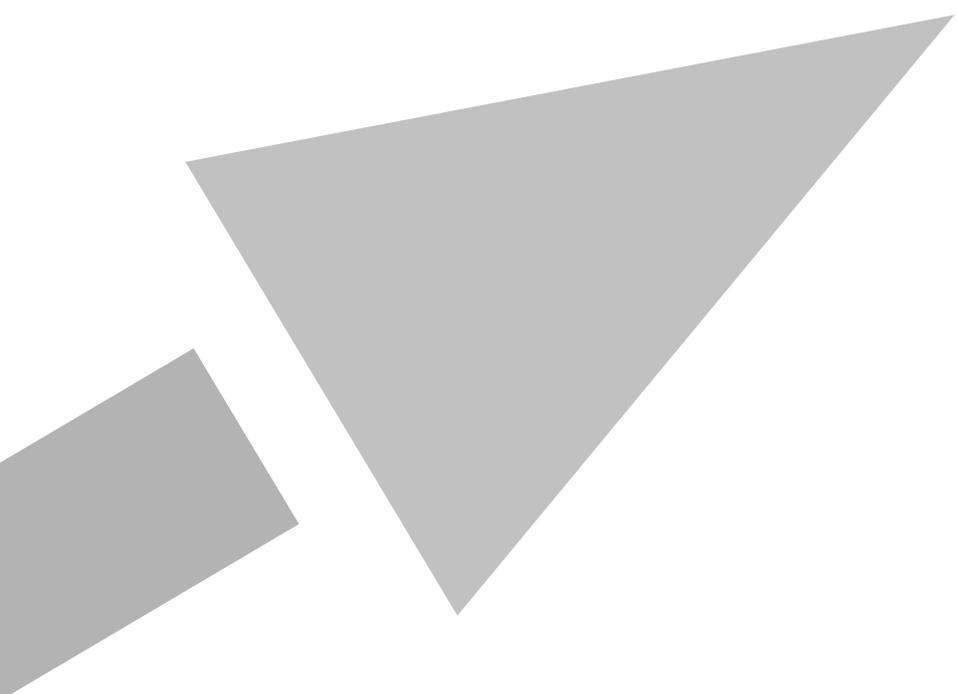


***Noncoding RNAs and
Risk Assessment Science
3 – 4 March 2016, Málaga***

Co-organised by ECETOC and Cefic-LRI

Workshop Report No. 32



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Noncoding RNAs and Risk Assessment Science

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SUMMARY

This report presents recent progress made on the basic research and the use of noncoding RNAs (ncRNAs) as biomarkers in regulatory toxicology and ecotoxicology, and as analytical and therapeutic agents, as discussed at a workshop held in Málaga, Spain, on 3 and 4 March 2016. Ten presentations provided an overview on the state-of-the art ncRNA-related research, i.e. (1) the spectrum of ncRNAs known to date; (2) their function and roles in physiology and mechanisms of action; (3) issues to be taken into consideration in linking ncRNA expression profiling to phenotypic events; (4) the role of ncRNAs during cellular adaptation to external stressors, the physiological interpretation of ncRNA expression profiles and their phenotypic anchoring for substance risk assessment; (5) a critical appraisal of the hypothesis of ‘cross-kingdom RNA effects’ especially with respect to the dietary uptake of RNAs; (6) possible correlations between changes in ncRNA expression and pathological phenotypes (‘cause or consequence’); (7) the relevance of dose levels, exposure durations, no effect levels and adverse effect levels on the interpretation of ncRNA expression profiles; (8) upcoming medical applications of ncRNA therapeutic agents; (9) an overview of ncRNA expression profiling techniques; and (10) considerations for risk assessment of ncRNAs present in agricultural products and applications. These topics were reflected in further detail in a panel discussion and in extensive plenary discussions. There was agreement that an abundance of data may be obtained from ncRNA profiling, but that these data require careful evaluation and ncRNA verification to determine the utility of specific ncRNAs in hazard identification and characterisation or the role of ncRNA expression profiles in the identification and quantification of apical effects, i.e. substance-induced observable effects identified in animal experiments.

Linking changes at the molecular level (such as ncRNA expression profiling) to toxicological outcomes is considered to offer great benefits for the safety assessment of substances. Such data may facilitate moving from regulatory decision-making substantially based on apical effects to decision-making based on an understanding of toxicological processes. Furthermore, there is the potential to use ncRNAs in combination with other molecular parameters to identify early molecular / cellular events *in vitro* and be able to link these with apical endpoints that may be observed *in vivo*. Thus, ncRNA-based technologies may assist the innovation and development of new chemical products, and reduce reliance on tests using experimental animals and the costs of developing and assessing potential adverse effects of novel molecules and the time required to market innovative substances. Clearly, these goals may only be reached in the long-term since many aspects of the roles that ncRNAs play in the maintenance of cellular homeostasis or the development of phenotypic alterations are still unknown. However, basic molecular understanding of the science in this field is developing rapidly, and ncRNA-based tools are already being applied in the areas of pharmaceuticals and agrochemicals. Since the identified goals are of outstanding relevance, they should be pursued with high priority.

On the second day of the workshop, breakout sessions served to specify research needs to advance the current understanding of the role of ncRNAs in regulatory toxicology and risk assessment in order to make a first step towards the ultimate goal of using a mode of action approach in toxicity testing. Specifically, the following research topics were identified to be forwarded to the Cefic Long-range Research Initiative (LRI) research programme as priority areas for funding:

- Conduct comprehensive literature reviews and bioinformatic reanalyses of existing data sets using a consistent methodology with the aims (1) to set up a list of candidate ncRNAs (in particular miRNAs and functionally well understood ncRNAs for which research is currently most advanced) for further assessment as relevant biomarkers in toxicity studies; and (2) to identify areas of toxicology where ncRNA profiling may provide the opportunity to overcome prevailing scientific deficiencies in obtaining information that is relevant for risk assessment. Such areas of toxicology include, but are not restricted to the analysis of non-genotoxic carcinogens and reproductive / transgenerational toxins (including *in utero* exposure leading to adult diseases), as well as the effects of long-term low-dose exposure and challenges in addressing mixtures in toxicology.
- Develop consensus on how to conduct ncRNA expression profiling in a toxicological context, including best scientific practices (and guidelines) to collect and curate data, to analyse data and to report the outcome of studies to support applicability of the data for regulatory decision-making. This topic should include the standardisation and validation of currently available technologies in order to improve the quantitative and qualitative comparability of ncRNA datasets obtained with different technologies and to reconcile the extreme sensitivity of profiling technologies. This project should link with a currently ongoing exercise to develop a framework for the analysis of transcriptome data.
- Conduct experimental projects to evaluate the toxicological relevance of the expression profiles of selected ncRNAs. Preferably, surplus samples from, e.g. control groups existing regulatory studies should be utilised. As necessary, such retrospectively analyses of ncRNA expression profiles may be supplemented by newly conducted (e.g. 90-day) rat oral toxicity studies. The project should aim at establishing 'normal' (i.e. physiological) profiles that cover the biological variability of healthy individuals (the range of intra- and inter-individual variability in the levels of ncRNA expression, incl. polymorphic differences), which is likely to be affected by a multitude of parameters, including the diet. To substantiate the relevance of key ncRNAs for cell homeostasis or pathogenesis, the experimental approaches must link measured molecular events with substance-induced pathological or apical effects in a dose-dependent manner, and they should strive to differentiate between causative changes and consequential (adaptive) responses. The data from the experimental studies should be used to integrate knowledge on ncRNAs into adverse outcome pathways. Since ncRNA-based technologies are closely related to other modern technologies, such as '-omics' and epigenetics, a holistic approach should be pursued to ensure that all new insights are jointly exploited in identifying adverse outcome pathways.

1. INTRODUCTION

1.1 Background

Non-(protein-)coding RNAs (ncRNAs) are broadly classified according to their length as long noncoding RNAs (lncRNAs; >150-200 nucleotides (nt)) and short ncRNAs (<150-200 nt) (Wright and Bruford, 2011). Between 4 and 10% of lncRNAs are processed to shorter RNAs (Kapranov *et al*, 2007; Derrien *et al*, 2012). Major classes of short ncRNAs include (1) endogenous microRNAs (miRNAs; approximately 19-25 nt) that are first produced as double-stranded 'miRNA duplex' (in which form they may also be used as therapeutic agents), whereas mature miRNAs are single-stranded and associated with RNA-induced silencing complexes (Kim, 2005; Winter *et al*, 2009); (2) endogenous Piwi-interacting RNA (piRNA; single-stranded, 24-31 nt; Piwi: P-element induced wimpy testis in *Drosophila*) that are involved in long-term transgenerational messenger RNA (mRNA) suppression in reproductive organs; (3) endogenous or synthetically produced short interfering RNAs (siRNAs; double-stranded, 20-24 nt) that induce degradation of transposable elements or mRNA targets, the latter being used as research tools in mammals (Watanabe *et al*, 2006, 2008; Arrigo and Pulliero, 2015; Wright and Bruford, 2011). Since knowledge on the existence and functionalities of different forms of ncRNAs is evolving rapidly, this classification of different forms of ncRNA is most likely not yet definite. Further classes of short ncRNAs are known and continue to be discovered, e.g. small nuclear RNA, small nucleolar RNA, vault RNA, or Y RNA (Martens-Uzunova *et al*, 2013; Kowalski and Krude, 2015).

Generally, endogenous ncRNAs have been found to be involved in post-transcriptional gene silencing, epigenetic regulation and mediation of physical and chemical environmental signals. However, although knowledge on how substances may influence the functionality of ncRNAs is growing, the potential role of ncRNAs in regulatory toxicology and the risk assessment (RA) of such substances is unclear. (Throughout this report, the term 'substance' is used as defined in *Regulation (EC) No 1907/2006 on the Registration, Authorisation, Evaluation and Restriction of Substances (REACH)*, i.e. a chemical element and its compounds in the natural state or obtained by any manufacturing process, including, as applicable, necessary stabilising additives and impurities deriving from the given production process.)

As Alan Poole, Secretary General of the European Centre for the Ecotoxicology and Toxicology of Chemicals (ECETOC, Belgium), outlined in his welcome to the workshop participants, ECETOC aims at generating knowledge that regulators can use for action. In pursuing this goal, ECETOC collaborates closely with the European Chemical Industry Council Long-range Research Initiative (Cefic LRI) that funds research to generate science-based knowledge that may be used to assess and ensure the safety of substances. The ECETOC workshop *ncRNAs and risk assessment science* aimed at reaching a common understanding on the state-of-the-art research on ncRNAs, on the implications of ncRNA expression profile changes for the evolution of apical effects, i.e. the observable outcomes of substance exposure to test animals (OECD, 2012), and the current and potential future role of ncRNAs in regulatory toxicology and RA. The deliverables of this workshop were not only to prepare a report that would be further processed into a manuscript to be submitted to a peer-reviewed open-access journal, but also to outline proposals for Cefic LRI-funded research to be put forward for funding in 2016 and, possibly, proposals for research to be suggested for public funding, e.g. under the European Commission programme for research and innovation *Horizon 2020*.

1.2 Workshop structure and aims

Approximately 20 experts attended the two-day workshop representing academia (Harvard Medical School (USA), Imperial College London (UK), John Hopkins University School of Medicine (USA), Leipzig University (DE), Regensburg University (DE), Technical University Munich (DE), VU University Medical Centre (NL), University of Surrey (UK)), authorities (European Food Safety Authority, Public Health England), research institutions (National Centre of Scientific Research - CNRS (F), Helmholtz Centre for Environmental Research – UFZ (DE)), industry (BASF, Dow, Monsanto, Sumitomo Chemical Europe), independent consultants (BioMath GmbH (DE), Scientific Consultancy – Animal Welfare (DE)), and the hosts of the workshop (ECETOC and Cefic LRI). The full list of workshop participants is provided in Appendix B.

Day 1 consisted of a series of talks and case studies with room for discussions, focusing on (Sessions 1 and 2) understanding ncRNAs, i.e. their physiology and mechanisms of action as well as their functional relevance and possible role in human pathology; and (Session 3) understanding the relevance of (including technical issues) ncRNA in toxicology and the RA of chemicals.

Day 2 consisted of brainstorming activity in which participants were divided into 3 breakout groups to (1) obtain clarity on data analysis and interpretation (what is relevant for regulatory toxicology and RA?); (2) assess whether current regulatory toxicity testing should be re-evaluated in light of current knowledge of ncRNA; and (3) develop a prioritised research agenda to further the use of ncRNAs (e.g. as biomarkers) in the regulatory toxicity testing of chemicals (*cf.* Appendix A for detailed workshop programme).

2. PRESENTATION SUMMARIES

2.1 Session 1: Understanding ncRNAs

2.1.1 What are miRNAs?

Tim Gant, Head of Toxicology, Centre for Radiation, Chemical and Environmental Hazards, Public Health England (PHE) and University of Surrey, UK

MiRNAs regulate gene expression at the post-transcriptional level predominantly by mRNA degradation or the inhibition of mRNA translation by binding to the 3'-untranslated regions (3'-UTR) of specific mRNAs. Further, miRNAs appear to play a role in the transgenerational transmission of altered phenotypes through the male germ line. Compared with mRNAs, only a small number of miRNAs have been identified, i.e. 4552 unique human miRNAs (at the time of writing) as compared to 30,000 known human mRNAs. However, one miRNA may regulate the expression of many mRNAs (by partial complementary base pairing). Hence, even though miRNAs are smaller in number, their effects on the phenotype of the cells may be substantial (Hannon and Rossi, 2004). By controlling mRNA translation, miRNAs elicit effects on the cellular protein levels, and the more miRNA is present in the cell, the less protein will be translated (Selbach *et al*, 2008). MiRNAs may be tissue specific, e.g. miR-124 in the brain, miR-133a in the heart, or miR-122 in the liver, the latter being well explored in regard to drug overdose-induced hepatotoxicity (Laterza *et al*, 2009). Tissue-specific miRNAs may be relevant biomarkers for RA, even more so since they are stable and may be found in all body fluids. Finally, miRNA may play a role in the evolution of chromosome aberrations (Calin and Croce, 2006).

In the human genome, the majority of miRNAs is located in between genes (i.e. they are intergenic), where miRNAs are organised into cistronic and polycistronic regions, i.e. expressing one or more miRNAs, respectively (He *et al*, 2005). Nevertheless, miRNAs may also be found in the introns of genes, whereby they are under the control of a mRNA promoter and enhancer region. Such miRNAs often act as feedback loops to the genes in which they are contained. Finally, the smallest group of miRNAs are exonic (Gant *et al*, 2015). During embryonic development, miRNAs may be involved in the very early stages of gene transcription that begins in zygotes during the eight-cell stage. If such gene transcription processes are disturbed, e.g. by environmental or epigenetic factors, there is the potential (currently hypothetical) for the phenotype of the resulting embryo to be affected (Trerotola *et al*, 2015).

In the search for ncRNA biomarkers, some priority should be given to the class of miRNA as their functions in terms of controlling gene expression in a wide variety of cellular processes (including proliferation, differentiation and development) and of involvement in both toxicity and disease is currently best understood. Furthermore, some miRNA exhibit organ-specific expression, and they can be detected in the blood plasma thereby acting as distant biomarkers of adverse events. This focus should not exclude other forms of ncRNAs, which will most likely assume an equal status as their roles in physiology and disease are better understood.

Q&A

Are changes in miRNA possibly molecular initiating events (MIE)? – This depends on the context: They could constitute a MIE, but they could also constitute downstream steps of an adverse outcome pathway (AOP; Ankley *et al*, 2010).

In respect to tissue-specific expression patterns of miRNAs, what are the (patho-)physiological implications of the high contents in body fluids? – This is not yet understood.

Will miRNA levels change over life stages of animals or humans? – Yes, and in response to changes in environment.

2.1.2 What do ncRNAs do? Mechanisms of action, functional relevance, importance of negative results

Gunter Meister, Biochemistry Center Regensburg, Regensburg University, Germany

Small regulatory ncRNAs are found in all higher eukaryotes. They play key regulatory roles in cellular processes that are as diverse as embryonic development, stress response or transposon silencing. All small ncRNA species are generated from longer precursor molecules and are finally incorporated into effector protein complexes. Of the small ncRNAs, recent research has focused on miRNAs that guide post-translational, temporary gene silencing. This is mediated by members of the so-called argonaute (AGO) protein family that bind to the miRNAs (Huntzinger and Izaurralde, 2011; Meister, 2013; Schraivogel and Meister, 2014; Dueck and Meister, 2014; Jonas and Izaurralde, 2015). Second, **lncRNAs** are being extensively studied. In mammals, lncRNAs are found in the nucleus and in the cytoplasm. In the nucleus, they mainly associate with chromatin and regulate the expression of specific genes. In the cytoplasm, they may bind to mRNAs thereby influencing post-transcriptional gene expression. lncRNAs can function as guides (enhancers of proper localisation of protein complexes), sponges (by binding to complementary RNAs thereby inhibiting their functionality), scaffolds (adaptors to bring two or more proteins into discrete complexes) or even nucleators for higher order chromatin structures (Ebert and Sharp, 2010; Salmena *et al*, 2011; Rinn and Chang, 2012). Similar to small RNAs, lncRNA expression is frequently altered in human diseases. Very recently, **circular RNAs** have been characterised (Hentze and Preiss, 2013). Under specific conditions or in specific tissues (e.g. the brain), circular RNAs can be abundant. They are generated by alternative splicing and may fulfil functions such as sponges for RNAs and proteins. Apparently, some circular RNAs even have coding potential and may contribute to the diversification of gene products. Finally, **epitranscriptome, i.e. sugar or base modifications** that are introduced after synthesis of the RNA transcript constitute procedures that, similar to epigenetics, exist 'on top' of coding and non-coding transcripts. Generally, RNAs may be modified at particular bases, as has been known for many years for ribosomal and transfer RNAs. Recently, it was found that such modifications are also present on mRNAs where they can influence gene expression. RNA-binding proteins may associate with transcripts, thereby changing their function independently of their sequence, and apparently RNAs can cross-talk by hybridisation (Lin and Gregory, 2014). All of these factors should be taken into account when analysing gene expression. Most likely, to date, only the 'tip of the iceberg' has been uncovered and many more such modifications with important biological functions remain to be detected.

Q&A

What triggers changes in gene expression? – These are complex mechanisms. At the molecular level, triggers could be transcription factors or modifying enzymes.

In terms of AOPs, what are the key issues one should focus on? – One should focus on the level of the transcript and determine if changes are sensitive. Very often, changes are small (e.g. five-fold increases of singular molecules), in which case they will be difficult to detect.

2.1.3 Expression of macroscopic phenotypes for miRNA-guided regulation at the molecular level

Hervé Seitz, Institut de Génétique Humaine (IGH), Centre National de la Recherche Scientifique (CNRS), Montpellier, France

MiRNAs are small regulatory RNAs that repress specific target genes. According to the current view, each miRNA regulates hundreds of genes. Computational tools aim at identifying miRNA targets, looking for seed matches in 3' UTRs (Bartel, 2009). Usually, this is performed by selecting evolutionarily conserved miRNA binding sites. However, such predictions are often biologically irrelevant, since short matches are very frequent. Sixty per cent of human coding genes seem to be targeted (Friedman *et al*, 2009), and miRNAs are implicated in every physiological process in animals. Nevertheless, miRNA-mediated gene repression is usually very modest, i.e. less than two-fold, which is lower than well-tolerated fluctuations in gene expression. Focusing on miR-223-guided gene repression, Baek *et al* (2008) observed that, in wild-type mice, gene repression was smaller than the inter-individual variability for 150 out of 192 predicted targets. Hence, most predicted targets appear functionally insensitive to the miRNAs. Moreover, many conserved miRNA binding sites appear to be conserved in a miRNA-independent fashion. It is unclear, why miRNA binding sites have been conserved since they do not appear to be functional. Sequence elements may be conserved for other reasons, while being fortuitously complementary to miRNAs. Accordingly, comparative genomics may yield a high proportion of false positive results.

In revisiting the definition of a 'miRNA target' it is concluded that not every measurable change in gene expression translates into a macroscopic, evolutionarily selectable phenotype. The role of miRNAs in normal and pathological conditions may have been over-estimated, and the very notion of 'gene regulation' should be reconsidered taking into account the robustness of cellular homeostasis to external insults. It appears difficult to reconcile such observations with the extreme sensitivity required for genetic fine-tuning. Most likely, the 'butterfly effect' (indicating that a small dose may trigger a substantial consequence) has been counter-selected for.

Q&A

How similar are ncRNA targets in rats and mice, i.e. the predominant animal species used in toxicology? – Generally, they appear to be very similar, but concordances are not yet fully understood.

Is it necessary to address macroscopically observable effects for RA (which may counteract the modern toxicological paradigm to strive for short-term assays that focus on mechanisms of toxicity and MIEs)? – One should search for dose-sensitive effects that seem to be tightly controlled during gene regulation.

2.1.4 Cellular adaptation: Relevance for risk assessment, physiological interpretation: Phenotypic anchoring

Emma Marczylo, PHE, UK

The vast majority of the mammalian genome is composed of so-called noncoding DNA, with only approximately 1-2% of the mammalian genome coding for proteins. The majority of noncoding DNA is transcribed into ncRNAs, which play major roles in regulating gene expression. Whilst lncRNAs do this in a variety of ways, including chromosome remodelling and transcriptional or post-transcriptional regulation, short ncRNAs predominantly regulate gene expression at the post-transcriptional level. ncRNAs interact with epigenetic mechanisms to form a robust regulatory epigenetic network (Marczylo *et al*, 2016). Many ncRNAs, particularly miRNAs, have been shown to be involved in both toxicity and disease. Specifically, ncRNA play a central role during cellular adaptation in response to changes in the environment. Following an environmental exposure, cellular processes are activated in an attempt to regain homeostasis. These processes may be transient, returning to the original state once the insult is removed, or they may become established as the ‘new homeostasis’. Such adaptations may be positive, providing protection against further stress (Wheeler and Jong, 2007; Jain *et al*, 2014); or they may be negative, resulting in an increased susceptibility to further stress (Greathouse *et al*, 2012; Wong *et al*, 2015). If cells are unable to adapt to an environmental exposure, adverse effects may evolve that may either be reversible (leading back to homeostasis) or irreversible which ultimately results in cell death. For RA, it is vital to distinguish between cellular adaptation and adverse effects.

In determining cellular ‘normality’ with the aim of making use of ncRNAs for RA, it is important to understand and characterise the variability in ncRNAs both within individuals (tissue specificity) and between different individuals and different species that are present in the same steady state (normal environment). For instance, more than 500 different miRNAs were present in the plasma of 18 disease-free human volunteers of which approximately 50 miRNAs were present in all samples. Further, 10 of the most highly expressed miRNAs accounted for 90% of the total miRNA expression, and 5 of these were blood cell-associated. Finally, 10 of the most stably expressed miRNAs included 5 of the most highly expressed ones (Tonge and Gant, 2016). Variability in ncRNA expression should also be understood in response to changes in the normal environment that do not exert adverse effects both within individuals and between individuals. This will enable identification of genetic susceptibilities, normal ranges of ncRNA expression, and adaptations with increased susceptibilities.

In determining the relevance of miRNA-initiated cellular adaptations to environmental stressors, it is important to distinguish whether the change in ncRNA expression is the cause or the consequence of an apical effect. Yet, whilst a robust, dose-dependent relationship between specific ncRNA(s) and environmental stressor(s) or subsequent effect(s) is vital for RA, establishing causality is not necessarily essential. ncRNAs that do not in themselves directly induce adverse effect(s), but instead act as markers of

exposure and/or predictors of future toxicity, may also be useful in regulation (Marczylo *et al*, 2016). Increasingly, the potential use of miRNAs as biomarkers is being investigated since they are secreted in multiple body fluids (including blood, semen, saliva), and they are stable (secreted within exosomes), accessible, and easily measured (Gant *et al*, 2015). Spermatozoal miRNAs are of particular interest for regulatory purposes since they can be altered by environmental exposures, transmitted across generations, involved in the physiological or pathophysiological development of subsequent progeny and are easily collected and analysed (Liu *et al*, 2012; Marczylo *et al*, 2012; Rodgers *et al*, 2013, 2015; Gapp *et al*, 2014; Stowe *et al*, 2014). To be used as biomarkers, ncRNAs should further be sensitive, specific, and linearly related to exposure and effect.

With regard to the physiological interpretation (i.e. phenotypic anchoring) of alterations in ncRNA expression, these molecules pose unique challenges since they act at multiple levels forming part of an interactive network. For example, miRNAs have multiple mRNA targets, just as potential mRNA targets are targeted by multiple miRNAs. Consequently, the interpretation of observations may be challenging. To facilitate phenotypic anchoring, ncRNA expression profiles may be correlated with other profiles and phenotypic endpoints (Akinjo *et al*, 2016). In this respect, '-omics' technologies allow simultaneous profiling of multiple variables using a systems biology approach.

In conclusion, ncRNAs are important regulators of gene expression and represent novel mechanisms and markers of toxicity that might be useful for regulatory purposes. To explore such use, a greater mechanistic understanding should be obtained, e.g. by performing additional analyses on surplus biological samples from existing regulatory studies, thereby avoiding the use of extra animals. It may also be considered to adapt existing testing guidelines to incorporate ncRNA analyses, as appropriate, to begin collecting data on ncRNA in a regulatory context.

Q&A

Considering the possible use of miRNAs as indicators for exposure in live animals: How well established is the correlation between alterations recorded in the blood, urine or other body fluids and changes seen in target organs? – Many studies have investigated plasma miRNAs as markers of tissue/organ-specific toxicity. Nevertheless, to date, there is very little information regarding the variation of expression in a given biological fluid and how this expression alters in response to factors such as age, gender, obesity and smoking status. Indeed, a number of studies have reported correlations between organ-specific toxicities and miRNAs that are actually predominantly associated with blood cells. Therefore it may be questioned whether these correlations are instead a consequence of blood sample preparation. Thus, robust correlations have yet to be established.

Is it possible to observe changes in gene expression before histopathological alterations can be observed, and are there changes in gene expression that are unrelated to histopathological findings? – There is a large collection of literature on gene expression changes at sub-toxic doses, which will include changes that do not directly relate to the ultimate apical effects. To determine the relevance of such expression changes, it is important to understand mechanisms of toxicity and to perform full dose-responses.

2.1.5 Stability and transport: Cross-kingdom RNA effects, modes of ncRNA exposure, exosomes and protein

Kenneth Witwer, Johns Hopkins University School of Medicine, USA

In cross-kingdom RNA communication, the nematode *Caenorhabditis elegans* is the known champion, using ingested environmental RNA as a type of immune system (Jose, 2015). Such cross-kingdom communication has also been described or proposed in various host-pathogen interactions, e.g. in the relationship of retroviruses with their hosts (Klase *et al*, 2007; Wagschal *et al*, 2012). Applied to mammals, the so-called ‘dietary RNA hypothesis’ suggests that intact RNAs present in the food may enter the ingesting organism and exert gene expressional functions in its cells (Witwer and Hirschi, 2014). Studies of mammalian uptake have focused mostly on miRNAs, or ‘xenomiRs’ to denote their foreign origin. However, in the meantime, enthusiasm about the absorption and function of xenomiRs has been diminished by negative findings and evidence of contamination and experimental design flaws that account for apparently positive results (Mlotshwa *et al*, 2015). Nevertheless, despite scant and suspect evidence for the hypothesis, interest is likely to continue into the foreseeable future. Focusing on mammals exposed to plants and milk (Dickinson *et al*, 2013; Snow *et al*, 2013; Witwer *et al*, 2013; Baier *et al*, 2014; Tosar *et al*, 2015), experimental results and current understanding of RNA stability, transport, and function do not appear to be consistent with proposed forms of cross-kingdom communication. In many cases, presumably positive ncRNA findings were shown to result from, e.g. sample contaminations or artefacts caused by technical limitations of the applied technologies (Witwer, 2015).

Q&A

Why may viruses enter cells and induce miRNA production, whereas plant miRNA apparently are unable to enter the body? – Viruses actively infect (invade) cells. When they come into contact with host cells, they actually interact with the cell membranes, for example, by means of fusogenic proteins that are generally not found on miRNA-containing lipid complexes (van Dongen *et al*, 2016). Furthermore, miRNAs produced from viral transcripts are made entirely by the host machinery and incorporated into host silencing complexes. It is not known how miRNAs from the diet, such as plant miRNAs, would enter cells, and a mechanism for transfer of miRNA from a plant AGO to a host AGO has not been proposed. miRNAs are not known to be released from AGO once bound (Martinez *et al*, 2002).

Have differences in the stability of miRNAs been recorded? – Generally, miRNAs are stable. A miRNA that is found in any body fluid must be protected. For instance, if it is present in the extracellular plasma of the blood, it must be AGO-bound. Even if the AGO is undetectable with current techniques, such as within extracellular vesicles, a protein binding partner must nevertheless be present. Therefore, the detection of an increase or a decrease of a miRNA in a certain matrix requires careful interpretation since its functionality may depend on the protein binding partner.

2.1.6 Cause or Consequence? Links between changes in ncRNA expression and pathological phenotype

Jörg Hackermüller, Young Investigators Group Bioinformatics and Transcriptomics, Department of Molecular Systems Biology, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany

Considering that mammalian cells are capable of producing a plethora of ncRNAs, the question arises to which extent non-coding transcription is functionally important. Are changes in ncRNA expression causal for diseased or adverse states or a consequence thereof? Even if the latter is the case, ncRNAs might still form a valuable pool for biomarkers of adversity or disease. However, if ncRNAs play in part causal roles, do we need to consider ncRNAs in mode-of-action (MoA) frameworks or AOPs, and, if so, is it already possible to define MoAs or AOPs where ncRNAs participate in key events? The example of interleukin 6 (IL-6) and signal transducer and activator of transcription 3 (STAT3)-controlled ncRNAs served to further elucidate these questions. Specific multiple myeloma (B cell malignancy) cells depend strictly on IL-6 and become apoptotic when IL-6 is not present. However, this cellular phenotype cannot be explained by an IL-6-enabled differential expression of protein-coding genes (Brocke-Heidrich *et al*, 2004). Instead, the miRNA miR-21 appears responsible for the anti-apoptotic effect of IL-6 and indeed shows strong differential expression in B cell malignancy cells (Löffler *et al*, 2007). Further, miR-21 has been observed to target the tumour suppressor genes ANP32A, PDCD4 and SMARCA4 (Schramedei *et al*, 2011). IL-6 and STAT3 have been observed to control a plethora of lncRNAs and to regulate a set of macroRNAs (including STAIRs; STAT3-induced RNAs) that are in part specific for multiple myeloma (Hackermüller *et al*, 2014). Some STAIRs seem to be tightly coupled with IL6 signalling via STAT3 and inherent components of this pathway.

Even though knowledge on such specific interactions is beginning to evolve, it can be difficult to ascertain the causality of the expression of a given ncRNA for several reasons: Its effects on the regulation of gene expression may depend on a given context. With the exception of some small RNA classes, limited conservation at the primary sequence level complicates tracing ncRNAs between model animals. Finally, ncRNAs have been found to interfere with pathways at multiple levels – with strong consequences on the entire pathway but little effect of individual interactions (e.g. Boll *et al*, 2013). Nevertheless, increasingly, the role of lncRNAs in toxicity testing is being addressed, such as the effects of chemicals on lncRNA H19 and other imprinted genes as well as Hox gene transcript antisense RNA (HOTAIR) (Croce, 2010; Bhan *et al*, 2014). These findings may form the basis for an AOP. Non-coding loci are also of increasing interest in environmental epidemiology. Deep methylome sequencing, i.e. an analysis of the methylation status of the genome, in a mother-child cohort study identified numerous differentially methylated regions of the DNA (DMRs) in children that are affected by their mothers' smoking habits. The majority of DMRs was associated with noncoding targets and differential methylation was in part found to persist over years (Bauer *et al*, 2016).

In conclusion, to a larger part, the differential expression of ncRNAs may be a consequence of disease or adverse effect, but many short ncRNAs do play a causal role in disease. Also for a growing number of lncRNAs, changes in either expression or mutation have been found causal for disease. Even though only few lncRNAs have so far been associated with pathways of toxicity and knowledge on their role in the involvement of apical effects is still limited, lncRNAs, and not only miRNAs, should be considered in AOP or MoA analyses.

2.1.7 Exposure effects? Relevance of dose level, time, no effect level, adverse effect level

Nigel Gooderham, Computational and Systems Medicine, Department of Surgery and Cancer, Imperial College London, UK

MiRNAs are an abundant class of genes in mammals that may control gene expression through translational repression and by induction of mRNA degradation. Since miRNAs regulate development, cell proliferation, apoptosis, and differentiation, they play a central role in processes that are potentially important in toxicology. Specifically, miRNAs have been shown to interact with cellular pathways that are relevant for carcinogenesis (Calin and Croce, 2006). Tumour-suppressor miRNAs may negatively regulate protein coding oncogenes, whereas oncogenic miRNAs may repress tumour suppressor genes. MiRNAs may also alter the epigenomics landscape by reprogramming a cell's epigenome (Moazed, 2009). For instance, miR-29 has been observed to inhibit the expression of DNA (cytosine-5-)-methyltransferase 3 (Fabbri *et al*, 2007), and miR-101 regulates the histone methyltransferase EZH2 (enhancer of zester homolog-2) (Floris *et al*, 2015). Further, miRNA combinations may cooperate to regulate multiple proteins within cancer-important pathways.

Currently, genotoxic carcinogens can be identified reliably and quickly using a weight-of-evidence approach that takes into account structural information, *in vitro* assays, and *in vivo* DNA damage assays. By contrast, there are no short-term tests having received regulatory acceptance that allow predicting non-genotoxic carcinogenicity. To investigate if changes in miRNA expression may constitute early indicators of substance-induced carcinogenicity with adequate sensitivity and specificity and provide information on mechanisms of toxicity, a spectrum of liver carcinogens was investigated in 90-day oral toxicity studies in Fisher rats. These substances were applied at carcinogenic doses, whereas additional non-carcinogenic substances were applied at the respective maximum tolerated dose. In none of the test groups did tumours become evident by the end of the exposure period. Total RNA was extracted from the liver, labelled and profiled using the Agilent miRNA microarray platform (Agilent Technologies, USA). MiRNA expression was normalised to the 75th percentile, and miRNAs that were not detected in at least 50% of the samples of any test group were excluded from the evaluation. Thereby, 21 miRNAs were identified as differentially expressed, and hierarchical clustering revealed specific patterns of miRNA expression (Koufaris *et al*, 2012). These miRNAs appeared to regulate pathways that are frequently disrupted during chemical carcinogenesis or implicated in the progression or suppression of carcinogenesis; these same miRNAs had previously been found to be dysregulated in tissue-specific tumours. Bioinformatic analysis indicated that specific pathways (such as phosphoinositide 3-kinase or epidermal growth factor) were targeted and over-represented in the analysis. This points to the need to assess the biological plausibility of the predicted miRNA-regulated pathways in respect to cancer development.

Further, a 14-day rat oral toxicity study was conducted to investigate whether the tumour promoter phenobarbital elicits miRNA-related effects in a temporal and dose-dependent manner. Again, total RNA was extracted from the liver, labelled and profiled using the Agilent microRNA microarray platform. While there were no obvious statistically significant microarray responses during the first 7 days of treatment, within 14 days, clustering could be observed and a distinct dose-related time-dependent effect on the miRNA expression profiles could be shown (Koufaris *et al*, 2013). In summary, the mentioned 90-day and 14-day studies suggest that both genotoxic and epigenetic carcinogens can dysregulate miRNA expression to

produce a ‘fingerprint’ that can be detected long before tumours develop in the treated animals. This miRNA ‘fingerprint’ appears to be compound, dose and temporally regulated, and details of the miRNA ‘fingerprint’ may offer insight into potential MoAs. Consequently, the concept that miRNAs are biomarkers of toxicity such as chemical-induced carcinogenesis is highly attractive and offers the potential of translational biomarkers of progression of the disease (Koufaris *et al*, 2013; Gooderham and Koufaris, 2014).

Q&A

How was differential expression of miRNAs quantified? Which specific statistically measurable differences in miRNA expression were recorded? - Between 3-5 animals per treatment group were used. RNA quality was determined using an Agilent Bioanalyser, and expression was assessed using the Agilent miRNA microarray platform and microarray scanner. The data were collected using the Agilent Feature Extraction software, for miRNA hybridisation signals a threshold of 1 was set, and the signals were log-2 transformed and normalised to the 75th percentile using the GeneSpring GX software. One-way ANOVA with Benjamini-Hochberg multiple testing correction followed by Tukey’s test was used to identify dysregulated miRNAs. Targeted quantitative real-time polymerase chain reaction (qRT-PCR) was used to confirm differential miRNA expression between control and test groups.

Are there miRNA reference databases against which to anchor data, such as the ones collected during the mentioned studies? – At the Imperial College London, a number of bioinformatic packages and databases to interpret the miRNA data are applied. These include packages to predict miRNA targets (miRWALK, DIANA-mt, miRanda, miRDB, RNAhybrid, PICTAR, PITA, RNA22 and Targetscan), the KEGG database and PANTHER (both open source) to pathway map predicted targets and Gene Expression Omnibus for archiving miRNA expression data.

Could similar investigations also be performed for other organs? – Yes, in addition to the liver, similar analyses were performed on the kidneys, intestinal tissue, mammary tissue, cardiac tissue, biological fluids and numerous cell lines and culture media. In the presented animal studies, blood samples were also analysed. However, the sensitivity of detecting miRNA in the blood was lower than in, e.g. the liver tissue.

2.2 Session 2: Understanding possible links between changes in ncRNA and pathology

2.2.1 NcRNA therapy: Upcoming medical applications

Achim Aigner, Rudolf-Boehm-Institute for Pharmacology and Toxicology, Leipzig University, Germany:

In the past years, increasing insight has been gained into the physiological and pathophysiological roles of many important classes of ncRNAs. This also included the discovery of new gene silencing mechanisms, such as RNA interference (RNAi) or miRNA-mediated inhibition of specific protein synthesis. These discoveries substantially enhance the understanding of intra- and extracellular communication beyond proteins and provide important information on the basis of various diseases involving aberrant ncRNA expression. Importantly, they also allow for the exploration of these mechanisms for therapeutic purposes. Major players of RNA-based therapies include antisense oligonucleotides and siRNAs (that target all forms of RNA), locked nucleic acid anti-miRs, tiny locked nucleic acid anti-miRs, miRNA sponges and antagomiRs (i.e. chemically modified oligonucleotides that target specific miRNAs), miRNA mimics and ribozymes.

As a rule, RNA-based therapeutic agents are too large as molecules, negatively charged and instable in biological fluids. This constitutes technical disadvantages over many other drugs mainly with regard to poor pharmacokinetics. Therefore, chemical modifications and/or formulations have to be implemented to allow for therapeutic use. Despite many years of research, however, the issue of delivery is still a major obstacle in therapeutic RNA use. On the other hand, RNA-based therapeutics allow for broader target selection and offer new therapeutic strategies. Dependent on the RNA, they can address different MoAs that may be based on physiological processes. Generally, RNA-based therapeutic agents do not efficiently elicit antibody responses, even when they are bound to proteins (Ling *et al*, 2013). NcRNA-based therapies either aim at restoring normal ncRNA functionalities or at using small RNA molecules to trigger desired therapeutic effects. For example, RNAi allows for the inhibition of any target gene of choice. This provides the opportunity to develop novel concepts in therapy also with regard to otherwise 'undruggable' genes.

Consequently, since its discovery in the late 1990s, RNAi-based therapeutic drugs have been brought into more than 50 clinical trials involving 26 different siRNAs (Ling *et al*, 2013; Lundin *et al*, 2015; Wittrup *et al*, 2015). Specifically, two phase III trials are in progress to treat familial neurodegenerative and cardiac syndromes caused by transthyretin mutations (Singh and Peer, 2016). More recently, explorations on the potential of miRNA replacements or miRNA inhibitions have also been initiated, e.g. to reduce hypertrophic dermal scarring by targeting connective tissue growth factor or to treat pancreatic cancer (Singh and Peer, 2016). Outstanding major issues in ncRNA therapy, however, are targeted RNA-drug delivery to specific organs, specificity, efficacy and absence of side effects (Wittrup *et al*, 2015). Various RNA-delivery strategies, including the use of nanocarriers, and chemical RNA modifications have been developed and are currently under pre-clinical and clinical investigation (Dai and Tan, 2015; Grünweller and Hartmann, 2016; Singh and Peer, 2016).

Q&A

How are doses selected for phase I and II trials? - This will depend on *in vitro* and *in vivo* studies that provide an indication on relevant concentrations. As a rule, concentrations will be very low, and only few molecules will end up reaching the target.

How specific are the effects elicited by RNA-based therapeutic agents, and are side effects to be expected? - Traditional chemotherapy is a good example for non-specificity, whereas RNA-based therapeutic agents allow for sequence specificity as has been explored in the field of miRNA inhibition or siRNA-mediated gene knockdown. Sequence specificity increases with decreasing length of the miRNAs. Currently, the major problem is to ensure the delivery of the RNA-based therapeutic agents, and strategies also include approaches for targeted delivery to a specific organ. Independent of delivery issues, the selection of a ncRNA without off-target effects is crucial.

How can one validate that a specific miRNA was successfully inhibited in the test animals? – This is performed by evaluating the miRNA itself or by selecting and assessing relevant target genes. This also allows for *in vitro* - *in vivo* comparisons.

Which animal species is best suited for preclinical studies? – An important determinant is efficacy, which will depend on the pathology in the best matching species. To investigate tumour therapies, rats or mice are frequently well suited. For other pathologies, the selection of the best suited animal species may be less straight forward.

2.3 Session 3: Technical issues around ncRNA in risk assessment

2.3.1 Data analysis and biomarkers

Frank Slack, Department of Pathology, Director, Institute for RNA Medicine, Beth Israel Deaconess Medical Center - BIDMC Cancer Center Harvard Medical School, USA

MiRNAs have been found in all multicellular eukaryotes investigated so far, where they are important regulators of gene expression, usually acting post-transcriptionally. MiRNAs may be disease-related loci, e.g. oncogenic and tumour suppressor loci. As such, they may regulate the expression of important disease genes, e.g. oncogenes and tumour suppressors (Chen *et al*, 2008; Chin and Slack, 2008; Lawrie *et al*, 2008; Mitchell *et al*, 2008). Accordingly, miRNAs may be useful diagnostic and prognostic markers for diseases, and they are emerging as therapeutic and targeted therapeutics in different diseases, including cancer (Lu *et al*, 2005; Calin and Croce, 2006; Kasinski and Slack, 2011). For instance, RNA profiling points to miR-34a as important and prognostic in triple negative breast cancer (Kato *et al*, 2009; Adams *et al*, 2016).

A suitable starting point in investigating the applicability of miRNAs as therapeutic targets or agents is to measure their relative or absolute levels in normal and diseased tissues. MiRNA profiles may be obtained from (fresh, fixed or frozen) tissues, organs, cells and subcellular fractions as well as from all relevant body fluids. MiRNAs are stable in the blood and other body fluids (Weber *et al*, 2010; Mitchell *et al*, 2008). Technologies for detecting miRNAs include Northern blotting, *in situ* hybridization, (low density) qRT-PCR, microarray technologies, and miRNA sequencing (Johnson *et al*, 2007; Chiang, 2014). In a comparative assessment, each technology appeared to have its own strengths and limitations. Hence, even though miRNAs rankings were concordant between different technologies, their outcomes could not be compared quantitatively (Baker, 2010). One of the drawbacks of miRNA-sequencing is that many miRNAs have isomeric forms so that it is hard to directly compare with detection systems that are not always fully precise. Further challenges to the detection and diagnostic use of miRNAs include the small sizes of the molecules, delivery and specificity issues, the determination of 'normal' miRNA levels, and the fact that very many miRNAs are only expressed at very low levels (Cheng *et al*, 2015). Even though the available technologies allow detecting such low levels of miRNAs, their biological implications remain to be determined.

Generally, whereas miRNA expression profiling provides first information on biomarkers of interest, miRNA levels alone do not allow determining their functionalities (Pritchard *et al*, 2012). As a rule, such determinations require mouse knock-out or knock-in models or 'therapeutic' interventions, using, e.g. anti-miRs that inhibit miRNA function and or expression, miRNA mimics that increase miRNA expression, viral pre-miR and miRNA sponges or miRNA luciferase sensors.

Q&A

What are the implications that investigations on the functionality of miRNAs generally inhibit the miRNA? – Generally, when the miRNA is inhibited one sees that the corresponding target factors are increased in expression. This can be complemented with additional experiments where a miRNA mimic is used to increase the levels of a given miRNA. This should show the opposite effect on the expression of target factors.

What is the specificity of miRNA that can be detected in the blood? – The miRNA profiles of a given tumour and concordantly collected blood samples may not be the identical, because the tumour may release only some miRNAs. However, further comparative investigations are necessary to determine the biological implications of such findings.

How stable are miRNA in tissue samples or body fluids? – Generally, they are very stable and may be detected and recorded even after decades provided that samples were stored appropriately.

2.3.2 Risk assessment considerations for ncRNA in agricultural products and applications

Jan Verhaert (and Jay S. Petrick), Monsanto, Belgium

The RNA interference (RNAi) pathway has been recognised in crops for over a decade. Recent advances in technology have led to advances in agricultural applications based on the RNAi pathway. RNA-based traits are the basis for phenotypes in conventional crops, with soybean seed coat colour and maize stalk colour serving as examples of RNA-based gene regulation harnessed through selective breeding (Tuteja *et al*, 2004; Della Vedova *et al*, 2005; Koseki *et al*, 2005). In addition, RNA-based technologies have been successfully employed to introduce new traits in crops, such as virus resistance, altered oil composition in soybeans, and insect protection against corn rootworm. These types of traits have been risk assessed and approved by multiple regulators across the globe. RNA has an extensive history of safe consumption and humans and animals routinely consume small RNAs and longer double stranded RNAs in staple foods that have 100% sequence identity to the consuming human or animal without impact to health (Ivashuta *et al*, 2009; Jensen *et al*, 2013; Frizzi *et al*, 2014). This safe consumption results in part from extensive barriers to ingested RNAs, such as low gut pH, nucleases, multiple membrane barriers, and rapid renal elimination of RNA (Petrick *et al*, 2013). These barriers are also evidenced by drug delivery challenges faced by developers of oligonucleotide-based drugs (Juliano *et al*, 2009; O'Neill *et al*, 2011; Petrick *et al*, 2013). Further studies in mammals indicate that ingested double-stranded RNAs, even those targeting a gene in the test species, do not produce adverse health effects in these animals (Petrick *et al*, 2015). Whereas RNA-based technologies provide new tools to address agricultural challenges, the overall weight-of-evidence including historical knowledge as well as new empirical evidence shows that these technologies are safe and fall within the current safety assessment process.

3. PANEL DISCUSSION: DATA INTERPRETATION TO ENHANCE THE APPLICABILITY OF NCRNAS AS BIOMARKERS FOR RISK ASSESSMENT

Moderator: Saskia van der Vies, VU University Medical Centre, NL

Panel: Frank Slack, Harvard University, USA; Reza Rasoulpour, Dow, USA; Kerstin Schmidt, BioMath GmbH, Germany; Tim Gant, PHE, UK

3.1 Statements from the panel

How is the abundance of ncRNA currently managed? Which knowledge gaps and knowledge needs prevail? Which data are relevant for RA? What is key to data analysis and data evaluation?

F. Slack: It is equally evident that ncRNAs have great potential to be used as biomarkers and that extensive knowledge gaps must be addressed before this goal may be met. Current investigations focus on analysing data to determine the best suitable ncRNAs and how they fit into specific pathways. Generally, knowledge on ncRNAs is just beginning to evolve, and new classes of RNAs, such as circular RNAs, have only been discovered very recently. Therefore, also an understanding on their tissue-specific presence or functionalities is still at its very beginning. It is very likely that further classes of RNAs remain to be discovered, just as miRNAs may not be the smallest RNAs present in organisms.

R. Rasoulpour: The available knowledge on ncRNAs may indirectly or directly benefit substance RA, i.e. to concertedly design new molecules and to reveal specific ncRNA signatures in those animal species that are relevant for regulatory toxicology. In combining exposure and hazard assessment during RA, ncRNA profiling may provide biological explanations on mechanisms of toxicity that specific substances may affect. Eventually, such ncRNA profiling may provide opportunities to improve regulatory toxicity testing.

K. Schmidt: The time- and dose-dependent up- and down-regulation in response to toxic compounds qualifies ncRNAs as useful biomarkers for toxicological studies. NcRNA expression profiles might supplement or even substitute conventional parameters, obtained by, e.g. haematology or clinical biochemistry. NcRNAs as new endpoints could be integrated into consolidated test vs. control group comparisons, and, basically, the familiar principles to statistically analyse potential apical effects and dose-response relationships are also adoptable to ncRNA expression profiling. In fact, such approaches are very similar to current toxicological testing; even though only single parameters are measured in the current toxicity tests, these parameters are combined for an assessment of complex endpoints. Challenges of quantification and data interpretation are not limited to the harmonisation of techniques to measure ncRNAs. Standardised procedures are also needed for data normalisation and referencing. Further, standard statistical estimators must be established to ensure comparability and to facilitate assessment, also of the biological relevance of effects. Historical control data and effect sizes of potential toxicological interest (e.g. in respect to the up- or down-regulation of genes) have to be established. It has to be clarified whether the test group sizes indicated in current test guidelines have sufficient power to detect ncRNA effect sizes. Finally, modern statistical methodologies and

presentation methods should be implemented to enhance comprehensive analyses and interpretation of results (Schmidt *et al*, 2016).

T. Gant: Research on ncRNAs is a technology-driven process that yields an abundance of data. In making use of ncRNA expression profiles for RA, interpretation of the data is the biggest challenge, as is also known for ‘-omics’ technologies. Some of the information that these technologies provide is not necessarily fully understood. Nevertheless, it is beneficial to collect all data, even though it may be challenging to manage large datasets. Knowledge gaps in respect to evaluating data by applied bioinformatics prevail. It is not yet understood which of the data are relevant for toxicological RA, which changes in ncRNA expression are causal and which are consequential, or how ncRNAs are involved in toxicological mechanisms. Such an understanding, however, is a prerequisite to selecting ncRNAs as biomarkers for RA. Presumably, different types of substances and different patterns of change are related to specific mechanisms of toxicity.

3.2 Panel and plenary discussion

Alterations in ncRNA expression profiles may indicate a cell’s attempt to regain homeostasis. As such, they may be recorded before the cell is irreversibly damaged. Accordingly, relevant ncRNAs may be early predictors of different toxicological pathways making them potentially useful biomarkers for regulatory toxicology. The establishment of dose-response relationships is essential to building mechanistic understandings. Cells may counterbalance external insults with sufficient efficiency until a certain level or time of exposure. To understand mechanisms of toxicity, the point at which the system is irreversibly disrupted should be identified.

Extensive discussions addressed the question whether ncRNA expression changes were ‘cause or consequence’ of adverse effects and which implications this may have in making use of ncRNA technologies for RA. While ncRNA expression changes themselves may not be causative, their subsequent downstream effects can induce toxicity. Such changes can be thought of as causal. Those ncRNA expression changes that occur in response to a particular exposure and/or toxicity, but are not involved in the induction of adverse downstream effects, can be thought of as consequential. Also such changes may be useful in RA as biomarkers of exposure. Therefore, even without a full understanding of whether ncRNA expression changes are ‘cause or consequence’, ncRNAs may nevertheless be applied as biomarkers for RA.

From a toxicological point of view, concerns regarding the applicability of ncRNA profiling for RA are similar to the ones that have been voiced in respect to gene mapping. Data may indicate that, e.g. specific carcinogenic genes are enhanced or expressed. Upon too simplistic evaluation, this may be interpreted as giving rise to concern, even if no apical effect results from the genetic alteration. It is essential to understand the phenotypic consequences of a given ncRNA change, i.e. to perform a functional verification and validation of the ncRNA expression profile. At best, the physiological and pathological roles of all ncRNAs that rank high in expression profiles should be known. To date, such functionalities are investigated rather randomly by changing the expression of a specific ncRNA, e.g. by using knockout animals (or genetically modified cell lines) that lack a specific gene or ncRNA and searching for phenotypic (or cellular) alterations.

A way forward in identifying ncRNA fingerprints that may be applicable to determine, e.g. a substance’s carcinogenic potential, may be to comparatively assess ncRNA profiles from different animal species for

substances with known carcinogenicity. Generally, the most relevant animal species and animal model for a potential human issue should be identified, just as the human health relevance of findings should be ensured. Research should also aim at investigating the biological implications of (different levels of) ncRNAs present in body fluids. Even though ncRNAs (just as other biomarkers in the blood) may be exceptionally stable when bound to proteins, they may nevertheless be removed from the blood very quickly, i.e. before sampling can be performed. It is unclear from which organs or tissues the miRNAs present in body fluids come from, or if they are truly specific to the process under investigation. Importantly, research reports should not only include all data that were collected, but they should also clearly describe how the data were collected and analysed. Further investigations should aim at identifying the technology that is best suited to determine biologically relevant ncRNA alterations (that are not merely technological artefacts or sample contaminations).

4. BREAKOUT SESSIONS AIMING AT DEVELOPING RECOMMENDATIONS FOR RESEARCH PROPOSALS

4.1 Introductory discussion

Generally, there are three important drivers of ncRNA research. First, the 3Rs principle to reduce the number of studies and the number of animals used in the studies (Russell and Burch, 1959) that has been implemented in *Directive 2010/63/EU on the protection of animals used for scientific purposes*. Second, the motivation to accelerate substance development and the RA process. Third, the incentive to gain knowledge that is of increased biological and toxicological relevance. Accordingly, research on ncRNA should not merely strive to replace apical endpoints by earlier molecular / cellular effects but aim to create a new toxicological paradigm that provides much more comprehensive information. Even though this goal may only be reached in the long term, it is of high scientific relevance since an improved biological understanding of mechanisms of toxicity will serve to improve the RA of substances and products. Therefore research should also address scientific deficiencies of standard toxicity tests and opportunities for ncRNAs to contribute to overcoming such deficiencies (e.g. for specific endpoints, such as non-genotoxic carcinogens and reproductive / transgenerational toxins, including *in utero* exposure leading to adult diseases). Nevertheless, to begin with, it may be beneficial to include ncRNA profiling, just as other ‘-omics’ technologies, in the control groups used in Good Laboratory Practice-compliant toxicological studies. Such investigations should preferably be conducted as collaborative studies and will enable a prospective comparative assessment of the new tools and the collection of ncRNA data within a regulatory context.

4.2 Breakout session 1: Role of ncRNAs in toxicology and risk assessment

Questions:

1: How to make use of ncRNA technology in toxicology and chemicals risk assessment?

What is relevant and not relevant? What do ncRNAs really tell us? How far do ncRNAs really add information to toxicology and RA? What role for ncRNAs in toxicology and chemicals RA in the next 5-10 years?

2: In light of what we know about ncRNAs – should we evolve and adapt current toxicology and the risk assessment of substances?

What do changes in ncRNA expression mean regarding the phenotype? What is relevant (e.g. cross-kingdom RNA effects)? What are the implications for industry/regulations? Are the changes in the expression of ncRNAs helpful in our understanding of MoAs?

Outcome breakout group 'green'

Moderator: Helmut Greim, rapporteur: Alan Poole

Research activities should aim at collecting and merging available relevant information on ncRNAs, at best, by pooling data from different companies and institutions. For the time being, ncRNA-related research aiming at identifying biomarkers for regulatory toxicology and RA should not be restricted to promising miRNAs, even though current research is most advanced for this class of ncRNAs. Instead, it should also be open to possibly emerging evidence on the suitability of other classes of ncRNAs. The relevance of specific ncRNAs should be defined in studies that include both ncRNA expression analysis and phenotype assessments. This will serve to identify key ncRNAs that are relevant for cell homeostasis or pathogenesis. For this purpose, the range of normal variations of ncRNA expression should be identified, most importantly, by setting up historical control group databases. The variability observed in historical control datasets will provide an overview on the ranges of normal and abnormal expression levels, just as relevant ncRNAs may be identified. Further, the dose-response relationships of deviations from normality should be determined. Ideally, the knowledge on ncRNAs should be integrated into AOPs, e.g. by identifying the specific consequences of ncRNA alterations on different levels, including protein levels, enzyme compositions, and deviations from the phenotype. Finally, the standardisation and validation of ncRNA expression profiling technologies should be advanced, and guidelines on the reporting of data and results from ncRNA studies should be set up.

Outcome breakout group 'red'

Moderator: Tim Gant, rapporteur: Madeleine Laffont

MiRNAs were identified as the currently most developed class of ncRNAs to be subjected to research projects investigating their applicability for RA. A comprehensive literature review should be conducted to determine specific miRNAs, and also lncRNA candidates. Criteria to determine the relevance of ncRNAs include pathological functionality, e.g. tumour promotion for oncogenic ncRNAs, and organ specificity. Such information should be evaluated to draw up a list of ncRNA candidates that may be suitable biomarkers. Research addressing the possible relevance of changes in the expression of selected miRNAs for toxicological assessments should strive to distinguish between physiological variations and pathophysiological alterations. To date, it is unclear which order of magnitude is relevant, i.e. which changes may lead to downstream effects on established target genes. Further, specific MoAs have not yet been discerned. These questions should be pursued both in literature reviews and in specific research projects. Research should also aim at determining relevant test systems. For the time being, ncRNA expression profiling should be integrated into established test methods, such as Good Laboratory Practice-compliant rat 90-day oral toxicity studies that include properly defined kinetics and (histo-)pathological evaluations. Thereby, ncRNA assessments will be performed in parallel with other readouts.

Relevant body compartments for ncRNA analysis need to be defined. This includes non-invasive (blood, urine, and other body fluids) and invasive assessments (classical additional target organs depending on prior non-invasive analysis serving to identify representative miRNAs for specific organs or for specific early events, e.g. in respect to tumour development). As the example of miRNAs involved in paracetamol hepatotoxicity reveals, miRNA effects may be investigated in a time-dependent manner already during early

stages after substance administration when conventional parameters are not yet affected. In fact, this constitutes one of the advantages of miRNA profiling over conventional techniques. Also with respect to substance-induced carcinogenicity, different miRNA expression profiles are to be expected during pre-tumour and post-tumour investigations. Hence, changes in ncRNA expression should always be investigated in a time-dependent manner and they should always be related to normal expression levels.

Finally, ncRNA-related research should not be restricted to the investigation of carcinogenicity, but it should cover a wide range of toxicological endpoints. The pathological consequences and respective changes in ncRNA expression of long-term low-dose substance applications should also be addressed. An understanding on mechanisms of toxicity involving ncRNAs will form a basis to determine animal species-specific and tissue-specific differences in susceptibility to substance-induced effects.

[Outcome breakout group 'blue'](#)

Moderator: Roland Buesen, rapporteur: Achim Aigner

Research on ncRNAs should serve to use the respective technologies to modify systems (plants, therapeutic agents) and to advance the applicability of ncRNAs as biomarkers for the toxicological assessment of (non-ncRNA-based) substances. With respect to the latter, ncRNA-related tools will most likely not be beneficial in isolation, but they will add to a weight-of-evidence during RA, for instance as biomarkers to predict carcinogenicity. It will be of great value for RA to identify ncRNAs that are related to the development of specific apical effects and to trace them *in vitro* and *in vivo* and across animal species and strains. Eventually, a given ncRNA's predictivity of apical effects may be more important than its causality.

Research efforts to advance the applicability of modern technologies, including ncRNAs and '-omics', for RA should strive for application-oriented validation. In identifying relevant ncRNAs, research should focus on their potential to serve as non-invasive biomarkers released into various body fluids. A targeted approach should also be applied in order to advance knowledge in areas of toxicology where current test methods are scientifically deficient.

4.3 Breakout session 2: Where are the research priorities and focus areas for industry and other funders?

Bruno Hubesch (Cefic LRI, Belgium) presented the LRI, a global research programme jointly conducted by the International Council of Chemical Associations (ICCA), the American Chemical Council (ACC), and Cefic. Within the Cefic LRI research programme, projects are funded in specific priority areas. The present workshop falls under the priority area 'innovating chemical testing'. Here, Cefic LRI contributes to the development of toxicological methods and testing strategies that allow linking molecular data to human health and environmental impacts. In breakout session 2, the workshop participants were invited to formulate specific project ideas for Cefic LRI to advance within its research programme.

In the long term, all proposed project ideas should serve the 3Rs principle by eventually reducing the need for animal testing, and they should improve cost efficiency and the timely access to new product developments.

Project ideas from breakout group 'green'

Moderator: Alan Poole, rapporteur: Nigel Gooderham

1. Identification of gaps in toxicology that ncRNAs may contribute to filling

In certain areas of toxicology, the experimental approaches on how to obtain data are scientifically deficient. Such knowledge gaps include, but are not restricted to ways to assess non-genotoxic carcinogens and reproductive / transgenerational toxins, including *in utero* exposure leading to adult diseases, the effects of long-term low-dose exposure and problems in addressing mixtures in toxicology. Specific ncRNAs may contribute to filling such knowledge gaps. To determine their applicability as biomarkers for regulatory toxicology, the phenotypic consequences of different ncRNA expression levels, including a profile of the 'normal' situation and its variations in maintaining cellular homeostasis, remain to be elucidated.

A comprehensive literature review should be conducted to establish the state-of-the-art ncRNA research for promising topics, taking into account the areas of toxicology listed above. The systematic review should cover all available relevant information on ncRNAs (with relevant studies covering both ncRNA expression analysis and phenotype assessments). The literature review should form the basis for the design of subsequent experimental projects to identify toxicologically relevant ncRNAs. These experimental projects may be conducted in the form of case studies investigating, e.g. changes in the expression of specific ncRNAs taking into account dose-response relationships and temporal aspects. Such case studies should address whether the polymorphism of ncRNAs is likely to have functional consequences and whether specific epigenetic events govern other epigenetic events.

2. Data fusion approaches

Data fusion approaches should be used to advance a meaningful and comprehensive interpretation of ncRNA expression profiles, '-omics', epigenetics, and classical toxicological endpoints including (histo-)pathology. Even though the precise relationships between these data (or technologies) are not yet understood, they all merge into specific AOPs. Data fusion approaches will provide added value from existing information.

3. Standardisation, verification and validation

Consensus should be developed on how to conduct ncRNA expression profiling in a toxicological and regulatory context; including best practice of reporting the outcome of such studies. This should include initiatives to improve standardisation of the respective technologies to form a basis for their verification and validation and to set up guidelines for reporting the outcome of ncRNA studies.

Taken together, the three projects serve to identify key ncRNAs that are relevant for cell homeostasis and/or pathogenesis and to advance a mechanistic understanding of toxicological pathways. The outcomes of the projects should be used to integrate knowledge on ncRNAs into AOPs, e.g. to identify the consequences of changes in the expression of a given ncRNA on the subsequent steps of a relevant AOP, including protein levels, enzyme compositions, and deviations from the phenotype.

Project ideas from breakout group 'red'

Moderator: Reza Rasoulpour, rapporteur: Saskia van der Vies

1. Literature survey

A comprehensive literature survey should review the state-of-the-art of the role that ncRNAs play in, e.g. tumour development and to identify key ncRNAs as predictive biomarkers of tumour formation. At least initially, well-established ncRNAs should be addressed with priority, which will most likely mainly include miRNAs. Nevertheless, the survey should not be limited to miRNAs, and optimal candidates will have to be defined with regard to tissue specificity and pathological functionality, i.e. especially tumour promoting properties. As an outcome of the literature survey, a list of candidate ncRNAs to choose from for the subsequent experimental project should be drawn up. This list may also include new, hitherto untested ncRNAs. Beyond the assessment of differences that will truly be relevant for toxicological assessments (physiological variations vs. pathological changes; see above), optimal testing systems have to be defined with regard to pathology / readout and time kinetics.

2. Experimental project

A 90-day rat study should be performed to substantiate the relevance of selected ncRNAs as biomarkers for toxicity studies. For instance, it might be shown that a hepatotoxic substance does not change the ncRNA profile in other organ systems. Substances that are non-genotoxic carcinogens in different tissues should be selected as test substances. The study should be designed to allow the determination of dose-response relationships, and it should include ncRNA profiling of blood samples. Further, the choice of rat strain should be justified, just as the selection of 2-3 target organs (e.g. liver, thyroid, and kidney). All experiments should be run in parallel in multiple laboratories, and blinded samples should be used for miRNA analysis. The study should aim at linking ncRNA expression profiles to pathological findings (phenotypic alterations). Subsequent follow-up research should include a verification and validation of the ncRNA expression profiling by using unspecific chemicals, the inclusion of further organ systems, and a comparative analysis of miRNA expression profiles with '-omics' and epigenetics techniques.

Project ideas from breakout group 'blue'

Moderator: Jörg Hackermüller, rapporteur: Madeleine Laffont

1. Literature or experimental project to investigate possible exposure-effects from exogenous ncRNAs

A literature survey or, if the available data is insufficient, an experimental project should further elucidate if ncRNAs that are known to affect many gene expression pathways may be taken up systemically upon oral exposure, and, if so, if they may exert gene expressional functions in the cells of the ingesting organism.

2. Mining of existing data and literature survey to identify ncRNAs as potential biomarkers for RA to be progressed to a validation project

This project consists of 3 phases, i.e. (1) data mining and identification of candidate ncRNAs; (2) testing and validation with a focus on *in vitro* test systems; (3) increase of functional understanding of the candidate

ncRNAs. Applying a targeted approach, the expression profiles of specific ncRNAs, e.g. miR-155, miR-122, or HOTAIR, should be determined and evaluated to improve an understanding of MoAs. Such work may also address areas of toxicology where current methods to understand MoAs are poor, (e.g. non-genotoxic carcinogenesis, immunotoxicity, reproductive / intergenerational toxicity, or low-dose and long-term toxicity). Normal ncRNA expression profiles and inter-individual variations in control groups should be determined and used to assist in the interpretation of substance-induced changes in ncRNA profiles. Generally, the project should take into account the results from relevant disease-related genomics projects, and data-sharing between different companies and public health departments should be encouraged. As an outcome of this project, new predictive biomarkers of toxicity and disease states should be identified.

3. Best practices in bioinformatics for analysis of ncRNA data

This project should be linked with the ECETOC ‘-omics’ programme with separate consideration of ncRNA-specific principles for data analysis, as necessary.

4. Workshop: Microbiome

Changes in the microbiome (i.e. the spectrum of (genes of) microorganisms present in a given organism) may adversely affect human health. Accordingly, external stressors that may change the microbiome may also elicit adverse effects. Against this background, a workshop may be convened to identify research needs that serve to elucidate how the microbiome may modify substance exposure by metabolising or chemically modifying these substances, and how the microbiome is modified by specific substances, including short ncRNAs.

5. CLOSE OF THE WORKSHOP

Wrapping up the workshop, *Helmut Greim, Munich Technical University, Germany*, recollected that ncRNAs play important roles in maintaining cellular homeostasis and in the evolvement of phenotypic alterations. To make available ncRNAs as biomarkers for regulatory toxicology and RA, normal and adverse ncRNA profiles and dose-response relationships of effects should be determined, and ncRNA expression profiles should be linked to phenotypic alterations. Further, it should be determined whether ncRNA levels in specific body fluids reflect levels in specific target tissues. Even though a number of research projects demonstrated a lack of toxicologically relevant uptake and activity of ingested RNAs, bioavailability of ingested ncRNAs and potential impacts to the consuming organism may merit further investigation.

In the long-term, knowledge of ncRNAs may serve to understand specific mechanisms of toxicity, e.g. to assess genotoxic and non-genotoxic carcinogenicity, reproductive / transgenerational toxicity, mixture toxicology, and long-term low-dose effects. All ncRNA methodologies should be standardised and validated and guidance for the reporting of data should be established. NcRNA analysis tools can be incorporated into standardised *in vitro* assays and *in vivo* repeated-dose studies used for RA applying a holistic approach that may take epigenetic mechanisms, gene methylation and acetylation and ‘-omics’ technologies into account. Importantly, all those involved in regulatory toxicology and the RA of substances should be continuously informed on the potential applicability of ncRNA data for RA. In the long term, ncRNA expression profiling may improve RA by enhancing hazard predictions at earlier stages, and it may serve the goals to reduce animal testing as well as reduce the costs and time required to market innovative products.

ABBREVIATIONS

3'-UTR	3'-untranslated regions (of mRNAs)
AGO	Argonaute
AOP	Adverse outcome pathway
Cefic	European Chemical Industry Council
ECETOC	European Centre for the Ecotoxicology and Toxicology of Chemicals
esiRNA	Endogenous short interfering RNA
HOTAIR	Hox gene transcript antisense RNA
IL	Interleukin
lncRNA	Long noncoding RNA
LRI	Long-range Research Initiative
MIE	Molecular initiating event
miRNA; miR	MicroRNA
MoA	Mode-of-action
mRNA	Messenger RNA
ncRNA	Non-(protein-)coding RNA
nt	Nucleotide
qRT-PCR	Quantitative real-time polymerase chain reaction
piRNA	Piwi interacting RNA
Piwi	P-element induced wimpy testis in Drosophila
RA	Risk assessment
RNAi	RNA interference
siRNA	Short interfering RNA
STAT3	Signal transducer and activator of transcription 3
xenomiR	Xenobiotic microRNA

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APPENDIX A: WORKSHOP PROGRAMME

PROGRAMME DAY 1 – MORNING

10:00-10:30 Registration and welcome coffee

10:30-10:40 Welcome and Introduction

Alan Poole

ECETOC Secretary General

Session 1: Understanding ncRNAs

10:40-11:00 What are ncRNAs?

Tim Gant

PHE, UK

15 minutes + - Definitions
5 min Q&A - Spectrum of ncRNA (sizes, biogenesis)

11:05-12:00 What do they do?

15 minutes + - Mechanism of action, Functional Relevance
5 min Q&A - Importance of negative results

Gunter Meister

Regensburg University, Germany

15 minutes + - Expression of macroscopic phenotypes for miRNA-guided
5 min Q&A regulation at the molecular level

Hervé Seitz

CNRS, France

15 minutes + - Cellular adaptation – relevance for risk assessment
5 min Q&A - Physiological interpretation – phenotypic anchoring

Emma Marczylo

PHE, UK

12:00-12:20 Stability and transport

15 minutes +
5 min Q&A - Cross-kingdom RNA Effect

Kenneth Witwer

Johns Hopkins University
School of Medicine, USA

PROGRAMME DAY 1 – MORNING (CONT'D)

12:20-12:40	Cause or Consequence?	Jörg Hackermüller UFZ, Germany
<i>15 minutes + 5 min Q&A</i>	- What are the links between ncRNA, changes in ncRNA, expression and pathological phenotype	

12:40-13:00	Exposure effects?	Nigel Gooderham Imperial College London, UK
<i>15 minutes + 5 min Q&A</i>	- Relevance of dose level / time / no effect level / adverse effect level	

13:00-14:00 *Lunch*

PROGRAMME DAY 1 – AFTERNOON

Session 2: Understanding possible links between changes in ncRNA and pathology

14:00-14:20	ncRNA Therapy	Achim Aigner Leipzig University, Germany
<i>15 minutes + 5 min Q&A</i>	- Upcoming medical applications - What are the current medical applications of ncRNAs? - How can this help inform toxicology and risk assessment science? - Species differences: do animal models inform about human pathology?	

14:20-14:40	Biomarkers	Mohamed Benahmed UNICE, France
<i>15 minutes + 5 min Q&A</i>	- Technical issues around ncRNAs as biomarkers, (biomonitoring – fluids, and link to pathology – cells/tissues/organs)	

PROGRAMME DAY 1 – AFTERNOON (CONT'D)

Session 3: Technical issues around ncRNA in Risk Assessment

14:40-15:00	Data analysis	Frank Slack BIDMC Cancer Center Harvard Medical School, USA
<i>15 minutes + 5 min Q&A</i>	<ul style="list-style-type: none"> - What are the key analysis methods currently employed? - Is there a need for harmonisation? - Contamination: is what you are measuring actually what you think you are measuring? 	

15:00-15:30 *Coffee*

15:30-16:00	Panel Discussion	Moderator: Saskia van der Vies VU University Medical Centre, NL
<i>20 minutes discussion + 10 min Q&A</i>	<p>Data Interpretation of ncRNAs as biomarkers</p> <ul style="list-style-type: none"> - There is an explosion of ncRNA data available, how is it currently managed? - What are the gaps/needs? - What data are relevant for toxicology and risk assessment? (What data are relevant for regulations?) 	<p>Panel:</p> <p>Tim Gant, PHE CRCE Reza Rasoulpour, Dow Kerstin Schmidt, BioMath</p>

16:00-16:20	Risk Assessment considerations for ncRNA in agricultural products/applications	Jan Verhaert Monsanto, Belgium
<i>15 minutes + 5 min Q&A</i>	<ul style="list-style-type: none"> - Current agricultural applications of ncRNA 	

16:20-17:00	Conclusions of Day 1 Details for Networking Cocktail and Day 2	Alan Poole ECETOC SG
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17:00 *Close*

18:00-19:00 *Networking Cocktail*

PROGRAMME DAY 2 – MORNING

09:30-09:40 **Welcome, Proceedings for Day 2** **Alan Poole**
ECETOC, Belgium

09:40-10:00 **Summary of Day 1** **Saskia van der Vies**
VU University Medical Center, NL

10:00-11:00 **Breakout Session 1:**
Role of ncRNAs in toxicology and risk assessment

Each breakout group brainstorms both these questions. Sub-questions are a guide to prompt discussion

Moderator: Tim Gant / Rapporteur: Madeleine Laffont
Moderator: Roland Buesen / Rapporteur: Achim Aigner
Moderator: Helmut Greim / Rapporteur: Alan Poole

1. How to make use of ncRNA technology in toxicology and chemicals risk assessment?

- What is relevant and not relevant?
- What do ncRNAs really tell us?
- How far do ncRNAs really add information to toxicology and risk assessment?
- What role for ncRNAs in toxicology and chemicals risk assessment in the next 5-10 years? (What can they do and what cannot they do?)

2. In light of what we know about ncRNAs – should we re-evaluate current toxicology and chemicals risk assessment?

- What do changes in ncRNA mean regarding the phenotype?
- What is relevant (cross-kingdom RNA effects)?
- What are the implications for industry/regulations?
- Are the changes in the expression of ncRNA helpful in our understanding of MoA?

11:00-11:30 **Coffee**

PROGRAMME DAY 2 – MORNING (CONT'D)

11:30-12:30 **Plenary:**
Moderators share findings from breakout sessions, with room for audience discussion

12:30-12:45 Afternoon session explained **Madeleine Laffont**
ECETOC

12:45-13:00 Cefic LRI Programme explained **Bruno Hubesch**
Cefic-LRI

13:00-14:00 *Lunch*

PROGRAMME DAY 2 – AFTERNOON

14:00-15:15

**Breakout Session 2:
What don't we know?
Research Priorities for (i) industry (ii) other funders**

Each breakout group brainstorms the same question.

Separate Moderators / Rapporteurs for each group.

30 minutes discussion followed by 45 minutes drafting RfP outline(s).

Moderator: Alan Poole / **Rapporteur:** Nigel Gooderham

Moderator: Jorg Hackermüller / **Rapporteur:** Madeleine Laffont

Moderator: Reza Rasoulpour / **Rapporteur:** Saskia van der Vies

- Which answers are still needed? How can we provide the answers?
- What is currently unsatisfactory about the use of ncRNAs?
- What needs to change?
- What are the priorities?
- Complete outline RfP.

Complete RfP template(s)

15:15-15:45

Coffee

15:45-16:30

Plenary:

Moderators share RfP proposals & outcomes from Session 2. Room for audience participation.

16:30

Conclusions and Close

Helmut Greim

Munich Technical University, Germany

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