimiro D, Gurunathan S & DeRosa F (2020) The promise of mRNA vaccines: a biotech and industrial perspective. NPJ Vaccines 5: 11. DOI: 10.1038/s41541-020-0159-8

Janas, M.M., Harbison, C.E., Perry, V.K., Carito, B., Sutherland, J.E., Vaishnaw, A.K., Keirstead, N.D. and Warner, G., 2018. The nonclinical safety profile of GalNAc-conjugated RNAi therapeutics in subacute studies. Toxicologic pathology, 46(7), pp.735-745.

Jain KK (2008) Stability and delivery of RNA via the gastrointestinal tract. Curr Drug Deliv 5: 27-31. DOI: 10.2174/156720108783331023

Janout V, Cline LL, Feuston BP, Klein L, O'Brien A, Tucker T, et al. 2014. Molecular umbrella conjugate for the ocular delivery of siRNA. Bioconjug Chem 25, 197-201.

Jensen PD, Zhang Y, Wiggins BE, Petrick JS, Zhu J, Kerstetter RA, Heck GR & Ivashuta SI (2013) Computational sequence analysis of predicted long dsRNA transcriptomes of major crops reveals sequence complementarity with human genes. GM Crops and Food 4: 90-97. DOI: 10.4161/gmcr.25285

Jiang L, Ding L, He B, Shen J, Xu Z, Yin M, Zhang X (2014) Systemic gene silencing in plants triggered by fluorescent nanoparticle-delivered double-stranded RNA. Nanoscale 6: 9965-9969

Joga MR, Zotti MJ, Smagghe G & Christiaens O (2016) RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: What we know so far. Front Physiol 7: 553. DOI: 10.1007/s13744-015-0291-8

Jonas DA, Antignac E, Antoine JM, Classen HG, Huggett A, Knudsen I, Mahler J, Ockhuizen T, Smith M, Teuber M, Walker R & De Vogel P (1996) The safety assessment of novel foods: Guidelines prepared by ILSI Europe Novel Food Task Force. Food Chem Toxicol 34: 931-940.

Judge AD, Sood V, Shaw JR, Fang D, McClintock K & MacLachlan I (2005) Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nature Biotechnol 23: 457-462. DOI: 10.1038/nbt1081

Juliano RL (2016) Survey and summary: The delivery of therapeutic oligonucleotides. Nucleic Acids Res 44(14): 6518-481. DOI: 10.1093/nar/gkw236

Juliano RL, Ming X & Nakagawa O (2012) Cellular uptake and intracellular trafficking of antisense and siRNA oligonucleotides. Bioconjug Chem 23(2): 147−157. DOI: 10.1021/bc200377d

Kahn AA, Betel D, Miller ML, Sander C, Leslie CS & Marks DS (2009) Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs. Nature Biotechnol 27: 549-555. DOI: 10.1038/nbt.1543

Kamthan A, Chaudhuri A, Kamthan M & Datta A (2015) Small RNAs in plants: recent development and application for crop improvement. Front Plant Sci 6: 208. DOI: 10.3389/fpls.2015.00208

Kang W, Bang-Berthelsen CH, Holm A, Houben AJS, Müller AH, Thymann T, Pociot F, Estivill X & Friedländer MR (2017) Survey of 800+ data sets from human tissue and body fluid reveals xenomiRs are likely artifacts. RNA 23: 433–45. DOI: 10.1261/rna.059725.116

Kasiewicz LN & Whitehead KA (2019) Lipid nanoparticles silence tumor necrosis factor α to improve wound healing in diabetic mice. Bioeng Transl Med 4(1): 75–82. doi: 10.1002/btm2.10123

Katoch R, Sethi A, Thakur N & Murdock LL (2013) RNAi for insect control: current perspective and future challenges. Appl Biochem Biotechnol 170: 1-27. DOI: 10.1007/s12010-013- 0399-4

Kausik K, Ray KK, Wright RS, Kallend D, Koenig W, Leiter LA et al. (2020) Two Phase 3 trials of Inclisiran in patients with elevated LDL cholesterol. N Engl J Med 382: 1507-1519. DOI: 10.1056/NEJMoa1912387

Khajuria C, Vélez AM, Rangasamy M, Wang H, Fishilevich E, Frey ML, Carneiro NP, Gandra P, Narva KE, Siegfried BD. Parental RNA interference of genes involved in embryonic development of the western corn

rootworm, Diabrotica virgifera virgifera LeConte. Insect Biochem Mol Biol. 2015 Aug;63:54-62. doi: 10.1016/j.ibmb.2015.05.011. Epub 2015 May 22. PMID: 26005118.

Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi JZ, Albuquerque RJ, Yamasaki S, Itaya M, Pan Y, Appukuttan B, Gibbs D, Yang Z, Karikó K, Ambati BK, Wilgus TA, DiPietro LA, Sakurai E, Zhang K, Smith JR, Taylor EW & Ambati J (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 452(7187): 591-7. DOI: 10.1038/nature06765

Kobayashi, Y., Fukuhara, D., Akase, D., Aida, M. and Ui-Tei, K., 2022. siRNA Seed Region Is Divided into Two Functionally Different Domains in RNA Interference in Response to 2′-OMe Modifications. ACS omega, 7(2), pp.2398-2410.

Koch A & Kogel KH (2014) New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. Plant Biotechnol 12: 821-831. DOI:10.1111/pbi.12226

Kriegel C, Amiji M. 2011. Oral TNF-alpha gene silencing using a polymeric microsphere-based delivery system for the treatment of inflammatory bowel disease. J Control Release 150, 77-86

Kroes R, Kleiner J, Renwick A (2005) The Threshold of Toxicological Concern concept in risk assessment. Toxicol Sci 86: 226-230. DOI: 10.1093/toxsci/kfi169

Labiris, N.R. and Dolovich, M.B. (2003) Pulmonary drug delivery. Part I: physiological factors affecting therapeutic effectiveness of aerosolized medications. Br. J. Clin. Pharmacol. 56, 588–599

Ladewig, K., Niebert, M., Xu, Z.P., Gray, P.P. and Lu, G.Q., 2010. Efficient siRNA delivery to mammalian cells using layered double hydroxide nanoparticles. Biomaterials, 31(7), pp.1821-1829.

Ladewig, K., Xu, Z.P. and Lu, G.Q., 2009. Layered double hydroxide nanoparticles in gene and drug delivery. Expert Opinion on Drug Delivery, 6(9), pp.907-922.

Lai, S.K.; Wang, Y.Y.; Hanes, J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv. Drug Deliv. Rev. 2009, 61, 158–171.

Lam JK, Liang W & Chan HK (2012) Pulmonary delivery of therapeutic siRNA. Adv Drug Deliv Rev 64(1): 1-15. DOI: 10.1016/j.addr.2011.02.006

Langman CB, Ariceta G, Barrios K, Brown BD, Hoppe B, Rosskamp R (2021) Hepatic lactate dehydrogenase A is an RNA interference target for the treatment of all known types of primary hyperoxaluria. Kidney Int Rep (2021). DOI: 10.1016/j.ekir.2021.01.029

Lares MR, Rossi JJ & Ouellet DL (2011) RNAi and small interfering RNAs in human disease therapeutic applications. Trends Biotechnol 28(11): 570-579. DOI: 10.1016/j.tibtech.2010.07.009

Laroui H, Geem D, Xiao B, Viennois E, Rakhya P, Denning T, et al. 2014. Targeting intestinal inflammation with CD98 siRNA/PEI-loaded nanoparticles. Mol Ther 22, 69-80

Ledford H (2016) Investors flee as firm scraps RNA-interference drug candidate Struggling RNAi field faces another blow as its first treatments near the clinic. Nature Trend Watch 10 October 2016. DOI: 10.1038/nature.2016.20769

Levanova A & Poranen MM (2018) RNA Interference as a prospective tool for the control of human viral infections. Front Microbiol DOI: 10.3389/fmicb.2018.02151

Li, J. et al. (2018) Chitosan-based nanomaterials for drug delivery. Molecules 23, 2661

Li M, Chen T, Wang R, Luo JY, He JJ, Ye RS et al (2019) Plant MIR156 regulates intestinal growth in mammals by targeting the Wnt/beta-catenin pathway. Am J Physiol Cell Physiol 317: C434–C448. DOI: 10.1152/ajpcell.00030.2019

Li BJ, Tang Q, Cheng D, Qin C, Xie FY, Wei Q et al. (2005) Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. Nat Med 11: 944-951. DOI: 10.1038/nm1280

Link J, Thon C, Schanze D, Steponaitiene R, Kupcinskas J, Zenker M, Canbay A, Malfertheiner P & Link A (2019) Food-derived Xeno-microRNAs: Influence of diet and detectability in gastrointestinal tract—proof-of-principle study. Mol Nutr Food Res 63: 1800076 (1-11). DOI: 10.1002/mnfr.201800076

Liu CH, Sun Y, Li J, Gong Y, Tian KT, Evans LP, et al. 2015. Endothelial microRNA-150 is an intrinsic suppressor of pathologic ocular neovascularization. Proc Natl Acad Sci U S A 112, 12163-12168

Liu H, Liu Y, Ma Z, Wang J, Zhang Q. 2011. A Lipid Nanoparticle System Improves siRNA Efficacy in RPE Cells and a Laser-Induced Murine CNV Model. Invest Ophthalmol Vis Sci 52(7):4789-94

Liu R, Mu L-M, Bai J, Du Y-F, Xie Y & Lu W-L (2019) Development of double strand RNA mPEI nanoparticles and application in treating invasive breast cancer. RSC Adv 9: 13186-13200. DOI: 10.1039/c9ra01889a

Liu S, Jaouannet M, Dempsey DA, Imania J, Coustau C & Kogel K-H (2020) RNA-based technologies for insect control in plant production. Biotechnol Adv 39: 107463 (13 pp). DOI: 10.1016/j.biotechadv.2019.107463

Lopatin U (2019) Drugs in the Pipeline for HBV. Clin Liver Dis 23: 535–555. DOI: 10.1016/j.cld.2019.04.006

Lopez, S. B. G., Guimarães-Ribeiro, V., Rodriguez, J. V. G., Dorand, F. A., Salles, T. S., Sá-Guimarães, T. E., et al. (2019). RNAi-based bioinsecticide for Aedes mosquito control. Sci. Rep. 9:4038.

Loretz B, Föger F, Werle M & Bernkop-schnürch A (2006) Oral gene delivery: strategies to improve stability of pDNA towards intestinal digestion. J Drug Target 14: 311-319. DOI: 10.1080/10611860600823766

Lundgren JG & Jurat-Fuentes JL (2012) Physiology and ecology of host defense against microbial invaders. In Vega FE & Kaya HK, eds. Insect Pathology, 2nd Ed. Academic Press, pp 461–480.

Luscombe NM, Greenbaum D & Gerstein M (2001) What is bioinformatics? A proposed definition and overview of the field. Methods Inf Med 40(4): 346-58.

Lusk RW (2014) Diverse and widespread contamination evident in the unmapped depths of high throughput sequencing data. PLoS ONE 9(10): e110808. DOI: 10.1371/journal.pone.0110808

Ma Z, Yang C, Song W, Wang Q, Kjems J, Gao S. 2014. Chitosan hydrogel as siRNA vector for prolonged gene silencing. J Nanobiotechnology 12, 23.

Machin N & Ragni MV (2018) An investigational RNAi therapeutic targeting antithrombin for the treatment of hemophilia A and B. J Blood Med 9: 135–140. DOI: 10.2147/JBM.S159297

Mao YB, Cai WJ, Wang JW, Hong GJ, Tao XY, Wang L-J et al (2007) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotechnol 25: 1307-1313. DOI: 10.1038/nbt1352

Mao YB, Tao X-Y, Xue X-Y, Wang L-J & Chen X-Y (2011) Cotton plants expressing CYP6AE14 double-stranded RNA show enhanced resistance to bollworms. Transgenic Res 20: 665-673. DOI: 10.1007/s11248-010-9450-1

Manjunath N & Dykxhoorn DM (2010) Advances in synthetic siRNA delivery. Discov Med 9: 418-430.

McArthur K, Feng B, Wu Y, Chen S, Chakrabarti S. 2011. MicroRNA-200b regulates vascular endothelial growth factor-mediated alterations in diabetic retinopathy. Diabetes 60, 1314-1323.

McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML (2018) Honey bee and bumble bee antiviral defense. Viruses 10:395. https:// doi. org/ 10. 3390/ v1008 0395

McNeill EM & Hirschi KD (2020) Roles of regulatory RNAs in nutritional control. Annu Rev Nutr 40: 77-104. DOI: 10.1146/annurev-nutr-122319-035633

Mead B and Tomarev S.2017. Bone Marrow-Derived Mesenchymal Stem Cells-Derived Exosomes Promote Survival of Retinal Ganglion Cells Through miRNA-Dependent Mechanisms. Stem Cells Transl Med 6(4):1273-1285

Mertens JJWM (2013) A 90-day feeding study in Sprague Dawley rats with grain from MON 87411 corn. MRID Number 49505806. Monsanto Company, St. Louis, Missouri.

Mezzetti B, Smagghe G, Arpaia, S, Christiaens O, Dietz-Pfeilstetter A, Jones H et al. (2020) RNAi: What is its position in agriculture? J Pest Sci 93: 1125–1130. DOI: 10.1007/s10340-020-01238-2

Mitter N, Worrall EA, Robinson KE, Li P, Jain RG, Taochy C, Fletcher SJ, Carroll BJ, Lu GQ & Xu ZP (2017) Clay nanosheets for topical delivery of RNAi for sustaines protection against plant viruses. Nature Plants 3: 16207. DOI: 10.1038/nplants.2016.207

Mitter N, Worrall EA, Robinson KE, Xu ZP, Carroll BJ (2017b) Induction of virus resistance by exogenous application of double stranded RNA. Current Opinion in Virology 26: 49-55

Mohammad Shadid, Mohamed Badawi & Abedelnasser Abulrob (2021) Antisense oligonucleotides: absorption, distribution, metabolism, and excretion, Expert Opinion on Drug Metabolism & Toxicology, 17:11, 1281-1292, DOI: 10.1080/17425255.2021.1992382

Molitoris BA, Dagher PC, Sandoval RM, Campos SB, Ashush H, Fridman E et al (2009) SiRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury. J Amer Soc Nephrol 20: 1754–1764. DOI: 10.1681/ASN.2008111204

Moschos SA, Frick M, Taylor B, Turnpenny P, Graves H, Spink KG et al. (2011) Uptake, efficacy, and systemic distribution of naked, inhaled short interfering RNA (siRNA) and locked nucleic acid (LNA) antisense. Mol Ther 19, 2163-2168

Neumeier J and Meister G (2021) siRNA Specificity: RNAi Mechanisms and Strategies to Reduce Off-Target Effects. Front. Plant Sci. 11:526455. doi: 10.3389/fpls.2020.526455

Ng, B. et al. (2019) Intratracheal administration of siRNA triggers mRNA silencing in the lung to modulate T cell immune response and lung inflammation. Mol. Ther. Nucleic Acids 16, 194–205

Numata K, Ohtani M, Yoshizumi T, Demura T, Kodama Y (2014) Local gene silencing in plants via synthetic dsRNA and carrier peptide. Plant Biotechnology Journal 12: 1027-1034

O'Driscoll CM, Bernkop-Schnürch A, Friedl JD, Préat V & Jannin V (2019) Oral delivery of non-viral nucleic acid-based therapeutics - do we have the guts for this? Eur J Pharm Sci 133: 190–204. DOI: 10.1016/j.ejps.2019.03.027

OECD (1993) Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles. Organisation for Economic Cooperation and Development

OECD (2002) Consensus document on compositional considerations for new varieties of maize (Zea Mays): Key food and feed nutrients, anti-nutrients and secondary plant metabolites. Organisation for Economic Co-operation and Development. ENV/JM/MONO(2002)25

OECD (2019) OECD Conference on RNA interference (RNAi) based pesticides. Organisation for Economic Co-operation and Development, 2, rue André Pascal, 75016 Paris, France. 10-12 April 2019. (http://www.oecd.org/chemicalsafety/pesticides-biocides/conference-on-rnai-based-pesticides.htm)

OECD (2020) Considerations for the environmental risk assessment of the application of sprayed or externally applied ds-RNA-based pesticides. Organisation for Economic Co-operation and Development. ENV/JM/MONO(2020)26

Okuda, T. et al. (2018) Development of spray-freeze-dried siRNA/PEI powder for inhalation with high aerosol performance and strong pulmonary gene silencing activity. J. Control. Release 279, 99–113

O'Neill, M.J, Bourre L, Melgar S & O'Driscoll CM (2011) Intestinal delivery of non-viral gene therapeutics: physiological barriers and preclinical models. Drug Discov Today 16: 203-218. DOI: 10.1016/j.drudis.2011.01.0032011

Ozcan G, Ozpolat B, Coleman RL, Sood AK & Lopez-Berestein G (2015) Preclinical and clinical development of siRNA-based therapeutics. Adv Drug Deliv Rev 87: 108-119. DOI: 10.1016/j.addr.2015.01.007

Pandey, A.K. and Verma, S., 2021. An in silico analysis of effective siRNAs against COVID-19 by targeting the leader sequence of SARS-CoV-2. Advances in cell and gene therapy, 4(2), p.e107.

Pardi N, Hogan M, Porter F & Weissman D (2018) mRNA vaccines — a new era in vaccinology. Nat Rev Drug Discov 17: 261–279. DOI: 10.1038/nrd.2017.243

Park NJ, Yang Li, Yu T, Brinkman BMN & Wong DT (2006) Characterization of RNA in saliva. Clin Chem 52: 988-994. DOI: 10.1373/clinchem.2005.063206

Parker KM, Barragán Borrero V, van Leeuwen DM, Lever MA, Mateescu B & Sander M (2019) Environmental fate of RNA interference pesticides: adsorption and degradation of double-stranded RNA molecules in agricultural soils. Environ Sci Technol 53: 3027-3036. DOI: 10.1021/acs.est.8b05576

Parrott W, Chassy B, Ligon J, Meyer L, Petrick J, Zhou J, Herman R, Delaney B & Levine M (2010) Application of food and feed safety assessment principles to evaluate transgenic approaches to gene modulation in crops. Food and Chemical Toxicology 48: 1773-1790. DOI: 10.1016/j.fct.2010.04.017

Parsons, K. H., Mondal, M. H., McCormick, C. L., and Flynt, A. S. (2018). Guanidinium-functionalized interpolyelectrolyte complexes enabling RNAi in resistant insect pests. Biomacromolecules 19, 1111–1117. doi: 10.1021/acs. biomac.7b01717

Pastrello C, Tsay M, McQuaid R, Abovsky M, Pasini E, Shirdel E, Angeli M, Tokar T, Jamnik J, Kotlyar M, Jurisicova A, Kotsopoulos J, El-Sohemy A & Jurisica I (2016) Circulating plant miRNAs can regulate human gene expression in vitro. Scientific Reports 6: 32773. DOI: 10.1038/srep32773; (Published online 08 September 2016; retracted 22 May 2017; see following citation)

Pastrello C, Tsay M, McQuaid R, Abovsky M, Pasini E, Shirdel E, Angeli M, Tokar T, Jamnik J, Kotlyar M, Jurisicova A, Kotsopoulos J, El-Sohemy A & Jurisica I (2017) Retraction: Circulating plant miRNAs can regulate human gene expression in vitro. Scientific Reports 7: 46826. DOI: 10.1038/srep46826;

Patel, A.K. et al. (2019) Inhaled nanoformulated mRNA polyplexes for protein production in lung epithelium. Adv. Mater. 31, e1805116

Perez AP, Mundina-Weilenmann C, Romero EL, Morilla MJ. 2012. Increased brain radioactivity by intranasal P-labeled siRNA dendriplexes within in situ-forming mucoadhesive gels. Int J Nanomedicine 7, 1373-1385.

Perl M, Chung CS, Lomas-Neira J, Rachel TM, Biffl WL, Cioffi WG, et al. 2005. Silencing of Fas, but not caspase-8, in lung epithelial cells ameliorates pulmonary apoptosis, inflammation, and neutrophil influx after hemorrhagic shock and sepsis. Am J Pathol 167, 1545-1559.

Petrick JS, Brower-Toland B, Jackson AL & Kier LD (2013) Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: A scientific review. Regul Toxicol Pharmacol 66: 167–176. DOI: 10.1016/j.yrtph.2013.03.008

Petrick JS, Moore WM, Heydens WF, Koch MS, Sherman JH & Lemke SL (2015) A 28-day oral toxicity evaluation of small interfering RNAs and a long double-stranded RNA targeting vacuolar ATPase in mice. Regul Toxicol Pharmacol 71: 8-23. DOI: 10.1016/j.yrtph.2014.10.016

Petrick JS, Frierdich GE, Carleton SM, Kessenich CR, Silvanovich A, Zhang Y, Koch MS (2016) Corn rootworm-active RNA DvSnf7: Repeat dose oral toxicology assessment in support of human and mammalian safety. Regul Toxicol Pharmacol 81: 57-68. DOI: 10.1016/j.yrtph.2016.07.009

Pioneer Hi-Bred International, Inc (2020) Petition for the determination of nonregulated status for insect resistant and herbicide-tolerant DP23211 maize. Volume 1- Petition. Posted by the US Animal and Plant Health Inspection Service on Nov 3, 2020. [CBI-deleted copy accessed and downloaded at https://www.regulations.gov/document/APHIS-2020-0098-0002 on 18 February 2021.]

Pridgeon JW, Zhao L, Becnel JJ, Strickman DA, Clark GG & Linthicum KJ (2008) Topically applied AaeIAP1 double-stranded RNA kills female adults of Aedes aegypti. J Med Entomol 45: 414-420. DOI: 10.1093/jme/tjv192

Qin B, Liu J, Liu S, Li B, Ren J. 2016. MiR-20b targets AKT3 and modulates vascular endothelial growth factor-mediated changes in diabetic retinopathy. Acta Biochim Biophys Sin (Shanghai) 48, 732-740

Qiu, Y. et al (2016). Delivery of RNAi Therapeutics to the Airways—From Bench to Bedside. Molecules. 21, 1249.

Qiu, Y. et al. (2017). From pulmonary surfactant, synthetic KL4 peptide as effective siRNA delivery vector for pulmonary delivery. Mol. Pharm. 14, 4606–4617

Qiu, Y. et al. (2019). Effective mRNA pulmonary delivery by dry powder formulation of PEGylated synthetic KL4 peptide. J. Control. Release 314, 102–115

Ramon M, Devos Y, Lanzoni A, Liu Y, Gomes A, Gennaro A & Waigmann E (2014) Commentary: RNAi-based GM plants: food for thought for risk assessors. Plant Biotechnol J 12: 1271–1273. DOI: 10.1111/pbi.12305

Rangel G (2015) A short history of GMOs, from prehistoric times to today. Genetic Literacy Project, Science in the News, August 12, 2015. https://geneticliteracyproject.org/2015/08/12/gmos-from-ancient-history-to-the-future/ [Accessed 23 June 2021]

Rehman A, Sina C, Gavrilova O, Hasler R, Ott S, Baines JF, Schreiber S & Rosenstiel P (2011) Nod2 is essential for temporal development of intestinal microbial communities. Gut 60: 1354–1362. DOI: 10.1136/gut.2010.216259

Reich SJ, Fosnot J, Kuroki A, Tang W, Yang X, Maguire AM, Bennett J, Tolentino MJ. 2003. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Molecular Vision 9:210-6

Robbins M, Judge A & MacLachlan I (2009) siRNA and innate immunity. Oligonucleotides 19: 89–102. DOI: 10.1089/oli.2009.0180

Rodrigues TB & Petrick JS (2020) Safety considerations for humans and other vertebrates regarding agricultural uses of externally applied RNA molecules. Front. Plant Sci. 11: 407-418. DOI: 10.3389/fpls.2020.00407

Roh. et al. (2022). Inhalable mRNA vaccines for respiratory diseases: a roadmap. Current Opinion in Biotechnology. 74:104–109

Rosas-Taraco AG, Higgins DM, Sanchez-Campillo J, Lee EJ, Orme IM, Gonzalez-Juarrero M. 2009.Intrapulmonary delivery of XCL1-targeting small interfering RNA in mice chronically infected with Mycobacterium tuberculosis. Am J Respir Cell Mol Biol 41, 136-145

Roy S, Nasser S, Yee M, Graves DT. 2011. A long-term siRNA strategy regulates fibronectin overexpression and improves vascular lesions in retinas of diabetic rats. Mol Vis 17, 3166-3174.

Sahin U, Karikó K & Türeci Ö (2014) mRNA-based therapeutics - developing a new class of drugs. Nat Rev Drug Discov 13: 759–780. DOI: 10.1038/nrd4278

Salathe, M. et al (2007). Regulation of mammalian ciliary beating. Annu. Rev. Physiol. 69, 401–422.

Scott LJ & Keam SJ (2021) Lumasiran: First Approval. Drugs 81(1). DOI: 10.1007/s40265-020-01463-0

Senoo T, Hattori N, Tanimoto T, Furonaka M, Ishikawa N, Fujitaka K et al (2010) Suppression of plasminogen activator inhibitor-1 by RNA interference attenuates pulmonary fibrosis. Thorax 65: 334-340. DOI: 10.1136/thx.2009.119974

Schiroli D, Gomara MJ, Maurizi E, Atkinson SD, Mairs L, Christie KA, Cobice DF, McCrudden CM, Nesbit MA, Haro I, Moore T. 2019. Effective In Vivo Topical Delivery of siRNA and Gene Silencing in Intact Corneal Epithelium Using a Modified Cell-Penetrating Peptide. Mol Ther Nucleic Acids 17:891-906

Sherman JH, Munyikwa T, Chan SY, Petrick JS, Witwer KW & Choudhurie S (2015) Commentary: RNAi technologies in agricultural biotechnology: The Toxicology Forum 40th Annual Summer Meeting. Reg Toxicol Pharmacol 73(2): 671-680. DOI: 10.1016/j.yrtph.2015.09.001

Sioud M (2005) On the delivery of small interfering RNAs into mammalian cells. Expert Opin Drug Deliv 2: 639-651. DOI: 10.1517/17425247.2.4.639

Sioud M (2015) Overcoming the challenges of siRNA activation of innate immunity: Design better therapeutic siRNAs. In: RNA interference: Challenges and therapeutic opportunities (1st ed, Vol. 1218, pp. 304–305. New York: Humana Press.

Smith CIE & Zain R (2019) Review: Therapeutic oligonucleotides: State of the art. Annu Rev Pharmacol Toxicol 59: 605–630. DOI: 10.1146/annurev-pharmtox-010818-021050

Snow JW, Hale A, Isaacs SK, Baggish AL & Chan SY (2013) Ineffective delivery of diet-derived microRNAs to recipient animal organisms. RNA Biology 10: 1107-1116. DOI: 10.4161/rna.24909

Soriano V, Barreiro P, Cachay E, Kottilil S, Fernandez-Montero JV & de Mendoza C (2020) Advances in hepatitis B therapeutics. Ther Adv Infectious Dis 7: 1–11. DOI: 10.1177/2049936120965027

Sorrentino S, Naddeo M, Russo A & D'Alessio G (2003) Degradation of double-stranded RNA by human pancreatic ribonuclease: crucial role of noncatalytic basic amino acid residues. Biochem 42: 10182–10190. DOI: 10.1021/bi030040q

Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M et al (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432: 173-178. DOI: 10.1038/nature03121

Spisák S, Solymosi N, Ittzés P, Bodor A, Kondor D G. Vattay DG, Barták BK et al. (2013) Complete genes may pass from food to human blood. PLoS One 8(7): e69805. DOI: 10.1371/journal.pone.0069805

Srivastava A, Nikamo P, Lohcharoenkal W, Li D, Meisgen F, Xu Landen N, et al. 2017. MicroRNA-146a suppresses IL-17-mediated skin inflammation and is genetically associated with psoriasis. J Allergy Clin Immunol 139, 550-561

Stein CA & Castanotto D (2017) Review: FDA-Approved Oligonucleotide Therapies in 2017. Mol Ther 25(5): 1069-1075. DOI: 10.1016/j.ymthe.2017.03.023.

Study to Evaluate the Safety and Tolerability of MRT5005 Administered by Nebulization in Adults with Cystic Fibrosis. ClinicalTrials.gov, https://clinicaltrials.gov/ct2/show/NCT03375047?term=mrt5005.

Sullenger BA & Nair S (2016) From the RNA world to the clinic. Science 352(6292): 1417-1420. DOI: 10.1126/science.aad8709

Suhr OB, Coelho T, Buades J, Pouget J, Conceicao I, Berk J, Schmidt H, Waddington-Cruz M, Campistol JM, Bettencourt BR, Vaishnaw A, Gollob J & Adams D (2015) Efficacy and safety of patisiran for familial amyloidotic polyneuropathy: a phase II multi-dose study. Orphanet J Rare Dis 10, 109- DOI: 10.1186/s13023-015-0326-6

Sun CQ & Ou Y (2018) New Developments in the medical treatment of glaucoma. Curr Ophthalmol Rep 6: 73–78. DOI: 10.1007/s40135-018-0166-3

Tavitian B, Terrazzino S, Kuhnast B, Marzabal S, Stettler O, Dolle F, et al. 1998. In vivo imaging of oligonucleotides with positron emission tomography. Nat Med 4, 467-471.Winkler J, Stessl M, Amartey J, Noe CR. 2010. Off-target effects related to the phosphorothioate modification of nucleic acids. ChemMedChem 5, 1344-1352.

Thanki K, Blum KG, Thakur A, Rose F & Foged C (2018) Formulation of RNA interference-based drugs for pulmonary delivery: challenges and opportunities. Ther Deliv 9: 731-749. DOI: 10.4155/tde-2018-0029

Tiwari, P.M. et al. (2018). Engineered mRNA-expressed antibodies prevent respiratory syncytial virus infection. Nat. Commun. 9, 3999

Thompson JD, Kornbrust DJ, Foy JW-D, Solano ECR, Schneider DJ, Feinstein E, Molitoris BA & Erlich S (2012) Toxicological and pharmacokinetic properties of chemically modified siRNAs targeting p53 RNA following intravenous administration. Nucleic Acid Ther 22: 255-264. DOI: 10.1089/nat.2012.0371

Tseng Y-C, Mozumdar S & Huang L (2009) Lipid-based systemic delivery of siRNA. Adv Drug Deliv Rev 61: 721-731. DOI: 10.1016/j.addr.2009.03.003

Turchinovich A, Zoidl G, Dermietzel R. 2010. Non-viral siRNA delivery into the mouse retina in vivo. BMC Ophthalmol 10, 25.

Turner CT, Davy MW, MacDiarmid RM, Plummer KM, Birch NP & Newcomb RD (2006) RNA interference in the light brown apple moth, Epiphyas postvittana (Walker) induced by double-stranded RNA feeding. Insect Mol Biol 15: 383–391. DOI: 10.1111/j.1365-2583.2006.00656.x

Ui-Tei, K., Naito, Y., Nishi, K., Juni, A. and Saigo, K., 2008. Thermodynamic stability and Watson–Crick base pairing in the seed duplex are major determinants of the efficiency of the siRNA-based off-target effect. Nucleic acids research, 36(22), pp.7100-7109.

Ui-Tei, K., 2013. Optimal choice of functional and off-target effect-reduced siRNAs for RNAi therapeutics. Frontiers in Genetics, 4, p.107.

Urgard E, Lorents A, Klaas M, Padari K, Viil J, Runnel T, et al. 2016. Pre-administration of PepFect6-microRNA-146a nanocomplexes inhibits inflammatory responses in keratinocytes and in a mouse model of irritant contact dermatitis. J Control Release 235, 195-204.

USEPA (2004) Potential implications of genomics for regulatory and risk assessment applications at EPA. Science Policy Council, US Environmental Protection Agency, Washington DC20460. EPA 100/B 04/002, December 2004

USEPA (2013) White paper on RNAi technology as a pesticide: Problem formulation for human health and ecological risk assessment. (Submitted to the FIFRA Science Advisory Panel for review on comment. October 29, 2013) Downloadable at https://www.regulations.gov/document/EPA-HQ-OPP-2013-0485-0011

USEPA (2014) A set of scientific issues being considered by the Environmental Protection Agency regarding: RNAi Technology: Program formulation for human health and ecological risk assessment. Scientific Advisory Panel Minutes No. 2014-02 (Arlington, VA), 1–77. Available online at: https://www.epa.gov/sites/production/files/2015-06/documents/012814minutes.pdf

USEPA (2016a) FIFRA Scientific Advisory Panel Minutes No. 2016-02. A set of scientific issues being considered by the Environmental Protection Agency regarding: RNAi technology: Human health and ecological risk assessments for SmartStax PRO. September 27-28, 2016. FIFRA Scientific Advisory Panel Meeting, EPA Conference Center, One Potomac Yard Arlington, Virginia

USEPA (2016b) Human Health Risk Assessment: Review of Product Characterization and Protein Expression Analysis Data in support for a Sec. 3 Registration of Combination Plant-Incorporated Protectant (PIP): MON 89034 x TC 1507 x MON 8741 l x DAS-59122-7 20% structured refuge product [EPA Reg. No. 524-AGE] and MON 89034 x TC 1507 x MON 87411xDAS-59122-79515% Seed Blend [EPA Reg. No. 524-AGR]; submitted by Monsanto Company. (Report accessed at https://www.regulations.gov/document/EPA-HQ-OPP-2014-0293-0401 on 16 June 2021.)

USEPA (2017) Proposed registration decision for commercial use corn products containing the DvSnf7 dsRNA plant-incorporated protectant (Event MON 87411). Downloadable at https://www.regulations.gov/document?D=EPA-HQ-OPP-2014-0293-0406

US-FDA (2006) Guidance for industry: Questions and answers regarding food allergens, including the Food Allergen Labeling and Consumer Protection Act of 2004 (Edition 4). Docket No. FDA-2005-D-0227. Downloadable at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-questions-and-answers-regarding-food-allergens-edition-4

US-FDA, 2018. Center for Drug Evaluation and Research. Application number 210922Orig1s000

US-FDA, 2020. Center for Drug Evaluation and Research. Application number 214103Orig1s000

Vaishnaw AK, Gollob J, Gamba-Vitalo C, Hutabarat R, Sah D, Meyers R, de Fougerolles T & Maraganore J (2010) A status report on RNAi therapeutics. Silence 1: 14. DOI: 10.1186/1758-907X-1-14

Wahle BS (2014) A 28-day oral study in the mouse with DvSnf7_968 RNA. MRID Number 49505804. Monsanto Company, St. Louis, Missouri.

Wang K, Li H, Yuan Y, Etheridge A, Zhou Y, Huang D et al. (2012) The complex exogenous RNA spectra in human plasma: an interface with human gut biota? PLoS One 7(12): e51009. DOI: 10.1371/journal.pone.0051009

Weissman D (2015) mRNA transcript therapy, Expert Rev Vaccines 14(2): 265-281. DOI: 10.1586/14760584.2015.973859

Whitehead KA, Dahlman JE, Langer RS & Anderson DG (2011) Silencing or Stimulation? siRNA delivery and the immune system. Annu Rev Chem Biomol Eng 2: 77-96. DOI: 10.1146/annurev-chembioeng-061010-114133

Witwer KW (2015) Contamination or artifacts may explain reports of plant miRNAs in humans. J Nutr Biochem 26: 1685. DOI: 10.1016/j.jnutbio.2015.09.004

Witwer KW & Halushka MK (2016) Towards the promise of microRNAs - Enhancing reproducibility and rigor in microRNA research. RNA Biol 13(11): 1103-1116. DOI: 10.1080/15476286.2016.1236172

Witwer KW & Hirschi KD (2014) Transfer and functional consequences of dietary microRNAs in vertebrates: concepts in search of corroboration. Bioessays 36: 394-406. DOI: 10.1002/bies.201300150

Witwer KW, McAlexander MA, Queen SE & Adams RJ (2013) Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs: Limited evidence for general uptake of dietary plant xenomiRs. RNA Biology 10: 1080-1086. DOI: 10.4161/rna.25246

Xiao B, Laroui H, Viennois E, Ayyadurai S, Charania MA, Zhang Y, et al. 2014. Nanoparticles with surface antibody against CD98 and carrying CD98 small interfering RNA reduce colitis in mice. Gastroenterology 146, 1289-1300 e1281-1219.

Xu, S. et al. (2020). Blockade of endothelial, but not epithelial, cell expression of Pd-L1 following severe shock attenuates the development of indirect acute lung injury in mice. Am. J. Physiol. Lung Cell Mol. Physiol. 318, L801–L812

Yang C & Yang B (2016) siRNA-Induced RNAi therapy in acute kidney injury. In: Biochemistry, Genetics and Molecular Biology: RNA Interference. Chapter 11. Abdurakhmonov IY (Ed). ISBN 978-953-51-2272-2. DOI: 10.5772/61838 https://www.intechopen.com/books/rna-interference/sirna-induced-rnai-therapy-in-acute-kidney-injury

Youngren-Ortiz SR, Gandhi NS, España-Serrano L & Chougule MB (2017) Aerosol delivery of siRNA to the lungs. Part 2: Nanocarrier-based delivery systems. Kona 34: 44-69. DOI: 10.14356/kona.2017005

Yuen M-F, Schiefke I, Yoon J-H, Ahn SH, Heo J, Kim JH, Chan HLY, Yoon KT, Klinker H, Manns M, Petersen J, Schluep T, Hamilton J, Given BD, Ferrari C, Lai C-L, Locarnini SA & Gish RG (2020) RNA Interference therapy with ARC-520 results in prolonged Hepatitis B surface antigen response in patients with chronic Hepatitis B infection. Hepatol 72(1): 19-31. DOI: 10.1002/hep.31008

Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y et al (2012) Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res 22(1): 107–26. DOI: 10.1038/cr.2011.158

Zhang L, Jiang J, Xia X. 2014. Suppression of retinal neovascularization by small interfering RNA targeting PGC-1alpha. Int J Mol Med 33, 1523-1530.

Zhang H, Demirer GS, Zhang H, Ye T, Goh NS, Aditham AJ, Cunningham FJ, Fan C, Landry MP (2019) DNA nanostructures coordinate gene silencing in mature plants. Proc Natl Acad Sci USA 116 (5): 7543-7548

Zhang, Y., Cui, J., Zhou, Y., Cao, J., Gong, H., Zhang, H., et al. (2018). Liposome mediated double-stranded RNA delivery to silence ribosomal protein P0 in the tick Rhipicephalus haemaphysaloides. Ticks Tick Borne Dis. 9, 638–644. doi: 10.1016/j.ttbdis.2018.01.015

Zhou R, Wei H, Sun R & Tian Z (2007) Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice. J Immunol 178: 4548–4556. DOI: 10.4049/jimmunol.178.7.4548

Zhou XH, Dong Y, Xiao X, Wang Y, Xu Y, Xu B, et al. 2011. A 90-day toxicology study of high-amylose transgenic rice grain in Sprague-Dawley rats. Food Chem Toxicol 49, 3112-3118.

Zhou XH, Dong Y, Zhao YS, Xiao X, Wang Y, He YQ, et al. 2014. A three generation reproduction study with Sprague-Dawley rats consuming high-amylose transgenic rice. Food Chem Toxicol 74, 20-27.

Zhuang X, Teng Y, Samykutty A, Mu J, Deng Z, Zhang L, et al. 2016. Grapefruit-derived Nanovectors Delivering Therapeutic miR17 Through an Intranasal Route Inhibit Brain Tumor Progression. Mol Ther 24, 96-105.

Zotti M, Dos Santos EA, Cagliari D, Christiaens O, Taning CNT & Smagghe G (2018) RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. Pest Manag Sci 74: 1239–1250. DOI: 10.1002/ps.4813

Zuckerman JE, Gritli I, Tolcher A, Heidel JD, Lim D, Morgan R, et al. 2014. Correlating animal and human phase Ia/Ib clinical data with CALAA-01, a targeted, polymer-based nanoparticle containing siRNA. Proc Natl Acad Sci U S A 111, 11449-11454

New references cited since the OECD ERA document

Note: The following subset of references lists new and additional publications which have been accessed since the first document, Considerations for the Environmental Risk Assessment of the Application of

Sprayed or Externally Applied ds-RNA-Based Pesticides, was published. Note that all these references are included in the comprehensive reference list (above).

Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV et al (2018) Patisiran, an RNAi therapeutic, for hereditary Transthyretin Amyloidosis. N Engl J Med 379(1): 11-21. DOI: 10.1056/NEJMoa1716153

Arpaia S, Christiaens O, Giddings K, Jones H, Mezzetti B, Moronta-Barrios F, Perry JN, Sweet J, Taning CN, Smagghe G & Dietz-Pfeilstetter A (2020) Biosafety of GM crop plants expressing dsRNA: Data requirements and EU regulatory considerations. Frontiers in Plant Science 11: Article 940 (13 pp). DOI: 10.3389/fpls.2020.00940

Auer C & Frederick R (2009) Crop improvement using small RNAs: Applications and predictive ecological risk assessments. Trends Biotechnol 27(11): 644-651 DOI: 10.1016/j.tibtech.2009.08.005

Badri P, Jiang X, Borodovsky A, Najafian N, Kim J, Clausen VA, Goel V, Habtemariam B & Robbie GJ (2020) Pharmacokinetic and pharmacodynamic properties of Cemdisiran, an RNAi therapeutic targeting Complement Component 5, in healthy subjects and patients with Paroxysmal Nocturnal Hemoglobinuria. Clin Pharmacokinet (13 Oct 2020). DOI: 10.1007/s40262-020-00940-9.

Balwani M, Sardh E, Ventura P, Peiró PA, Rees DC, Stölzel U et al (2020) Phase 3 trial of RNAi therapeutic Givosiran for Acute Intermittent Porphyria. N Engl J Med 382: 2289-2301. DOI: 10.1056/NEJMoa1913147

Burnett JC, Rossi JJ & Tiemann K (2011) Current progress of siRNA/shRNA therapeutics in clinical trials. Biotechnol J 6(9): 1130–1146. DOI: 10.1002/biot.201100054

Burnett JC & Rossi JJ (2012) RNA-Based therapeutics: Current progress and future prospects. Chem Biol 19(1): 60-71. DOI: 10.1016/j.chembiol.2011.12.008

Cervantes A, Alsina M, Tabernero J, Infante JR, LoRusso P, Shapiro G et al (2011) Phase I dose-escalation study of ALN-VSP02, a novel RNAi therapeutic for solid tumors with liver involvement. J Clin Oncol 29 (15_suppl): 3025. DOI: 10.1200/jco.2011.29.15_suppl.3025

Chalbatani GM, Dana H, Gharagouzloo E, Grijalvo S, Eritja R, Logsdon CD, Memari F, Miri SR, Rad MR & Marmari V (2019) Small interfering RNAs (siRNAs) in cancer therapy: a nano-based approach. Int J Nanomedicine 14: 3111–3128. DOI: 10.2147/IJN.S200253

Chassy B, Hlywka JJ, Kleter GA, Kok EJ, Kuiper HA, McGloughlin M, Munro IC, Phipps RH & Reid JE (2004) Nutritional and safety assessments of foods and feeds nutritionally improved through biotechnology. Compr Rev Food Sci Food Saf 3: 38 - 104.

Chen C, Yang Z & Tang X (2018) Chemical modifications of nucleic acid drugs and their delivery systems for gene-based therapy. Med Res Rev 38: 829-869. DOI: 10.1002/med.21479

Chi X, Gatti P & Papoian T (2017) Safety of antisense oligonucleotide and siRNA-based therapeutics. Drug Discovery Today 22(5): 823-833. DOI: 10.1016/j.drudis.2017.01.013

Christensen J, Litherland K, Faller T, van de Kerkhof E, Natt F, Hunziker J et al. (2014) Biodistribution and metabolism studies of lipid nanoparticle-formulated internally [3H]-labeled siRNA in mice. Drug Metab Dispos 42(3): 431-440. DOI: 10.1124/dmd.113.055434

Codex Alimentarius Commission (2008) Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants. Codex Alimentarius, CAC/GL 45-2003

Cruz C, Tayler A & Whyard S (2018) RNA interference-mediated knockdown of male fertility genes in the Queensland Fruit Fly Bactrocera tryoni (Diptera: Tephritidae). Insects 9: 96 (13 pp). DOI: 10.3390/insects9030096

Dalakouras A, Wassenegger M, Dadami E, GanopoulosI, Pappas ML & Papadopoulou K (2020) Genetically Modified Organism-Free RNA Interference: Exogenous Application of RNA Molecules in Plants. Plant Physiology 182: 38–50. DOI: 10.1104/pp.19.

DEEM/FCID (2018) Dietary Exposure Evaluation Model – Food Commodity Intake Database, Version 4.02, Durango Software, LLC. United States Environmental Protection Agency, https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/deemfcidcalendex-software-installer

Dong K, Sun L, Liu JT, Gu S-H, Zhou JJ, Yang RN et al. (2017) RNAi-induced electrophysiological and behavioral changes reveal two pheromone binding proteins of Helicoverpa armigera Involved in the perception of the main sex pheromone component Z11–16:Ald. J Chem Ecol 43: 207-214. DOI: 10.1007/s10886-016-0816-6

Dyrbuś K, Gąsior M, Penson P, Ray KK & Banach M (2020) Inclisiran—New hope in the management of lipid disorders? J Clin Lipidol 14(1): 16-27. DOI: 10.1016/j.jacl.2019.11.001

EFSA Panel on Genetically Modified Organisms (GMO) (2011) Guidance for risk assessment of food and feed from genetically modified plants. EFSA J 9: 2150 (37 pp). DOI: 10.2903/j.efsa.2011.2150

EFSA Panel on Genetically Modified Organisms (GMO) (2019) Assessment of genetically modified maize MON 87427 x MON 89034 x MIR162 x MON 87411 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2017-144). EFSA Journal 17(11): 5848 33pp. DOI: 10.2903/j.efsa.2019.5848

FAO/WHO (1996) Biotechnology and food safety. Report of a Joint FAO/WHO Consultation, Rome, Italy, 30 Sept - 4 Oct 1996, Paper No. 61FAO/WHO (2000)

FAO/WHO (2000) Safety aspects of genetically modified foods of plant origin: Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, 29 May – 2 June 2000. World Health Organization, Geneva

FAO/WHO (2001) Safety assessment of foods derived from genetically modified microorganisms. Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology. Food & Agriculture Organization of the United Nations, World Health Organization

Flisiak R, Jaroszewicz J & Łucejko M (2018) siRNA drug development against hepatitis B virus infection, Expert Opin Biol Ther 18(6); 609-617. DOI: 10.1080/14712598.2018.1472231

Fortunato O & Iorio MV (2020) The Therapeutic potential of microRNAs in cancer: Illusion or opportunity? Pharmaceuticals (Basel) 13(12): 438. DOI: 10.3390/ph13120438

Foss FM, Querfeld C, Zain J, Pinter-Brown LC, Joyce R, Moskowitz A et al (2019) Phase I trial of cobomarsen, a mir-155 inhibitor, in HTLV-1 associated adult T cell lymphoma/leukemia. miRagen Therapeutics, Inc. poster, downloadable at http://www.viridiantherapeutics.com/publications/2019/01/Phase-I-Trial-of-Cobomarsen-a-MiR-155-Inhibitor-in-HTLV-1-Associated-Adult-T-cell-Lymphoma-Leukemia.pdf (accessed on 22 February 2021)

FSANZ (2013) Response to Heinemann et al on the regulation of GM crops and foods developed using gene silencing. Food Standards Australia New Zealand, http://www.foodstandards.gov.au/consumer/gmfood/Documents/Heinemann%20Response%20210513.pdf

FSANZ (2020) Safety assessment – Application A1202: Food derived from herbicide-tolerant and insect-protected corn line DP23211. Food Standards Australia New Zealand, 1 October 2020 [136-20] Supporting document 1, 48 pp. A1202 SD1.pdf (foodstandards.govt.nz (Accessed on 21 June 2021)

Goldstein DA, Shelton PE, Cullen MR, Easterday PA, Eppard PJ & Cabanilla BR (2004) Responding to the challenge of novel technology: An industrial hygiene and safety program for antibody production in maize. J Occup Env Med: 46(8): 784-790. DOI: 10.1097/01.jom.0000135691.69649.f8

Gonzalez V, Sadaba B, Moreno-Montanes J, Ruz V, Paneda C & Jimenez AI (2015) Pharmacokinetics and biodistribution of bamosiran in human and animal model, a novel treatment for glaucoma. Invest Ophthalmol Vis Sci 56: 5748. [Abstract, Annual Meeting of the Association for Research in Vision & Ophthalmology (ARVO), Annual Meeting Abstract. June 2015]

Gupta SK, Haigh BJ, Griffin FJ & Wheeler TT (2012) The mammalian secreted RNases: Mechanisms of action in host defence. Innate Immun 19(1): 86–97. DOI: 10.1177/1753425912446955

Habtemariam BA, Karsten V, Attarwala H, Goel V, Melch M, Clausen VA et al. (2020) Single-dose pharmacokinetics and pharmacodynamics of transthyretin targeting N-acetylgalactosamine–small interfering ribonucleic acid conjugate, vutrisiran, in healthy subjects. Clin Pharmacol Ther 109(2): 372-382. DOI: 10.1002/cpt.1974

Hill A, Valls AG, Griffin M, Munir T, Borodovsky A, Kawahata N et al. (2016) A subcutaneously administered investigational RNAi therapeutic (ALN-CC5) targeting complement C5 for treatment of PNH and complement-mediated diseases: preliminary phase 1/2 study results in patients with PNH. Blood 128: 3891. DOI: 10.1182/blood.V128.22.3891.3891

Hu B, Weng Y, Xia X-H, Liang X-J & Huang Y (2019) Clinical advances of siRNA therapeutics. J Gene Med e3097. DOI: 10.1002/jgm.3097

Hu B, Zhong L, Weng Y, Peng L, Huang Y, Zhao Y & Liang X-J (2020) Therapeutic siRNA: state of the art. Signal Transduct Target Ther 5: 101. DOI: 10.1038/s41392-020-0207-x

Hu X, Steimel JP, Kapka-Kitzman DM, Davis-Vogel C, Richtman NM, Mathis JP, Nelson ME, Lu AL & Wu G (2019) Molecular characterization of the insecticidal activity of double-stranded RNA targeting the smooth septate junction of western corn rootworm (Diabrotica virgifera virgifera). PLoS ONE 14(1): e0210491. DOI: 10.1371/journal.pone.0210491

Hu Z, Boeckman CJ, Cong B, Steimel JP, Richtman NM, Sturtz K, Wang Y, Walker CL, Yin J, Unger A, Farris C & Lu AL (2020) Characterization of DvSSJ1 transcripts targeting the smooth septate junction (SSJ) of western corn rootworm (Diabrotica virgifera virgifera). Sci Rep 10(11139): 1-13. DOI: 10.1038/s41598-020-68014-1

Huang H, Davis CD & Wang TTY (2018) Extensive degradation and low bioavailability of orally consumed corn miRNAs in mice. Nutrients 10: 215-227. DOI: 10.3390/nu10020215

Jackson NAC, Kester KE, Casimiro D, Gurunathan S & DeRosa F (2020) The promise of mRNA vaccines: a biotech and industrial perspective. NPJ Vaccines 5: 11. DOI: 10.1038/s41541-020-0159-8

Jonas DA, Antignac E, Antoine JM, Classen HG, Huggett A, Knudsen I, Mahler J, Ockhuizen T, Smith M, Teuber M, Walker R & De Vogel P (1996) The safety assessment of novel foods: Guidelines prepared by ILSI Europe Novel Food Task Force. Food Chem Toxicol 34: 931-940.

Juliano RL, Ming X & Nakagawa O (2012) Cellular uptake and intracellular trafficking of antisense and siRNA oligonucleotides. Bioconjug Chem 23(2): 147−157. DOI: 10.1021/bc200377d

Kausik K, Ray KK, Wright RS, Kallend D, Koenig W, Leiter LA et al. (2020) Two Phase 3 trials of Inclisiran in patients with elevated LDL cholesterol. N Engl J Med 382: 1507-1519. DOI: 10.1056/NEJMoa1912387

Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi JZ, Albuquerque RJ, Yamasaki S, Itaya M, Pan Y, Appukuttan B, Gibbs D, Yang Z, Karikó K, Ambati BK, Wilgus TA, DiPietro LA, Sakurai E, Zhang K, Smith JR, Taylor EW & Ambati J (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 452(7187): 591-7. DOI: 10.1038/nature06765

Kroes R, Kleiner J, Renwick A (2005) The Threshold of Toxicological Concern concept in risk assessment. Toxicol Sci 86: 226-230. DOI: 10.1093/toxsci/kfi169

Langman CB, Ariceta G, Barrios K, Brown BD, Hoppe B, Rosskamp R (2021) Hepatic lactate dehydrogenase A is an RNA interference target for the treatment of all known types of primary hyperoxaluria. Kidney Int Rep (2021). DOI: 10.1016/j.ekir.2021.01.029

Ledford H (2016) Investors flee as firm scraps RNA-interference drug candidate Struggling RNAi field faces another blow as its first treatments near the clinic. Nature Trend Watch 10 October 2016. DOI: 10.1038/nature.2016.20769

Levanova A & Poranen MM (2018) RNA Interference as a prospective tool for the control of human viral infections. Front Microbiol DOI: 10.3389/fmicb.2018.02151

Li M, Chen T, Wang R, Luo JY, He JJ, Ye RS et al (2019) Plant MIR156 regulates intestinal growth in mammals by targeting the Wnt/beta-catenin pathway. Am J Physiol Cell Physiol 317: C434–C448. DOI: 10.1152/ajpcell.00030.2019

Link J, Thon C, Schanze D, Steponaitiene R, Kupcinskas J, Zenker M, Canbay A, Malfertheiner P & Link A (2019) Food-derived Xeno-microRNAs: Influence of diet and detectability in gastrointestinal tract—proof-of-principle study. Mol Nutr Food Res 63: 1800076 (1-11). DOI: 10.1002/mnfr.201800076

Liu R, Mu L-M, Bai J, Du Y-F, Xie Y & Lu W-L (2019) Development of double strand RNA mPEI nanoparticles and application in treating invasive breast cancer. RSC Adv 9: 13186-13200. DOI: 10.1039/c9ra01889a

Lopatin U (2019) Drugs in the Pipeline for HBV. Clin Liver Dis 23: 535–555. DOI: 10.1016/j.cld.2019.04.006

Luscombe NM, Greenbaum D & Gerstein M (2001) What is bioinformatics? A proposed definition and overview of the field. Methods Inf Med 40(4): 346-58.

Lusk RW (2014) Diverse and widespread contamination evident in the unmapped depths of high throughput sequencing data. PLoS ONE 9(10): e110808. DOI: 10.1371/journal.pone.0110808

Machin N & Ragni MV (2018) An investigational RNAi therapeutic targeting antithrombin for the treatment of hemophilia A and B. J Blood Med 9: 135–140. DOI: 10.2147/JBM.S159297

McNeill EM & Hirschi KD (2020) Roles of regulatory RNAs in nutritional control. Annu Rev Nutr 40: 77-104. DOI: 10.1146/annurev-nutr-122319-035633

Mertens JJWM (2013) A 90-day feeding study in Sprague Dawley rats with grain from MON 87411 corn. MRID Number 49505806. Monsanto Company, St. Louis, Missouri.

Mezzetti B, Smagghe G, Arpaia, S, Christiaens O, Dietz-Pfeilstetter A, Jones H et al. (2020) RNAi: What is its position in agriculture? J Pest Sci 93: 1125–1130. DOI: 10.1007/s10340-020-01238-2

O'Driscoll CM, Bernkop-Schnürch A, Friedl JD, Préat V & Jannin V (2019) Oral delivery of non-viral nucleic acid-based therapeutics - do we have the guts for this? Eur J Pharm Sci 133: 190–204. DOI: 10.1016/j.ejps.2019.03.027

OECD (1993) Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles. Organisation for Economic Cooperation and Development

OECD (2002) Consensus document on compositional considerations for new varieties of maize (Zea Mays): Key food and feed nutrients, anti-nutrients and secondary plant metabolites. Organisation for Economic Co-operation and Development, ENV/JM/MONO(2002)25

OECD (2019) OECD Conference on RNA interference (RNAi) based pesticides. Organisation for Economic Co-operation and Development, 2, rue André Pascal, 75016 Paris, France. 10-12 April 2019. (http://www.oecd.org/chemicalsafety/pesticides-biocides/conference-on-rnai-based-pesticides.htm)

OECD (2020) Considerations for the environmental risk assessment of the application of Externally or Applied or externally applied ds-RNA-based pesticides. Organisation for Economic Co-operation and Development. ENV/JM/MONO(2020)26Tseng

Ozcan G, Ozpolat B, Coleman RL, Sood AK & Lopez-Berestein G (2015) Preclinical and clinical development of siRNA-based therapeutics. Adv Drug Deliv Rev 87: 108-119. DOI: 10.1016/j.addr.2015.01.007

Pardi N, Hogan M, Porter F & Weissman D (2018) mRNA vaccines — a new era in vaccinology. Nat Rev Drug Discov 17: 261–279. DOI: 10.1038/nrd.2017.243

Parker KM, Barragán Borrero V, van Leeuwen DM, Lever MA, Mateescu B & Sander M (2019) Environmental fate of RNA interference pesticides: adsorption and degradation of double-stranded RNA molecules in agricultural soils. Environ Sci Technol 53: 3027-3036. DOI: 10.1021/acs.est.8b05576

Parrott W, Chassy B, Ligon J, Meyer L, Petrick J, Zhou J, Herman R, Delaney B & Levine M (2010) Application of food and feed safety assessment principles to evaluate transgenic approaches to gene modulation in crops. Food and Chemical Toxicology 48: 1773-1790. DOI: 10.1016/j.fct.2010.04.017

Pioneer Hi-Bred International, Inc (2020) Petition for the determination of nonregulated status for insect resistant and herbicide-tolerant DP23211 maize. Volume 1- Petition. Posted by the US Animal and Plant Health Inspection Service on Nov 3, 2020. [CBI-deleted copy accessed and downloaded at https://www.regulations.gov/document/APHIS-2020-0098-0002]

Rangel G (2015) A short history of GMOs, from prehistoric times to today. Genetic Literacy Project, Science in the News, August 12, 2015. https://geneticliteracyproject.org/2015/08/12/gmos-from-ancient-history-to-the-future/ [Accessed 23 June 2021]

Rodrigues TB & Petrick JS (2020) Safety considerations for humans and other vertebrates regarding agricultural uses of externally applied RNA molecules. Front. Plant Sci. 11: 407-418. DOI: 10.3389/fpls.2020.00407

Sahin U, Karikó K & Türeci Ö (2014) mRNA-based therapeutics - developing a new class of drugs. Nat Rev Drug Discov 13: 759–780. DOI: 10.1038/nrd4278

Scott LJ & Keam SJ (2021) Lumasiran: First Approval. Drugs 81(1). DOI: 10.1007/s40265-020-01463-0

Smith CIE & Zain R (2019) Review: Therapeutic oligonucleotides: State of the art. Annu Rev Pharmacol Toxicol 59: 605–630. DOI: 10.1146/annurev-pharmtox-010818-021050

Spisák S, Solymosi N, Ittzés P, Bodor A, Kondor D G. Vattay DG, Barták BK et al. (2013) Complete genes may pass from food to human blood. PLoS One 8(7): e69805. DOI: 10.1371/journal.pone.0069805

Stein CA & Castanotto D (2017) Review: FDA-Approved Oligonucleotide Therapies in 2017. Mol Ther 25(5): 1069-1075. DOI: 10.1016/j.ymthe.2017.03.023.

Soriano V, Barreiro P, Cachay E, Kottilil S, Fernandez-Montero JV & de Mendoza C (2020) Advances in hepatitis B therapeutics. Ther Adv Infectious Dis 7: 1–11. DOI: 10.1177/2049936120965027

Suhr OB, Coelho T, Buades J, Pouget J, Conceicao I, Berk J, Schmidt H, Waddington-Cruz M, Campistol JM, Bettencourt BR, Vaishnaw A, Gollob J & Adams D (2015) Efficacy and safety of patisiran for familial amyloidotic polyneuropathy: a phase II multi-dose study. Orphanet J Rare Dis 10, 109- DOI: 10.1186/s13023-015-0326-6

Sun CQ & Ou Y (2018) New Developments in the medical treatment of glaucoma. Curr Ophthalmol Rep 6: 73–78. DOI: 10.1007/s40135-018-0166-3

Thanki K, Blum KG, Thakur A, Rose F & Foged C (2018) Formulation of RNA interference-based drugs for pulmonary delivery: challenges and opportunities. Ther Deliv 9: 731-749. DOI: 10.4155/tde-2018-0029

Tseng Y-C, Mozumdar S & Huang L (2009) Lipid-based systemic delivery of siRNA. Adv Drug Deliv Rev 61: 721-731. DOI: 10.1016/j.addr.2009.03.003

USEPA (2004) Potential implications of genomics for regulatory and risk assessment applications at EPA. Science Policy Council, US Environmental Protection Agency, Washington DC20460. EPA 100/B 04/002, December 2004

USEPA (2016b) Human Health Risk Assessment: Review of Product Characterization and Protein Expression Analysis Data in support for a Sec. 3 Registration of Combination Plant-Incorporated Protectant (PIP): MON 89034 x TC 1507 x MON 8741 l x DAS-59122-7 20% structured refuge product [EPA Reg. No. 524-AGE] and MON 89034 x TC 1507 x MON 87411xDAS-59122-79515% Seed Blend [EPA Reg. No. 524-AGR]; submitted by Monsanto Company.

US-FDA (2006) Guidance for industry: Questions and answers regarding food allergens, including the Food Allergen Labeling and Consumer Protection Act of 2004 (Edition 4). Docket No. FDA-2005-D-0227. Downloadable at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-questions-and-answers-regarding-food-allergens-edition-4

Wahle BS (2014) A 28-day oral study in the mouse with DvSnf7_968 RNA. MRID Number 49505804. Monsanto Company, St. Louis, Missouri.

Wang K, Li H, Yuan Y, Etheridge A, Zhou Y, Huang D et al. (2012) The complex exogenous RNA spectra in human plasma: an interface with human gut biota? PLoS One 7(12): e51009. DOI: 10.1371/journal.pone.0051009

Weissman D (2015) mRNA transcript therapy, Expert Rev Vaccines 14(2): 265-281. DOI: 10.1586/14760584.2015.973859

Witwer KW (2015) Contamination or artifacts may explain reports of plant miRNAs in humans. J Nutr Biochem 26: 1685. DOI: 10.1016/j.jnutbio.2015.09.004

Witwer KW & Halushka MK (2016) Towards the promise of microRNAs - Enhancing reproducibility and rigor in microRNA research. RNA Biol 13(11): 1103-1116. DOI: 10.1080/15476286.2016.1236172

Youngren-Ortiz SR, Gandhi NS, España-Serrano L & Chougule MB (2017) Aerosol delivery of siRNA to the lungs. Part 2: Nanocarrier-based delivery systems. Kona 34: 44-69. DOI: 10.14356/kona.2017005

Yuen M-F, Schiefke I, Yoon J-H, Ahn SH, Heo J, Kim JH et al (2020) RNA interference therapy with ARC-520 results in prolonged Hepatitis B surface antigen response in patients with chronic Hepatitis B infection. Hepatol 72(1): 19-31. DOI: 10.1002/hep.31008

Zotti M, Dos Santos EA, Cagliari D, Christiaens O, Taning CNT & Smagghe G (2018) RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. Pest Manag Sci 74: 1239–1250. DOI: 10.1002/ps.4813

9. Glossary and Abbreviations

A more extensive list of terms can be found in Section 12 of ENV/JM/MONO(2020)26.

ADME – absorption, distribution, metabolism and elimination; refers to the 4 key elements of pharmacokinetics.

ALAT - alanine aminotransferase

ApoB – Apolipoprotein B

Argonaute – proteins of the Argonaute family are essential components of the RNA-induced silencing complex (RISC). Argonaute proteins bind different classes of small non-coding RNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs). Small RNAs guide Argonaute proteins to their specific targets through sequence complementarity, leading to mRNA cleavage or translation inhibition. The repertoire of different Argonaute proteins varies among species. For example, there are more than 25 Argonautes in the nematode worm *Caenorhabditis elegans* compared with five in the fly *Drosophila melanogaster*.

Bioinformatics – One definition is as follows: Bioinformatics is conceptualizing biology in terms of macromolecules (in the sense of physical-chemistry) and then applying "informatics" techniques (derived from disciplines such as applied maths, computer science, and statistics) to understand and organize the information associated with these molecules, on a large-scale' (Luscombe et al, 2001).

cccDNA - covalently closed circular DNA, the replicative form of hepatitis B virus DNA.

Dicer - A member of the RNase III family of ribonucleases that cleaves double-stranded RNAs into small interfering RNAs, and precursor microRNAs and mirtrons (see definition below) into microRNAs. In most species, cleavage of longer dsRNAs by Dicer produces double-stranded siRNAs that are ~21 nucleotides long and have a two-nucleotide overhang at their 3′ end, as well as a 5′ phosphate and a 3′ hydroxyl group.

DLin-DMA - 1,2-dilinoleyloxy-3-dimethylaminopropane

Environmental RNAi - refers to sequence-specific gene silencing in response to environmentally encountered dsRNA.

Exportin-5 - a nuclear export receptor for certain classes of double-stranded RNA (dsRNA), including pre-micro-RNAs, viral hairpin RNAs, and some tRNAs.

2'-F - 2′-fluoro substitution (on an RNA base)

GalNac – N-acetylgalactosamine; see definition below.

GEM - genetically-engineered microorganism. GEM refers only to bacteria, fungi, yeast and other microorganisms.

GMO - genetically-modified organism. The acronym can apply to plants, animals or microorganisms, whereas the term genetically-engineered microorganism (GEM) refers only to bacteria, fungi, yeast or other microorganisms.

HAO1 – hydroxyacid oxidase 1

HIGS - Host-induced gene silencing (HIGS). HIGS is an RNA interference-based approach in which small interfering RNAs (siRNAs) are produced in the host plant and subsequently move into the pathogen to silence pathogen genes.

IVT mRNA - in vitro transcribed mRNA

KSP - kinesin spindle protein

LNA - Locked Nucleic acid. A modified RNA nucleotide in which the 2′-O and 4′-C atoms of the ribose are joined through a methylene bridge. This bridge limits the flexibility normally associated with the ring, locking the structure into a rigid conformation.

LC-MS – Liquid chromatography–mass spectrometry, an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the analysis capabilities of mass spectrometry (MS).

MALDI-MS imaging –Matrix-assisted laser desorption ionization - mass spectrometry imaging, a technique in which the sample, often a thin tissue section, is moved in two dimensions while the mass spectrum is recorded. The ability to measure the distribution of numbers of analytes at one time, without destroying the sample, make it a useful method in tissue-based studies.

miR168 –one of the most commonly-detected plant miRNAs, induced as a stress response during the course of infection by diverse plant pathogens. Mature miR168 is highly conserved across species and is a critical regulator of miRNA pathways by regulating ARGONAUTE1 (AGO1), a component of the RNA-induced silencing complex (RISC).

miRNA (microRNA or micro interference RNA) - a group of small, functional, non-protein coding RNA oligonucleotides universally found in microorganisms, plants, and animals. They have been shown to mediate ca. 30% of the post-transcriptional silencing in mammals and modulate a wide range of critical biological processes, including neuronal development, cell differentiation, apoptosis, proliferation, and the immune response.

miRNA* - The precursor microRNA (pre-miRNA) processed by Dicer generates a miRNA duplex containing the miRNA strand and the miRNA* strand, one of which is loaded into the RNA-induced silencing complex (RISC). The ratio of one strand to the other being loaded into RISC to mediate silencing activity can vary among species, tissues, and disease or developmental settings.

Mirtron - Mirtrons are a type of microRNA that are located in the introns of genes. Mirtrons arise from spliced-out introns. Mirtrons, first identified in Drosophila melanogaster and Caenorhabditis elegans, have also been described in mammals and plants. Plant miRNAs are derived from the sequential DCL1 cleavage from pri-miRNA to give pre-miRNA (precursor miRNA), but mirtrons bypass DCL1 cleavage and enter as pre-miRNA in the miRNA maturation pathway.

N-Acetylgalactosamine - N-Acetyl-D-galactosamine (GalNAc) is an aminosugar. It is a component of many O-linked and N-linked glycan structures. It is typically the first monosaccharide that connects serine or threonine residues in particular forms of protein O-glycosylation. In humans it is the terminal carbohydrate forming the antigen of blood group A. GalNac is necessary for intercellular communication and is concentrated in sensory nerve structures of both humans and animals.

ncRNA – non-coding RNA

Non-target Effects – see ‘Off-target’ effects

2'-OMe - 2′-methoxy group substitution (on an RNA base)

Off-target effects - Any detectible phenotypic change that is triggered by the RNAi treatment, other than those that are derived directly or indirectly from silencing the targeted mRNA. In the context of an RNAi-based pesticide, off-target effects occur when an siRNA processed by the RNA-Induced Silencing Complex

(RISC) down-regulates unintended targets; this could be in the target pest or in non-target species exposed to the pesticide.

PCSK9 - proprotein convertase subtilisin–kexin type 9

Precursor miRNAs (pre-miRNAs). Hairpin precursors of microRNAs formed by the cleavage of primary microRNAs by DCGR8 and Drosha in animals or by DCL1 in plants.

PS – phosphorothioate linkage

RES – reticuloendothelial system

RISC – see RNA-induced silencing complex

RNA-induced silencing complex (RISC) - RISC is composed of a group of proteins, including one of the Argonaute proteins, that induces target mRNA cleavage, based on loaded small interfering RNA or microRNA guide strands.

RNAses: enzymes that degrade RNA.

SIGS – Spray-induced gene silencing. SIGS refers to the spraying of dsRNAs or sRNAs that selectively target plant pests onto plant surfaces.

SNALP – see Stable nucleic acid lipid particle

sRNA – small RNA

Stable nucleic acid lipid particle (SNALP) - A lipid nanoparticle formulation for the systemic delivery of small interfering RNAs to tissues.

Toll-like receptors (TLRs) - A family of receptors that recognise pathogen-associated molecular patterns (PAMPs), including some DNA and RNA molecules.

Type I interferon response - An innate immune response to dsRNA, ssRNA, CpG DNA and other stimuli that triggers a protective antiviral response in host cells. Signalling elicits α- and β-interferon release, which activate multiple components of innate and adaptive immunity.

VEGF - vascular endothelial growth factor

XenomiR - xenomiRs are miRNAs derived from plants which are capable of transferring into human or animal tissue following dietary intake.

Appendix I: dsRNA-Based Therapeutic Products – Selected Examples

dsRNA-Based Therapeutic Products – Selected Examples						
Drug	**Action**	**Structure/Form**	**Dose Route**	**Adverse effects**	**Notes**	**Reference**
Target organ/tissue: *Liver*						
Fitusiran (ALN-AT3)	Haemophilia A & B	RNA modified with PS, 2'-OMe & 2'-F. GalNac-siRNA conjugate.	SC (weekly or monthly)	Well tolerated, with relatively minor local injection site reactions	GalNAc-siRNA conjugates are hepatotropic & long-acting and have the potential to treat a range of diseases involving liver-expressed genes. Once-monthly dosing induced dose-dependent antithrombin reductions and increased thrombin generation.	Machin & Ragni (2018); Hu et al, 2019b
Givosiran (GIVLAARI™)	Acute hepatic porphyrias. Gene silencing of delta-ALA synthase 1 (ALAS1)	RNA modified with PS, 2'-OMe & 2'-F. GalNac 3 conjugated to the siRNA passenger strand	SC	Higher frequency of hepatic and renal adverse events than placebo controls	Approved by the FDA in Nov 2019 to treat acute intermittent porphyria (AIP)	Balwani et al, 2020
Patisiran (ONPATTRO™)	Treatment of transthyretin-mediated amyloidosis, an inherited, progressively debilitating disease	Anti-transthyretin siRNA encapsulated in lipid nanoparticles (LNPs). RNA modified with 2'-OMe & 2'-F.	IV infusion	Mild - moderate infusion-related reactions	Treatment of transthyretin-mediated amyloidosis, an inherited,	Suhr et al, 2015; Adams et al, 2018

					progressively debilitating disease. The first RNAi drug to gain FDA and EMA approval (August 2018).	
Inclisiran	Hypercholesterolaemia. Inhibits hepatic synthesis of PCSK9 to provide sustained reductions in LDL cholesterol.	RNA modified with PS, 2'-OMe & 2'-F. GalNac 3 conjugated to the siRNA passenger strand.	SC	Mild – moderate injection-site events more frequent with than with placebo		Kausik et al, 2020; Dyrbuś et al, 2020
Lumasiran (ALN-GO1) (OXLUMO^{TM})	Primary Hyperoxaluria Type 1 (PH1). Targets HAO1 gene (encoding glycolate oxidase).	RNA modified with PS, 2'-OMe & 2'-F. GalNac-siRNA conjugate.	SC	An acceptable safety and tolerability profile was observed in phase 1 &2 studies	Approved in the EU to treat PH1 (all ages) on 19 Nov. 2020 & in the US on 23 Nov. 2020.	Hu et al, 2019b; Scott et al, 2021
Revusiran	Amyloidosis. Targets transthyretin (TTR).	RNA modified with PS, 2'-OMe & 2'-F. GalNac-siRNA conjugate.	SC	Several trial participants reported severe nerve pain; review found it unlikely to be the result of treatment.	Clinical investigations discontinued due to increased risk of mortality in treatment groups. More detailed investigation indicated that the deaths were not correlated with the drug.	Ledford, 2016; Hu et al, 2019b

Vutrisiran (ALN-TTRsc02)	Amyloidosis. Targets transthyretin (TTR).	RNA modified with PS, 2'-OMe & 2'-F. GalNac-siRNA conjugate.	SC	Acceptable safety profile; the most common adverse event was mild, transient injection site reactions in 6.7% of cases. Transient, asymptomatic elevations of ALAT were observed in some subjects		Habtemariam et al, 2020; Hu et al, 2019b
Cemdisiran (ALN-CC5)	Treatment of complement-mediated diseases by suppressing liver production of Complement 5 (C5) protein.	RNA modified with PS, 2′-OMe, 2′-F. GalNac-siRNA conjugate.	SC	Low immunogenicity and acceptable safety profile.	Achieved potent gene silencing for longer than a year after of single dose.	Badri et al, 2020; Hill et al, 2016;
ARB-1467	Hepatitis B infection. Targets HBV gene.	Three synthetic dsRNAs (targeting all four highly-conserved viral HBV RNA transcripts), packed inside proprietary LNPs.	IV infusion	Treatment well tolerated (monthly infusions over 3 months). With bi-weekly dosing at 0.4 mg/kg bw, treatment interruptions were rare (incl. one subject with a mild infusion reaction.	Clinical studies discontinued in 2020.	Flisiak et al, 2018; Hu et al, 2020b

ARO-HBV (JNJ-3989)	Hepatitis B infection. Targets HBV gene.	Two siRNAs, with PS, 2'-OMe, 2'-F, inverted base modifications. GalNac-siRNA conjugate delivery system – Arrowhead Pharmaceuticals TRiM™ platform.	SC	Single or multiple doses up to 400 mg were well tolerated in volunteers and patients.	Up to 2020, ARO-HBV was one of only 2 treatments resulting in Hep-B surface antigen (HBsAg) loss in significant numbers of patients.	Hu et al, 2019b; Lopatin, 2019; Soriano et al, 2020
Nedosiran (DCR-PHXC)	Primary Hyperoxaluria (all 3 genetic forms). Targets hepatic lactate dehydrogenase A (LDHA)	Chemical modifications to RNA undisclosed. GalNac-siRNA conjugate.	SC	After single doses up to 12 mg/kg bw, trial participants able to perform all activities of daily living. The most common AE was mild-to-moderate injection site reaction (18.8%).	Pre-clinical testing of GalNAc-conjugated siRNAs targetting liver-specific LDHA inhibition did not produce adverse effects in off-target (non-hepatic) tissues.	Hu et al, 2020b; Langman et al, 2021
ARC-520	Hepatitis B infection. Targets HBV gene.	2'-OMe, 2'-F, inverted base. 1:1 mix of 2 dsRNAs conjugated with cholesterol and mixed with two GalNAc-masked peptides targeting hepatocytes (Arrowhead Pharmaceuticals 'DPC2.0' proprietary technology)	IV infusion	Treatment was safe and well tolerated – AEs mild and none led to study discontinuation; Two cases of pyrexia within 1 h of infusion responded to paracetamol and did not recur.	The first RNAi therapeutic *vs* HBV to enter clinical testing. Studies terminated in 2016 due to deaths in a pre-clinical monkey study with another RNAi product using the same delivery agent used in ARC-520; the toxicity was not	Yuen et al, 2020; Hu et al, 2020b

					associated with the ARC-520 siRNA.	
ARC-521	Hepatitis B infection. Targets HBV mRNA transcripts from both cccDNA and integrated DNA.	As for ARC-520 (see above)	IV infusion	No deaths or drop-outs due to AEs or SAEs reported in healthy volunteers at single doses up to 6 mg/kg bw. No infusion reactions or lab. abnormalities, & no significant ALAT elevations.	Studies terminated in 2016 - see note above (ARC-520) *re* the siRNA delivery system.	Hu et al, 2019b; Hu et al, 2020b;
PRO-040201 (TKM-ApoB)	Targets ApoB expression for the treatment of hypercholesterolemia.	Unmodified siRNA formulated in lipid nanoparticles	IV	Well tolerated - showed no liver toxicity. One of 2 subjects at the highest dose reported flu-like symptoms, consistent with immune system stimulation.	Although well tolerated in Phase-I trials, & LDL cholesterol lowered, further development terminated due to the potential for immune stimulation, and only transient cholesterol reductions.	Burnett et al, 2011; Burnett & Rossi, 2012; Chen et al, 2018; Hu et al, 2019b; Ozcan et al, 2015
Target organ/tissue: *Tumours*						
ALN-VSP02	Solid tumours. Targets VEGF and KSP.	Two different siRNAs with PS, 2'-OMe modifications. Delivery system LNPs (DLin-DMA).	IV	Generally well tolerated (Phase I trials), with no dose-dependent trends in clinical or laboratory AEs		Cervantes et al, 2011; Hu et al, 2019b;

				up to doses of 0.7 mg/kg bw.		
Cobomarsen (MRG-106)	Blood cancers (cutaneous T cell lymphoma, adult T cell lymphoma/ leukaemia, diffuse large B cell lymphoma, chronic lymphocytic leukemia, mycosis fungoides). Targets miR-155, an oncomir that is highly expressed in a wide range of cancers e.g., leukemia, lung and breast cancer.	Locked nucleic acid (antimiR)	IV or SC	Well tolerated in 43 leukaemia patients in a safety and tolerability study. No evidence of immune suppression.		Foss et al, 2019; Fortunato & Iorio, 2020
***Target organ/tissue**: Eye*						
Bevasiranib	Treatment of age-related macular degeneration. Designed to target VEGF.	Naked siRNA	Intra-vitreal	Triggered significant activation of Toll-like receptor 3 (TLR3) and its adapter molecule TRIF, inducing the secretion of interleukin-12 and interferon-ϒ.	It was also demonstrated that 21 nt or longer siRNAs, regardless of their target gene products, can suppress choroidal neovascularisation (CNV) in mice. Further clinical investigations were terminated in 2009.	Kleinman et al, 2008; Hu et al, 2020b

Bamosiran (SYL040012)	Ocular hypertension, glaucoma. Targets beta2 adrenergic receptor mRNA.	Naked siRNA. No carrier.	Eye drops	AEs reported were not different from those reported for placebo. This correlates well with its low half-life in systemic tissues and plasma	bamosiran more stable in ocular fluids, with lower levels of RNases *cf*. plasma. Ocular admin. provides the opportunity for local gene silencing without systemic or immune exposure or RNase degradation.	Gonzales at al, 2015; Sun & Ou, 2018

Abbreviations not otherwise defined in the table: 2'-F - 2′-fluoro substitution; 2'-OMe - 2′-methoxy group substitution; ALAT - alanine aminotransferase; ApoB – Apolipoprotein B; cccDNA - covalently closed circular DNA, the replicative form of hepatitis B virus DNA; DLin-DMA - 1,2-dilinoleyloxy-3-dimethylaminopropane; HAO1 – hydroxyacid oxidase 1; KSP - kinesin spindle protein; PCSK9 - proprotein convertase subtilisin–kexin type 9; PS – phosphorothioate linkage; VEGF - vascular endothelial growth facto