New Approach Methodologies

EU-IN Horizon Scanning Report

February 2025
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Executive summary

This horizon scanning report describes current state of art and emerging trends of New Approach Methodologies (NAMs) development and explores considerations from a regulatory perspective encompassing both challenges and opportunities for the regulatory acceptance of NAMs and the advancement of the 3Rs principles in human medicine development and testing. NAMs refer to approaches incorporated into the assessment of the safety, efficacy and quality of a medicinal product to replace, reduce or refine (3Rs) the use of animals. NAMs encompass *in silico*, *in vitro*, *ex vivo* and *in chemico* approaches.

At present, the use of NAMs in regulatory submissions to the European Medicines Agency (EMA) is limited. This is owing to barriers such as developers' caution in sharing NAM data with regulators, driven by concerns over data protection and usage. Moreover, regulators have limited experience in assessing cutting-edge NAM data. A steep increase in the number of EMA early interactions with researcher indicate that new developments are on the horizon, approaching regulatory readiness aimed at wider adoption.

Limited awareness of regulatory frameworks and the complex terminology surrounding the regulatory acceptance of NAMs represent important challenges for NAMs developers. Increased collaboration, data sharing and mutual learning among stakeholders can accelerate progress and ensure that these innovations align with regulatory expectations. Therefore, developers of NAMs are encouraged to engage with regulators early in the development process.

Variations in regulatory requirements between different jurisdictions and regions impede the broader adoption of NAMs. Regulators must collaborate and harmonize their frameworks, provide ongoing support, communication and education.

1. Rationale and objectives of the report

Horizon scanning is the systematic examination of information to detect early signs of scientific and technological developments with previously unknown regulatory challenges or public health opportunities. It aims at enabling the European Medicines Regulatory Network (EMRN, a network comprised of over 50 regulatory authorities for medicines from the 30 European Economic Area countries, the European Medicines Agency and the European Commission), to proactively prepare for forthcoming challenges and opportunities. The European Medicines Agency (EMA) conducts horizon scanning in collaboration with experts and groups such as the EU-Innovation Network (EU-IN)[1]. Horizon scanning entails analysing and forecasting the future importance of selected topics and reporting their potential impact on the EMRN over the next 3 to 10 years. The reports include recommendations to adapt the EMRN (particularly in terms of work practice and capacity) to minimise regulatory bottlenecks, to support developers and to facilitate innovation reaching patients. Horizon scanning is an underlying action of the strategic goals in EMA's Regulatory Science Strategy to 2025[2] and the European Medicines Agencies Network (EMAN) Strategy to 2025[3]. Based on the continual screening of abstracts published by major scientific journals and following a consultation of EMA scientific coordination groups[4], the topic of the 3Rs (*replacement*, *reduction* and *refinement)* of animal use was identified. This report focuses on the regulatory acceptance of New Approach Methodologies (NAMs) in the development of human medicines. It should be noted that the 3Rs principles apply to both human and veterinary medicine development and that the challenges and opportunities highlighted in this report may apply to the veterinary field.

2. Introduction

In 2020, over 8 million animals were used for scientific purposes in the EU, with regulatory uses accounting for 1.4 million animals[5] (Figure 1). Of these regulatory uses, 54% were related to quality testing (most notably batch potency and batch safety testing), 40% related to toxicity and safety testing, including pharmacology, and the remainder (6%) were for other efficacy and tolerance testing. Animal models are currently considered the gold standard for supporting efficacy and safety of medicines prior to clinical trials. However, approximately 80–90% of medicines fail in clinical trials with 40 to 70% failing in phase II/III due to lack of clinical efficacy or toxicity[6]. A gradual shift towards alternative methods would address the ethical concerns associated with animal testing, could provide a more accurate representation of the human condition and allow for more accurate prediction of quality, safety and efficacy of medicines. Thus, progressively incorporating alternative methods to *Replace*, *Reduce* and *Refine* animal models offers significant potential. However, this should not compromise the established standards required for the assessment of medicines. It is essential to maintain a careful equilibrium - embracing these innovative methodologies while upholding the rigorous standards of the EMRN. A gradual and measured transition is key to this process with comprehensive assessment and understanding of these novel approaches.

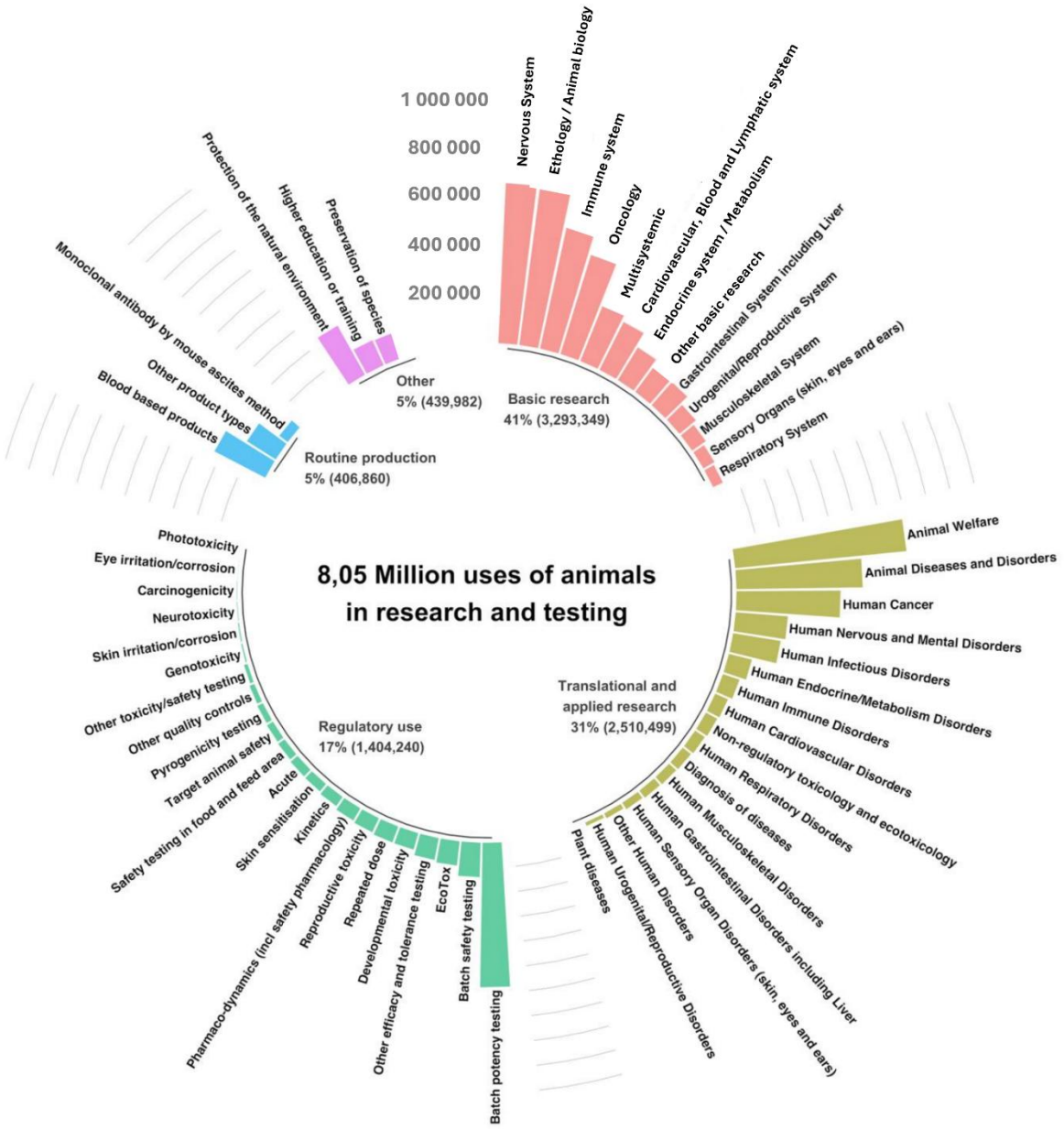

Figure 1. Modified from the Summary Report on the statistics on the use of animals for scientific purposes in the Member States of the European Union and Norway in 2020[5].

The principles of the 3Rs (*replacement*, *reduction* and *refinement* of animal models) were first defined by Russell and Burch in 1959. Since then, the definition has been updated and some variations may exist from one definition to another. Here, we define the 3Rs as follows[7,8]:

- ***Replacement***: refers either to the use of methods or models that avoid the use of live animals or to the replacement of vertebrates with animals having lower potential for pain perception.
- ***Reduction***: minimise the number of animals used per experiment or study, either by enabling researchers to obtain comparable levels of information from fewer animals, or to obtain more information from the same number of animals, thereby avoiding further animal use. Waiving animal studies that do not bring added value to the weight-of-evidence approach of medicine assessment and read-across strategies to avoid superfluous animal use fall under *reduction* strategies.

- ***Refinement***: minimise the pain, suffering, distress or lasting harm that may be experienced by the animals. *Refinement* applies to all aspects of animal use, from the housing and husbandry to the scientific procedures performed on them.

Alternative 3Rs-compliant methods, sometimes referred to as New Approach Methodologies (NAMs), have been under intense development over the past decades. The implementation of NAMs in a regulatory context has been pioneered in chemical toxicology testing, with the primary objective of safeguarding consumer health and safety[9]. In the context of medicines development, NAMs refer to 3Rs-compliant testing approaches to be used for regulatory testing of human and veterinary medicines. This encompasses *in silico*[10,11], *in vitro*[12–14], *ex vivo*[11,15] and *in chemico*[16] approaches. What is 'new' about NAMs is not necessarily the techniques *per se*, but rather their application to the regulatory decision-making process.

3. Current status and trends

3.1. Landscape of NAM research and development

Bibliometric Network Analysis of NAM Research & Development

NAMs intended for regulatory use in medicine development are currently advancing to technology readiness levels sufficient for initial engagement with regulatory authorities and inclusion in weight-of-evidence regulatory submissions[17] and/or in specific contexts of use. An extensive and comprehensive literature review on the state-of-art of NAM development is out of scope for this report. Instead, to prepare and inform the EMRN on developments within the field, an exploratory mapping exercise of the NAM research and development (R&D) landscape was performed. This involved a bibliometric network analysis of NAM-related scientific publications in PubMed from 2009 to 2024 (for details see *Section 6.1*). Briefly, author keywords from scientific articles identified through this literature search designed to capture NAM R&D were extracted. The level of similarity, a measure of interconnectedness of keywords, was calculated based on the co-occurrence of keywords within articles. Then, the most common keywords were plotted on a 2D-network with the proximity of two keywords representing their level of similarity. Clusters are computed automatically based on the level of interconnectedness of keywords. This exploratory bibliometric approach has been used to explore and analyse literature in various fields[18,19]. This provides a quantifiable overview of a research area and allows for the analysis of the morphology of nascent fields and visualization of scientific landscapes [20,21]. It is particularly valuable for obtaining a large picture of complex and multi-faceted fields, such as NAM R&D.

Results from a Bibliometric Network Analysis of NAM-related scientific publications

A total of 42,616 articles from March 01, 2009, until March 01, 2024, were identified. Since 2009, there has been an increasing number of articles related to NAM R&D, from 182 between 2009 and 2012 to 16,782 records between 2021 and 2024, showing a plateau in the last two time-bins 2018-2021 and 2021-2024 (Figure 2). The number of keywords related to NAMs saw a large rise between the 2012-2015 and 2015-2018 time-bins representing an expansion of the applications and technologies in NAM development.

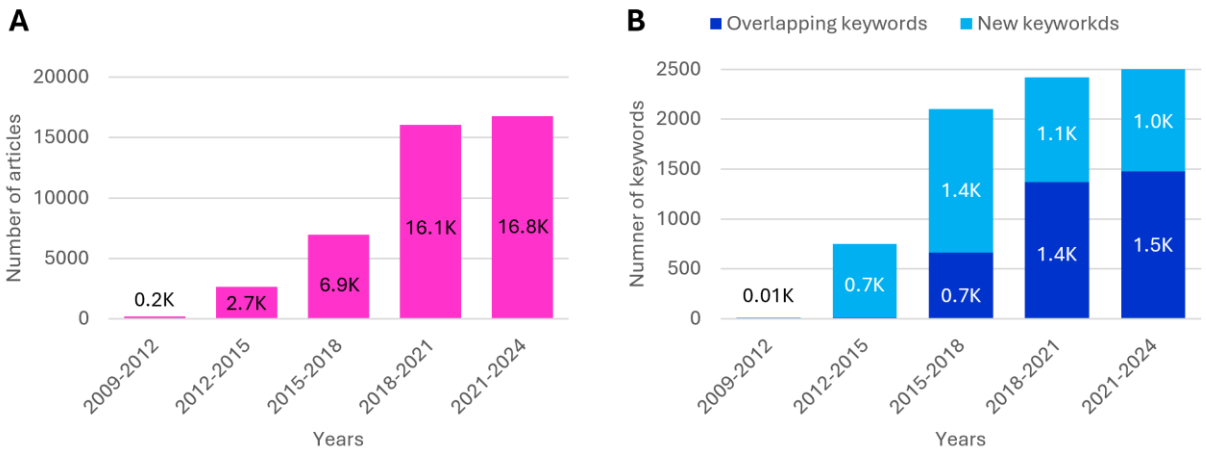

Figure 2. Articles (A) and their keywords (B) pertaining to NAM research and development between 2009 and 2024.

It is clear from the Bibliometric Network Analysis (Figure 3) that the types of NAMs and their applications in R&D are broad, spanning many research areas and medicine development aspects. The exploratory analysis shows multiple 'clusters' of NAM R&D emerging that represent the breadth of the field ranging from complex *in vitro* models (tissue engineering, organoids) to molecular docking *in silico* models. These sense-making clusters group highly connected co-occurring keywords. Generally, the network analysis shows a high level of inter-cluster interconnectedness showing the increasing trend of inter-disciplinary R&D to advance NAMs. The 12 most pertinent clusters are briefly presented and contextualised with keywords in bold in the Annex 8.2.

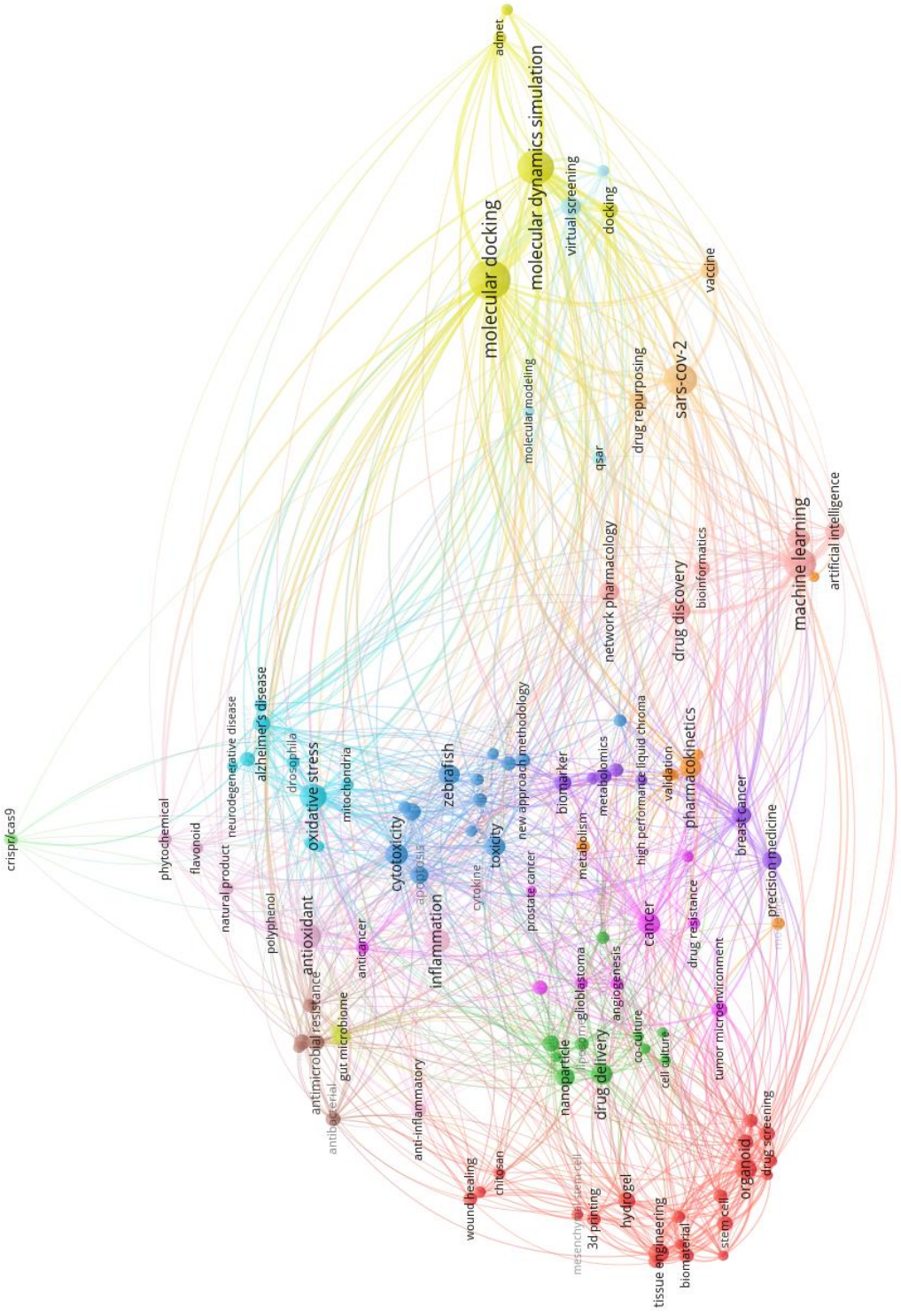

Figure 3. Bibliometric Network Analysis of NAM research and development from 2009 – 2024. 12 clusters appear: "Complex in vitro models" (red, left), "Drug delivery system testing" (green, middle-left), "Toxicology applications of NAMs" (dark blue, middle), "Gut Microbiome "(yellow, top left), "Precision Medicine" (purple, bottom-centre), "Neurodegenerative disorders" (light blue, top-centre), "Pharmacokinetics" (orange, centre), "Antimicrobial resistance" (brown, top-left), "Oncology" (pink, middle), "Machine Learning/Artificial Intelligence" (light red, middle), "Computational Drug Discovery", "Efficacy and Safety Assessment" (light orange, right) and "Computational Structure-based Drug Discovery, Efficacy and Safety Assessment" (light blue, right). For an interactive version follow: https://app.vosviewer.com/?json=https%3A%2F%2Fdrive.google.com%2Fuc%3Fid%3D1IzkM_UWcDNnKfassatJlsqm8bZNGpXno

3.2. 3Rs-related innovation from EMA early-interaction databases

To characterize 3Rs-related interaction and innovation currently reaching EMA, a search of internal EMA databases was performed. This consisted in identifying 3Rs-related topics from March 01, 2019, to January 01, 2024 present in:

(1) Innovation Task Force (ITF) briefing meeting requests

(2) Portfolio and Technology Meetings (PTMs, previously Business Pipeline Meetings)

(3) Scientific Advice (SA)

(4) Qualification of Novel Methodology (QoNM)

3.2.1. ITF briefing meeting requests

Increasing trend of 3Rs-related in ITF briefing meetings

EMA ITF briefing meetings provide a platform for early dialogue between developers and regulators on innovative aspects of medicine development. Since September 2021, the 3Rs have been designated as a special area of interest for ITF briefing meetings. This offers increased support for and promotion of 3Rs-related topics and developments as outlined in EMA's 2021 communication[22]. From March 2019 to December 2023, 45 out of 339 ITF briefing requests pertained to the 3Rs, showing an upward trend from zero in 2019 to 15 in 2023 (Figure 4A). In 2023, there was a noticeable rise in the proportion of requests where the 3Rs was a main topic (13 out of 15 requests) rather than a subtopic compared to previous years. This indicates that NAMs for regulatory use are steadily reaching technology readiness levels appropriate for more advanced discussions with regulators on the specifics of their development and their future regulatory acceptance. This trend could also be explained by an increased political and societal concerns on the use of animals as well as an increased awareness and support for the 3Rs. These ITF requests primarily addressed two overarching topics: (1) NAM implementation and development (80%, n=35) and (2) the feasibility of bridging studies or bypassing animal studies (20%, n=9) (Figure 4B). The former aligns with *replacement* of animal use (but could also contribute to reduction), while the latter pertains to *reduction*.

SMEs, Academia and EU-funded consortia leading 3Rs-innovation interactions at EMA

The majority of 3Rs-related ITF briefing meeting requests were filed by SMEs accounting for 31% of requests (Figure 4C). Large enterprises followed at 22%, with Academia and EU-funded consortia each contributing 18%. This trend suggests that SMEs are leading the way in regulator interactions relating to 3Rs innovation. In contrast, large enterprises tend to focus more on *reduction* of animal use, with half of their requests pertaining to the omission of animal studies or bridging programs. Large enterprises were also more likely to be directly referred to Scientific Advice (SA) (40% compared to 12% for all other applicant categories) (Figure 4D). Reasons for direct referral of a request to SA include a high level of maturity of the project and highly specific questions more suited for in depth answers provided through SA. EU-funded consortia emerge as particularly active in bringing forward NAM implementation and development topics with all 8 3Rs-related requests containing NAM-related questions. One such request was directly referred to QoNM. These findings indicate a lesser engagement of large enterprises with regulators on early-stage NAM development, as opposed to SMEs, Academia and EU-funded consortia, who appear to be leading in this area. Thus, these smaller developers are starting to liaise with regulators to receive support in the development of novel tools that could potentially be adopted by larger pharmaceutical companies.

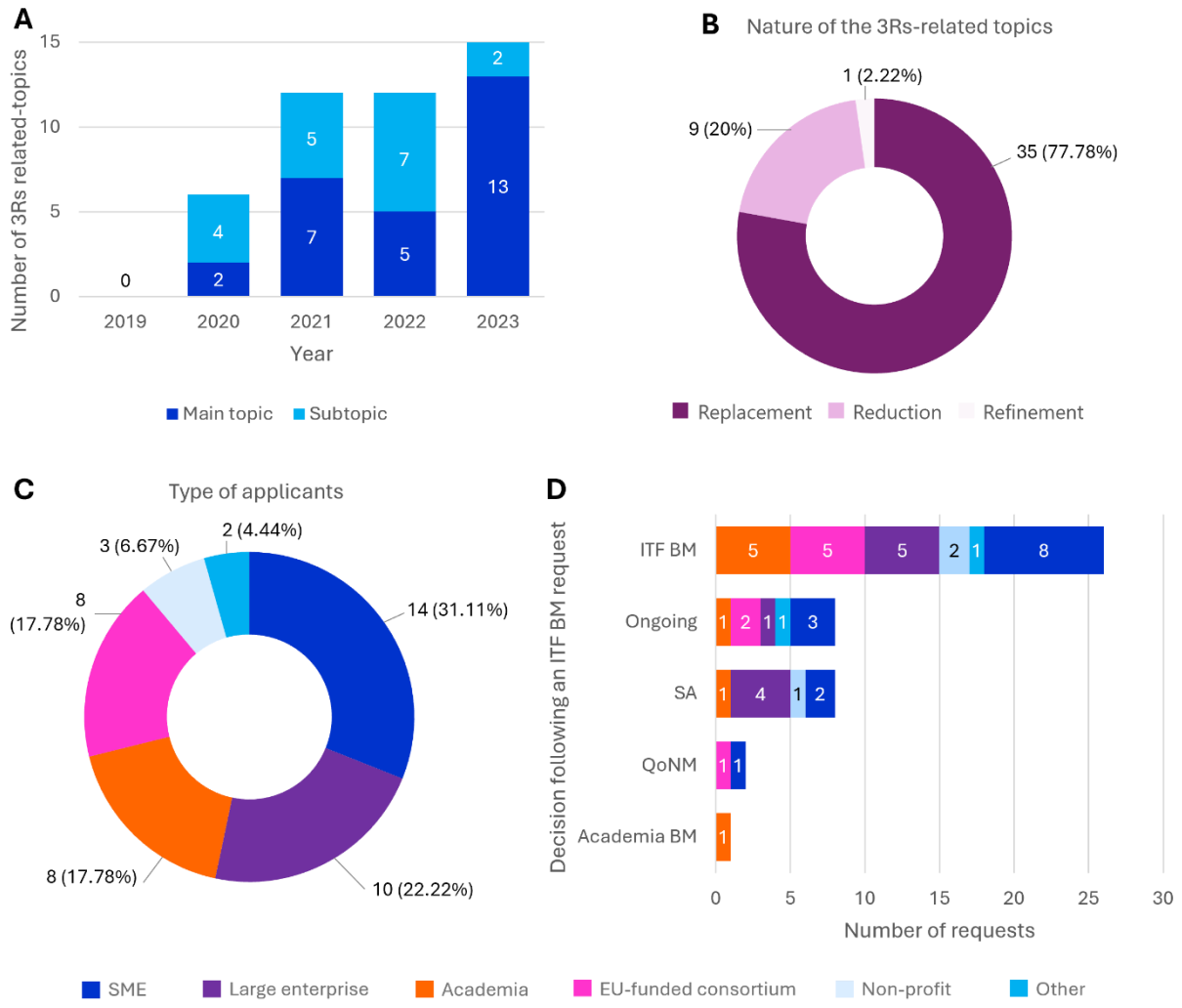

Figure 4. Number of 3Rs-related ITF briefing meetings requests received between 2019 and 2024 (A), main topics of discussion proposed (B), type of applicants (C) and advice provided by EMA ITF for the most appropriate regulatory interaction in response to the request (D).

Safety & Toxicology and Medicine Discovery & Efficacy most frequent aspects of discussion

Within the 45 ITF briefing meeting requests, 52 3Rs-related topics were put forward for discussion which were classified into six areas of medicine development (Figure 5). Safety & Toxicology dominated with more than half of requests proposing topics in this area (55%, n=24). Selected example topics include guidance for regulatory aspects of NAM development for the prediction of drug induced-liver injury (DILI) or cardiotoxicity assessment using *in vitro* models, and *in silico-in vitro* prediction of off-target effects of gene editing systems. Drug Discovery & Efficacy was the second most frequent area discussed (25%, n=11). This mainly involved non-animal disease models for efficacy assessment and facilitating medicines discovery. To note, none of the requests stemming from large enterprises contained Drug Discovery & Efficacy topics, rather this stage was made up of SMEs, EU-funded consortia and academia. The specificity of these models' use-cases was frequently ambiguous and lacked a defined context of use with the models or methods often broadly classified under 'drug discovery'. This encompassed a range of applications including medicine efficacy testing, hit-to-lead progression, lead optimization, elucidation of the mechanism of action, basic research and disease pathology studies. The next most frequently addressed area in the 3Rs-related ITF requests was Pharmacokinetics & Biodistribution (20%, n=9) which included topics such as physiologically-based pharmacokinetic (PBPK) modelling, dose estimation, dissolution and absorption pharmacokinetics and

biodistribution models. The last three areas in discussion-frequency were: Quality/Manufacturing (n=4), Formulation (n=3) and Environmental Risk Assessment (n=1). Quality/Manufacturing included questions relating to the use of novel biosafety testing, such as next generation sequencing methods, and Formulation included models for testing formulation performance.

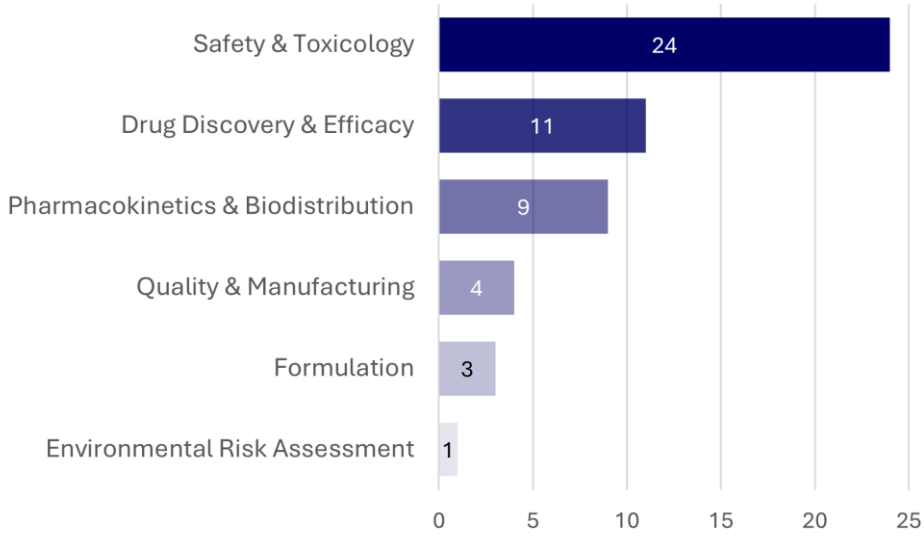

Figure 5. Medicine development topics addressed in 3Rs-related ITF briefing meeting requests.

NAM developments in ITF requests

Out of the 36 ITF meeting requests addressing a NAM, 46 NAMs were presented and further analysed to reveal that Liver, Brain, Heart and Musculoskeletal tissues were most frequently represented (n=10, 7, 5, 5, respectively) (Figure 6). While 2D *in vitro* models remained predominant (n=12), more innovative NAM types such as organ-on-chip (OoC) (n=11), 3D *in vitro* models (n = 7, including spheroids and organoids), *in silico* models (n=6) and combined *in vitro*/*in silico* models (n=3) were also proposed.

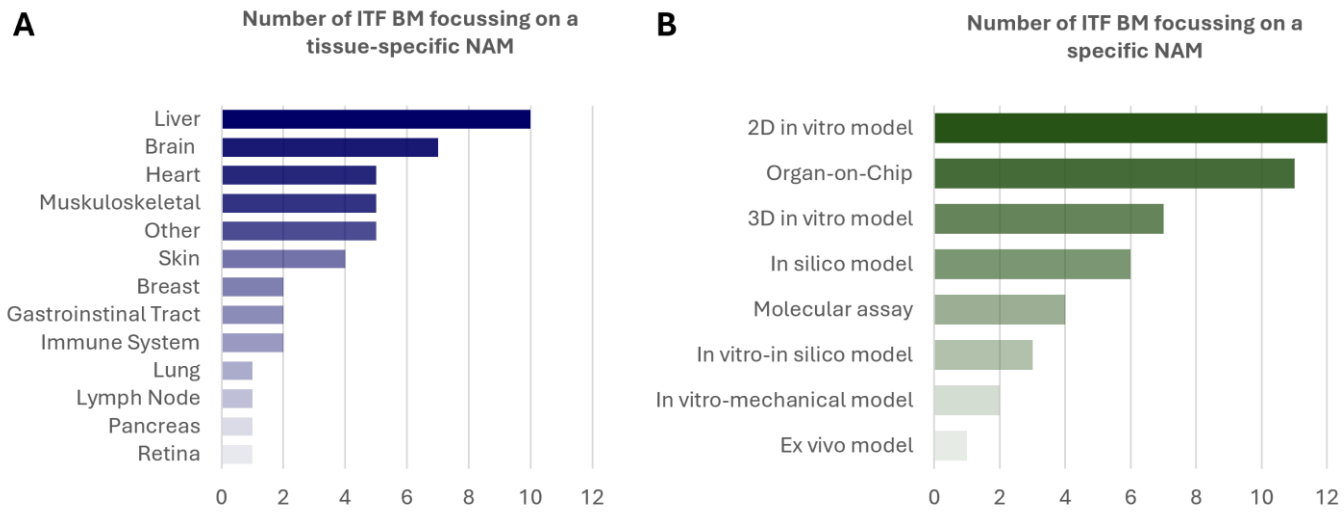

Figure 6. Number of ITF briefing meeting requests focussing on a specific tissue (A) or NAM (B).

3.2.2. Portfolio and technology meetings – increasing consideration of 3Rs principles in industry

3Rs-related topics increasing in trend in PTMs

EMA Portfolio and Technology meetings (PTMs, formerly called Business Pipeline meetings) were reviewed for 3Rs-related content from March 2019 to December 2023. These are free-of-charge, informal meetings between EMA and pharmaceutical companies with large medicines portfolios. They aim at identifying issues impacting product portfolios and development. They capture innovative and disruptive technologies and anticipate scientific and regulatory expertise required at EMA. Within this period, 12 PTMs incorporated 3Rs-related topics, demonstrating an upward trend from one meeting in 2019 to five in 2023 (Figure 7A). To note, these meetings were suspended in 2021 due to the COVID-19 pandemic.

Platform approaches; a hot topic relating to 3Rs

The most frequently discussed topics were related to leveraging platform approaches to allow for bridging programmes (n=5), particularly in the space of rare diseases (n=2) and Chemistry, Manufacturing, and Control (CMC) platforms (n=3). This indicates a drive from large pharmaceutical companies with extensive pipelines for regulatory facilitation of these platform approaches to accelerate development. The broader trend towards platform technologies offers a potential to reduce animal studies, particularly in the areas of safety, toxicology, pharmacokinetics and biodistribution studies.

Importance of manufacturing topics for large enterprises

Approaching the 3Rs from a large pharmaceutical company perspective, the emphasis significantly shifts towards CMC and manufacturing innovation as compared to academia and SMEs, as evidenced by the inclusion of 3Rs-related Quality/Manufacturing topics in 6 PTM meetings (Figure 7C). This included topics such as innovative animal-free ATMP manufacturing methods and a novel viral testing assay to be applied throughout the company's pipeline and various CMC platforms. This underscores the paramount importance of manufacturing innovation in the context of large-scale pharmaceutical operations.

NAMs presented in almost half of 3Rs-related PTMs

Discussions in PTMs are generally kept to a strategic high level. Nevertheless, 3Rs-intended NAMs were presented in 5 of the 12 meetings that included 3Rs topics (40%). This included both *in vitro* (n=5) and *in silico* models (n=4) applied across general toxicology, developmental and reproductive toxicology (DART), dosage determination, disease modelling and pharmacokinetic modelling (Figure 7D).

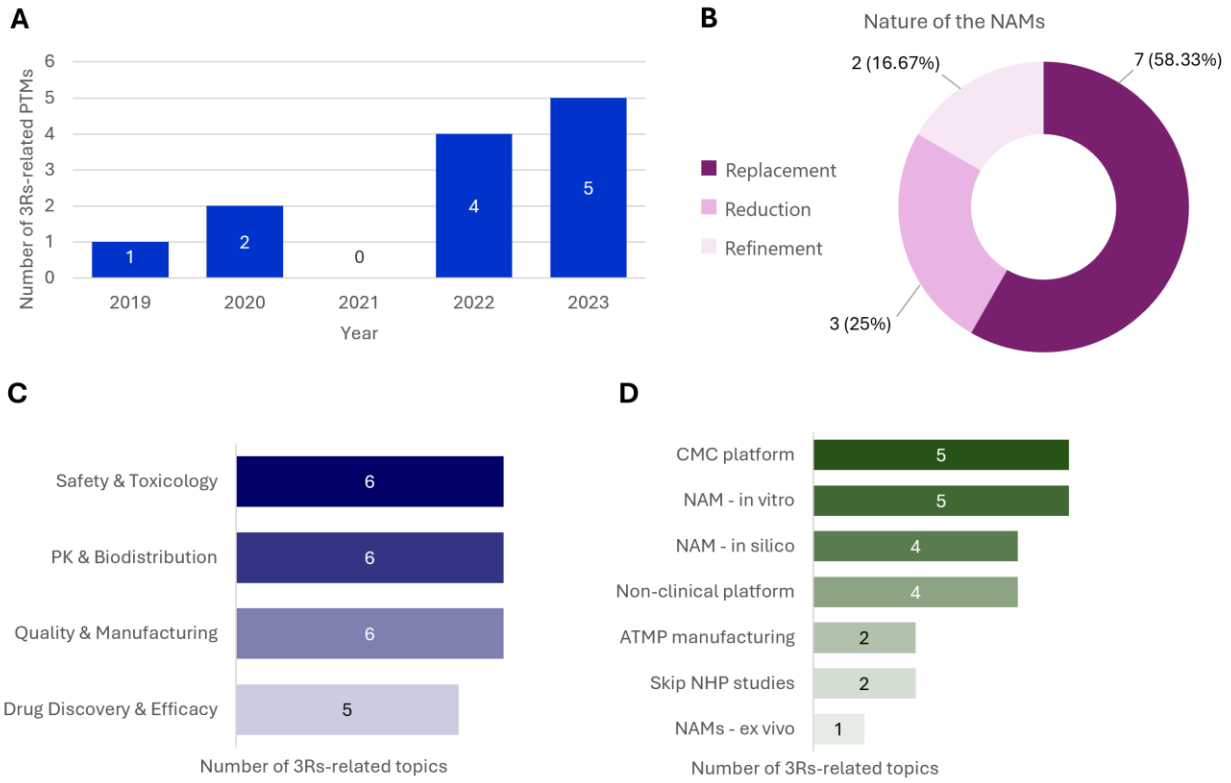

Figure 7. Number of 3Rs-related PTM held between 2019 and 2023(A), nature of the NAMs discussed (B), Medicine development topics broached (C) and specific NAMs in focus (D).

3.2.3. Scientific advice – leveraged for the 3Rs

Increasing trend of human SAs containing 3Rs-related terms

EMA Scientific advice (SA) is a consultative provision for medicine developers during medicine development. The Committee for Medicinal Products for Human Use (CHMP) provides specific scientific advice on methodological and study design via the Scientific Advice Working Party (SAWP). This ensures that robust efficacy, safety and quality data be generated with the appropriate tests and studies to mitigate major objections during the evaluation of any subsequent marketing authorisation applications (MAAs). The novel AI-based Scientific Explorer tool developed at EMA was utilized to identify human SA procedures incorporating 3Rs-related topics. Two search strategies were employed:

(1) a term search in all text fields for 3Rs-related terms:

"3R* principle*" OR "reduc* animal" OR "replace* animal" OR "refine* animal" OR "alternative* to animal"

(2) SA forms where applicants ticked the 3Rs principles box upon submission.

The combined approach yielded 192 SA procedures from March 2019 to December 2023. From 2019 to 2023, the number of SAs with 3Rs-related terms rose from 27 to 46. This not only signifies a growth in 3Rs-related interactions between EMA and medicine developers, but underscores the increasing importance attributed to the 3Rs principles in medicine development. However, it does not necessarily indicate proactive 3Rs innovation coming to the agency. For example, it could also relate to requests for study waivers or the CHMP commenting on the necessity for 3Rs considerations in the planned studies. Given the volume of identified cases, further analysis was conducted on SAs resulting from search method (2) equal to 36 3Rs-related SAs. Since the 3Rs principle tick-box was added to the application form of SA in 2020, it is assumed that SA applications with explicit 3Rs content would use

this feature to highlight the inclusion of 3Rs-related topics. Thus, approach (2) is anticipated to capture more innovative contributions to the agency regarding the 3Rs, as opposed to more general discussions or instances of CHMP advising applicants on 3Rs principles adherence.

Willingness of industry to incorporate reduction and replacement strategies in line with the 3Rs

From January 2021 to December 2023, 36 SAs pertained to the 3Rs, with no clear trend overtime (Figure 8A). Large enterprises submitted the majority of 3Rs-related SAs, accounting for nearly 70% of the identified SAs (Fig.8B). SMEs contributed to 28% and one submission came from a non-profit organisation. Notably, large enterprises were the applicants in three out of the four identified SAs that discuss NAM use for the 3Rs.

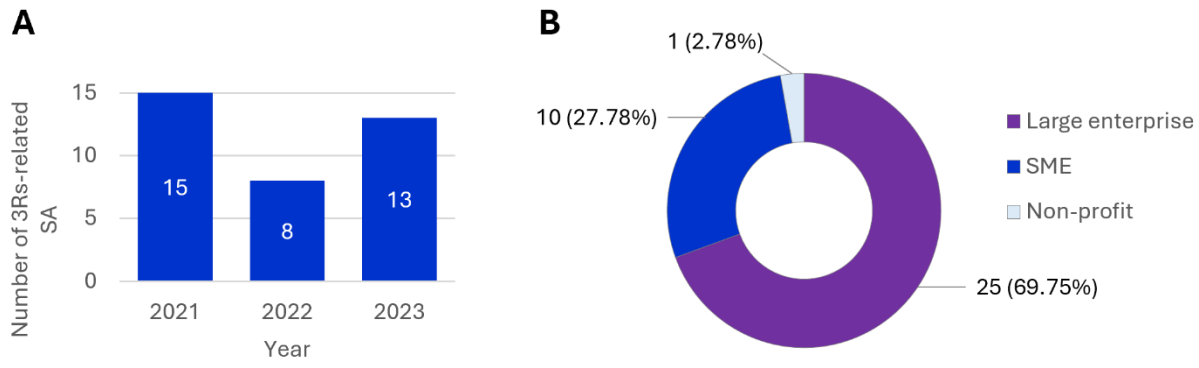

Figure 8. Number of 3Rs-related Scientific Advice (SA) procedures between 2021 and 2023 (A) and type of applicants for these procedures (B).

3Rs-related topics are mostly waivers for Safety & Toxicology studies

A total of 48 3Rs-related topics were identified within the 36 SAs (Figure 9A). In contrast to 3Rs-related topics in the ITF and PTMs, nearly all the SA 3Rs topics were related to Safety & Toxicology (93%). Most of the 3R-related SAs focused on *reduction* strategies (n=41) with only a few discussing *replacement* strategies using NAMs (n=7) (Figure 9B). Diving deeper into *reduction* topics, most topics related to asking the CHMP for waivers for animal studies (79%) and 3 topics related to study design optimization to *reduce* animal use (6%). Biosimilar comparability was the most frequent reason for requesting waivers for animal studies (n=14). The second most frequent sub-topic discussed was General Toxicology (n=13), mainly involving weight-of-evidence approaches asserting that a long term (3-month) repeat-dose toxicity in non-human primates (NHPs) or in rodents would not bring additional value to a future MAA. To note, one case included the CHMP recommending merging pharmacokinetic and general toxicity studies to *reduce* animal use in line with the 3Rs, rather than an applicant derived 3Rs topic. DART was the third most represented sub-topic discussed (n=8). This consisted of weight of evidence approaches to justify the omission of various DART studies including Enhanced Pre- and Postnatal Development studies (ePPND), embryofetal developmental toxicity studies, fertility studies and maternal-fetal medicine transfer. Other topics discussed from a *reduction* perspective included carcinogenicity, immunogenicity, on/off target toxicity, paediatric safety and omitting proof of concept in an animal disease model.

NAMs and replacement strategies in line with the 3Rs

7 NAM strategies were identified in 4 SA procedures (Fig.9B). These encompassed *in vitro* and *in silico* NAMs primarily employed to support weight of evidence approaches to justify the omission of specific Safety & Toxicology studies for a particular product and intended indication. For instance, an *in silico* model was used to support the replacement of an NHP toxicity study by addressing the identified risk of foetal exposure to the medicine. Another case proposed *in vivo* tumorigenicity studies. In alignment

with the 3Rs, the CHMP recommended the use of more sensitive *in vitro* assays over the *in vivo* studies. Furthermore, one case of a novel *in vitro* next generation sequencing assay for quality potency testing was presented.

This demonstrates engagement from industry to make changes that, if acceptable from a regulatory perspective based on scientifically pertinent and robust evidence, could substantially *reduce* animal use in medicine development.

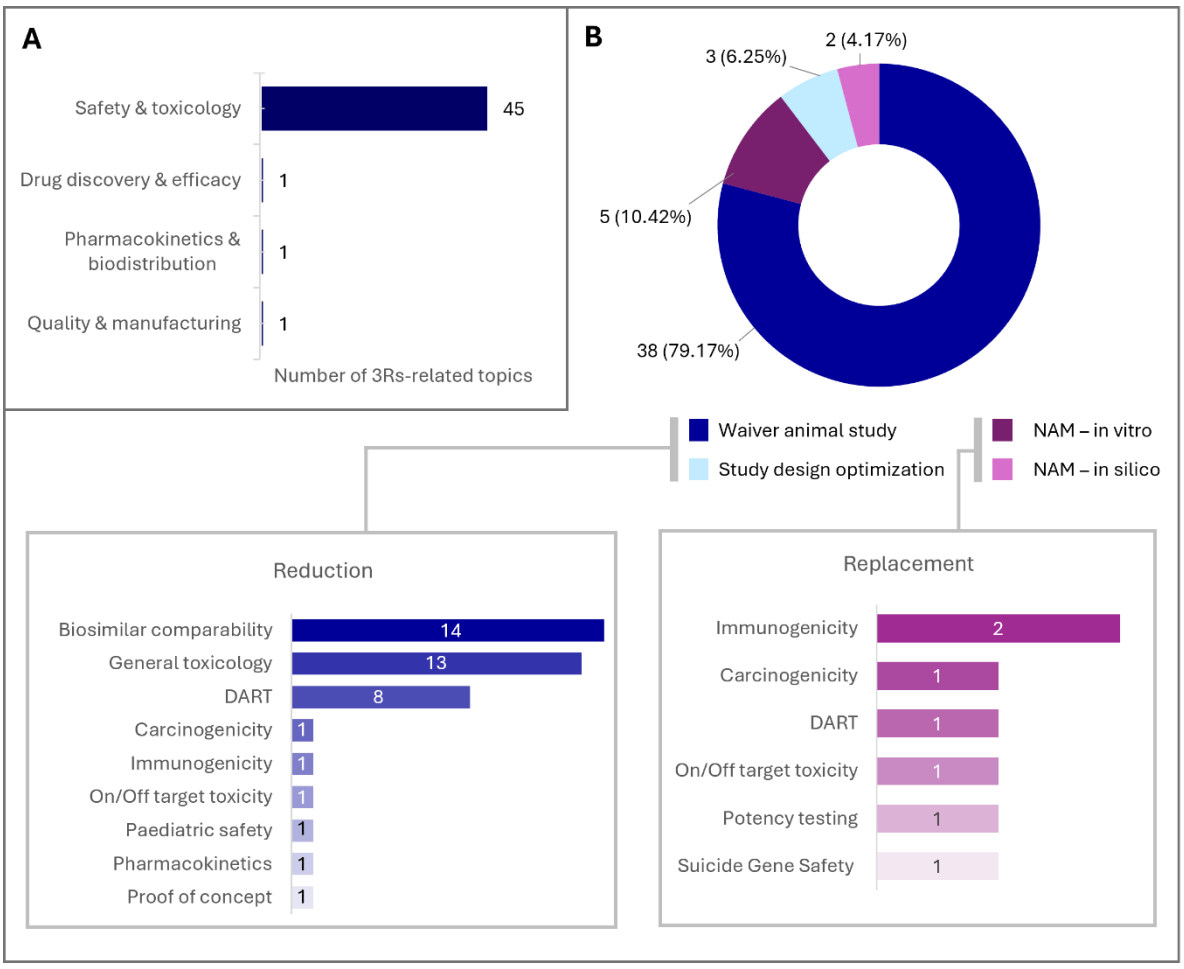

Figure 9. Medicine development domains addressed in 3Rs-related SA procedures (A) and topics of discussion (B).

3.2.4. Qualification of Novel Methodologies for Medicine Development

The Qualification of novel methodologies is an EMA procedure allowing developers of innovative medicine development methods such as new approach methodologies to request the qualification of these instruments within a pre-defined context of use. Currently, there are no NAMs that have been qualified by EMA for regulatory uses in new medicine development.

4. 3Rs Initiatives and Regulatory Preparedness

4.1. Current EU regulatory initiatives

The 2010/63/EU Directive on the protection of animals used for scientific purposes legally mandates the application of the 3Rs to animal experimentation in the EU. Thus, it is in the remit of the EMRN to embrace, support and promote the principles of the 3Rs in medicine development and medicine

regulatory applications, as highlighted in the Regulatory Science Strategy to 2025[23]. As a response to the 2010/63/EU Directive's legal mandate, an EMA Joint Expert Group on the application of 3Rs in the development of medicines (JEG 3Rs) was established in 2010 for a period of 6 years. This was followed by the formation of a Joint CVMP/CHMP Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products (J3RsWG) with a mandate running from 2017 to 2019 [24]. Finally, the EMA 3Rs Working Party (3RsWP) was formed in 2022 as a standing working group, mirroring the growth in interest in the 3Rs and advances in the field. The EMA 3RsWP is a multidisciplinary, strategic working party that monitors and supervises EMA's 3Rs activities in collaboration with other working groups and experts from the EMRN. Besides the 3RsWP, a number of working groups, platforms or expert groups have been established to reinforce the implementation of the 3Rs-compliants approaches and methods. The Non-Clinical and New Approach Methodologies European Specialised Expert Community (NC NAMs ESEC), established in 2023, is an important platform for information sharing between non-clinical and NAM experts from the ERMN and from academia[25]. The Batch Release Testing Operational Expert Group was established in 2024 to review batch release testing of human and veterinary medicines and identify and support the implementation of 3Rs-compliant methods. EMA is further promoting the 3Rs through the ITF, which provides an early contact point for 3Rs innovators with regulators. Finally, another important EMA channel made available to developers of NAMs is the Qualification of New Methodologies procedure. This procedure leads to an opinion on the acceptability of a proposed method such as a NAM in a research and development context and for a specific context of use.

Essential EMA documents related to the promotion of the 3Rs in a regulatory context include:

Guideline on the principles of regulatory acceptance of 3Rs (*replacement*, *reduction*, *refinement*) testing approaches (europa.eu).

Reflection paper providing an overview of the current regulatory testing requirements for medicinal products for human use and opportunities for implementation of the 3Rs

Consolidated 3-year work plan for the Non-clinical domain including the priorities for 2023 (europa.eu)

Concept paper on the revision of the Guideline on the principles of regulatory acceptance of 3Rs (*replacement*, *reduction*, *refinement*) testing approaches (EMA/CHMP/CVMP/JEG-3Rs/450091/2021 (europa.eu)

NC domain priorities for 2024 - final Dec 23 (europa.eu)

Several European initiatives are being undertaken beyond EMA, some of which are presented here. The European Directorate for the Quality of Medicines & HealthCare (EDQM) and the European Pharmacopoeia (Ph. Eur.) Commission have re-evaluated the relevance of animal tests mentioned in the Ph. Eur. texts and monographs and included alternative methods when deemed appropriate. The European Partnership for Alternative Approaches to Animal Testing (EPAA) seek to promote the 3Rs principles across industry sectors, including pharmaceutical, food and chemical sectors[26]. Finally, an European Commission Roadmap has been started in 2023 towards phasing out the use of animals for chemical safety assessments[27].

4.2. Current international regulatory initiatives

A number of international initiatives exist, such as the collaborative project between the UK National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) and the World Health Organization (WHO) aiming at updating WHO guidelines and recommendations to promote a more harmonised adoption of the 3Rs principles [28]. Another recent example of international initiative is the International Medicines Regulators' Working Group on 3Rs (IMRWG3R) which held a kick-off meeting in January 2024. The IMRWG3R, which gathers EMA, the Swiss Agency for Therapeutic Products (Swissmedic), the Japanese Pharmaceuticals and Medical Devices Agency (PMDA), the

Australian Therapeutic Goods Administration (TGA), Health Canada and the United States Food and Drug Administration (FDA), aims to achieve internationally harmonised recommendations in relation to the 3Rs, including agreement on regulatory acceptance criteria for NAMs, and to facilitate knowledge-sharing on experience and learnings from regulatory 3Rs initiatives in the various regions. Note that there are a number of other international initiatives and this list is not exhaustive.

4.3. Projects and Fundings

In the last two decades, the European Union has funded over 300 projects to support the development of alternatives to animal testing in the pharmaceutical sector and other sectors such as food, chemicals and pesticides. These projects, which include the Innovative Medicines Initiative (IMI) 1 and IMI 2-funded projects, reached a total spending of over 1 billion euros[29]. Of this, 120 million was destined for OoC research. Most EU funding went to research on alternatives for toxicology purposes led by repeat-dose testing (185 million EUR) followed by DART (36 million EUR), cardiac toxicity (33 million EUR) and immunotoxicity (20 million EUR). The methods funded encompassed *in vitro* cell cultures including tissues, organoids, OoCs and *in silico* modelling. In the following 2024 Horizon funding, there were 6 calls relating to 3Rs research totalling 102.5 million euros with a minimum of 14 and a maximum of 29 projects with 281 applicants (Annex 8.3). Funding for one project totalling 2 million euros is explicitly related to the regulatory perspective of 3Rs implementation titled "Gaining experience and confidence in NAMs for regulatory safety and efficacy testing - coordinated training and experience exchange for regulators" (HORIZON-HLTH-2024-IND-06-09).

At the international level, an example of an initiative is the C-Path's Predictive Safety Testing Consortium (PSTC), which was founded in 2006 to promote the development of novel safety tests accepted by regulators globally. There are many other international projects and fundings but drawing up an exhaustive list goes beyond the scope of this report.

5. Challenges and opportunities from a regulatory perspective

In this section, we present considerations from a regulatory perspective encompassing both challenges and opportunities for the regulatory acceptance of NAMs and the advancement of the 3Rs principles in human medicine development and testing. While significant progress and numerous achievements have been made at both the EU and international levels over the past decades, the highlighted challenges remain pertinent and require ongoing attention. These challenges are being progressively addressed through various 3Rs initiatives within the EMRN. The opportunities and recommendations provided herein offer direction for ongoing and new initiatives and serve as a catalyst for further discussion and facilitation of innovation.

5.1. Bridging the gap between stakeholders and regulators

One of the critical challenges in the development and implementation of NAMs is the lack of communication and data sharing between stakeholders and regulatory bodies. Many NAMs are initially developed within academic settings, primarily for academic purposes, which can hinder the development of NAMs that are suitable for regulatory use. The absence of regulatory perspectives in academic curricula and grant proposals exacerbates this issue, as researchers may not prioritize regulatory considerations in their work. Consequently, there is a pressing need to bridge this gap to ensure that NAMs are both scientifically robust and aligned with regulatory requirements from the outset. The uncertainty on the appropriate regulatory advice mechanism to be used for different types of development and the stage of development at which stakeholders can start seeking advice from regulators further impedes proactive 3Rs interactions. Transparent sharing of NAM data with regulators is also crucial for regulators to stay informed about the current status of NAMs' R&D and be aware of

possible challenges and opportunities related to their use in a regulatory context. Much expertise and knowledge on NAMs has been developed in-house within industry. In particular, large enterprises with large portfolios have pioneered NAMs for internal de-risking strategies in early-stage medicine discovery and development. This includes target identification, initial hits, lead optimization, candidate selection phases and safety assessment. The EMA database analysis of regulator-developer interactions shows that requests for discussions on NAM innovation predominantly stem from SMEs, Academia and EU-funded consortia, while large enterprises tend to share NAM data with regulators on a more need-to-know, case-by-case basis to support weight-of-evidence approaches, particularly for toxicological assessments. The concerns about the potential negative impact of submitted NAM data on regulatory application decisions partially accounts for this limited sharing.

Opportunities:

- Promote regulatory awareness, in particular in academic settings:

  It is advisable to incorporate regulatory expertise into 3Rs/NAM research centres of excellence, such as premier academic institutions, to streamline regulatory acceptance of NAM developments through early and proactive incorporation of these considerations. Incorporating educational material on regulatory perspectives into research environments should also be encouraged. For example, provide lecture material on regulatory frameworks and guideline flexibility, targeting biomedical research courses (MSc & PhD level) that often lack these perspectives. Intermediary structures should also be set-up to help disseminate and bridge the gap between developers and regulatory considerations, similar to the NC NAMs ESEC (see 3.1. Current EU regulatory initiatives).

- NAM developers should be encouraged via information campaigns and up-to-date websites to contact regulators at an early stage of development, before a full package has been generated. The various options for advice mechanisms and platforms for information sharing at the national and EU levels (e.g. national Innovation Offices, EMA ITF, European Specialized Expert Community for NAMs, national and EMA SA) should be better communicated to developers and clarity should be provided as to what is considered an appropriate timing to contact regulators.

- Strategies to encourage NAM developers and end-users, including large enterprises, to share their data with regulators should be adopted. In particular, the possibility to submit NAMs data through a voluntary submission of data approach (so called 'safe harbour') should be better advertised, and the way these data will be used by regulators should be clarified.

5.2. Cooperating at national and international levels

The international harmonisation of regulatory frameworks and acceptance criteria for NAMs is crucial for the application of the 3Rs in animal testing worldwide. This harmonisation is necessary because even if a single country or region requires traditional animal testing for product approval, the medicine developer will be forced to conduct the study and the overall goal of reducing animal use is not achieved. Thus, it is paramount to harmonise and be on the same page for regulatory acceptance of the 3Rs on the international stage.

It is also important to ensure cooperation and continuity in the application of the 3Rs principles at the member state at all levels, which encompasses more than just the EMRN. Directive 2010/63/EU sets out the regulatory framework for the protection of animal used for experimental and other scientific purposes. The competent authorities responsible for the implementation of the Directive and the competent authorities responsible for medicines regulation may differ within a Member State. This separation may lead to the conduct of animal studies that could have been avoided if cooperation was

improved between those reviewing applications for project authorisation applications under Directive 2010/63/EU and those reviewing the application under the medicine legislation.

Opportunities:

- Strive towards internationally harmonised regulatory acceptance criteria for NAMs in specific context of uses[30]. A potential route could be the proposal of harmonized guidances via the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).
- Leverage the IMRWG3Rs to ensure knowledge transfer and learnings from other regulatory regions' initiatives on the 3Rs.
- Further promote collaboration between all bodies with responsibility for 3Rs within EU member states.

5.3. Clarifying the terminology surrounding the regulatory acceptance of NAMs

It is clear from early EMA interactions with NAM developers, for example in ITF briefing meetings and multistakeholder workshops, that developers find it challenging to distinguish between the concepts of validation and qualification of their NAM development. Within scientific literature and grey literature explored in the context of this report, many views, definitions, strategies and frameworks on validation, qualification and standardisation of NAMs were seen coming from a wealth of stakeholders. As a developer, finding clarity within this wealth of initiatives and resources on appropriate terminology and expected steps for regulatory acceptance of a NAM could be challenging. Within the qualification procedure, a number of terms are also differently interpreted. This is for instance the case of terms such as "context of use" or "efficacy testing", which might refer to slightly different concepts depending on the stakeholder. It is crucial that terminology - what it means and what it delineates - is communicated clearly, comprehensively, and simply from the regulatory authority.

Opportunity:

- Revise the Guideline on the principles of regulatory acceptance of 3Rs testing approaches with the inclusion of updated, clarified terminology, and communicate clearly with developers the delineation of validation and qualification of a NAM.
- Bring clarify as to when qualification is needed and when NAM data can be submitted as part of a MAA without the need for a large scale qualification.

5.4. Providing evidence supporting NAM claims

In the development of NAMs, broad claims are often made to encompass a wide range of potential applications, aiming to increase utility and marketability of a product. However, for regulatory purposes, a specific context of use is required to assess the validity and reliability of a NAM. This discrepancy can lead to ambiguity as developers strive for versatility while regulators require specificity. This necessitates a collaborative approach where both parties work together: developers can provide detailed information about the intended application, data type and interpretation within the regulatory context, and regulators can offer clear guidelines and expectations for the necessary information. This cooperative effort can facilitate informed decisions about the suitability of a NAM for a particular use-case and intended framing within the medicine development process.

Opportunities:

- Continue and increase promotion of initiatives to encourage early interactions of NAM developers with regulators to educate and support the definition of a specific context of use and advise medicine development pipeline placement for regulatory uses of NAMs.

- Revise the Guideline on the principles of regulatory acceptance of 3Rs testing approaches with the inclusion of regulatory acceptance criteria for specific contexts of use for specific technologies.

5.5. Raising awareness of regulatory flexibility and efficient animal studies planning

Regulatory authorities evaluate medicines providing a benefit-risk analysis for the patients. This assessment is conducted in line with the ICH and EMA guidance and guidelines, which allow for flexibility. It is important to support developers to efficiently plan their animal studies and raise awareness of the regulatory flexibility, especially among first-time medicine developers. For instance, certain studies, such as a six-month chronic toxicology study, may be bypassed for certain therapeutic modalities if no risk is found in shorter toxicology studies. Furthermore, species lacking expression of human targets or pre-defining a specific target population of a therapy can justify omitting certain toxicological animal studies as they would not add value to the regulatory submission. Additionally, providing NAM data to support a weight-of-evidence approach shows the potential of guideline flexibility for the 3Rs. This flexibility, when appropriately leveraged, can streamline the medicine development process to *reduce* and start the transition towards the *replacement* of animal use.

Opportunities:

- Educate first-time developers about the flexibility of regulatory guidelines and clarify that regulatory agencies support deviation from these guidelines when justified. Disseminate information on the weight-of-evidence approach to support 3Rs.
- Promote regulator interaction / inclusion of regulatory considerations early in medicine development programmes.
- Raise awareness and educate regulators on new methodologies and paradigms that support innovative animal study design, e.g. via webinars and workshops.

5.6. Leveraging platform approaches for the 3Rs

Platform approaches are gaining traction in medicine development, particularly within large pharmaceutical companies, as evidenced by recent acquisitions of platform-based biotech firms[31]. This trend signifies an increasing interest in platform medicine development strategies as industry moves towards more efficient and innovative approaches in medicine development. Leveraging prior knowledge for regulatory dossiers based on previous approved products, including from developments stemming from platform technologies, is a strategy already employed by the pharmaceutical industry effectively reducing animal use[32]. However, this requires regulatory know-how which not all developers have access to, representing a challenge for more routine and widespread implementation.

Regulatory bodies have the opportunity to leverage this industry interest to promote the 3Rs principles to *reduce* regulatory animal use by providing an explicit framework for platform technologies that include 3Rs considerations. In the EMA database analysis on 3Rs interactions, platform technologies were the most frequently discussed topic in relation to the 3Rs in PTMs with Large Enterprises (see section 2.2.2.). This indicates that this topic is relevant and lacks clarity even for experienced developers. In the presented analysis of SA, it is clear that EMA is already implementing a case-by-case approach to advise on the necessity of animal studies for medicines derived from platform developments (section 2.2.3.). To formalise this process and increase transparency, a clear framework should be provided for medicines deriving from platform technologies outlining how this can be leveraged from the 3Rs perspective. This approach would enhance the impact of this strategy on the 3Rs allowing it to be incorporated from early stages of development, particularly benefiting smaller and

less experienced developers who may not be fully aware of these data bridging possibilities or have limited access to regulatory know-how.

Opportunities:

- Develop and provide an accessible framework for developers of all experience levels on using bridging data from platform technologies in medicine development to stimulate a *reduction* in animal use.

5.7. Developing representative models of diverse human populations

NAMs, in particularly cell-based models, present a unique set of challenges and opportunities in the representation of diverse human populations. Using one or a limited number of cell lines for cell-based NAMs fails to recapitulate the human population to account for, for example different metaboliser populations, paramount for safety and pharmacokinetic assessments[33]. In fact, generally, there is a lack of diversity in the cell lines used, which are predominantly derived from individuals of Caucasian descent, or unspecified ethnicity or gender[34,35]. This lack of genomic diversity misses opportunities to discover effects of variants that are common in underrepresented groups thus perpetuating medical inequalities and failing to predict possible efficacy or safety concerns in underrepresented groups[34].

On the flip side, the inherent flexibility of cell-based models offers an unprecedented opportunity to incorporate a variety of cell types from diverse populations, a feat that is not achievable in animal models or most clinical trials. This could significantly enhance the representativeness of these models, thereby improving their predictive power for human responses across different populations.

In silico and AI models, if built on robust and representative data, have the potential to control for population diversity[36]. This could further enhance the accuracy and applicability of these models across diverse human populations. However, if built on misrepresentative or non-inclusive data sets, *in silico* models could face similar issues to cell-based NAMs[37] .

Thus, while the current limitations in cell-based NAMs highlight the need for more inclusive practices in cell line derivation, the potential for improved representation through diverse cell types and *in silico* modelling underscores the promise of these methodologies in advancing personalised medicine[34,35].

Opportunities:

- Ensure equitable representation of human population in NAM developments.
- Include the necessity/consideration of representative models in guidance to developers.
- Strengthening the link between 3Rs and in silico domains, e.g. via the organization of joint workshops on toxicology prediction.

6. Recommendations

6.1. Communicating and engaging with stakeholders involved in the implementation of the 3Rs principles

- Communicating and engaging with patients, animal welfare and healthcare professional organisations to continuously build trust and awareness around activities being undertaken to replace, reduce, and refine animal models.

- Develop and promote closer interactions between regulators and stakeholders involved in NAM development to foster knowledge exchange and understanding of NAMs-related complexities and challenges.

Outline the regulatory flexibility in an EMA published document (reflection paper, position paper, roadmap, whitepaper) and advocate for regulatory flexibility within the EMRN to allow for the successful integration of NAMs.

6.2. Updating the regulatory framework

- Provide a clear framework for NAM qualification including a clear definition of a context of use, what is expected in terms of comparison to existing gold standards and highlight the importance of translational, clinically relevant endpoints.
- Provide clarity on the definition of validation and qualification and the expected requirements. Harmonise this, to the extent possible, internationally.
- Explore how new regulatory perspectives on platform approaches to medicines development can be leveraged in terms of the 3Rs principles.

6.3. Extending knowledge and expertise

- Ensure expert knowledge on the 3Rs within the EMRN covers all therapeutic areas and intended uses for NAMs and anticipate their use in combined or complex developments.
- Increase capability and knowledge of network assessors via training or sharing of case studies.
- Increase EMA/EMRN 3Rs capacity to ensure and maintain representation in international working groups, scientific meetings and conferences and advocate for prioritisation of NAM developments and 3Rs principles. Foster international cooperation and knowledge sharing.

6.4. Involving regulatory and scientific groups in further review and actions

- Continue fostering discussions at ICH level on the 3Rs.
- Engage with and promote formation of intermediaries for knowledge spreading and data sharing: identify honest brokers as facilitators, umbrella organisations, knowledge sources and intermediaries between regulators and developers. In this light, explore the role of learned societies or public-private partnerships as intermediaries.
- Strengthen cooperation between assessors from national competent authorities for medicines on one side and experts responsible for the application of the Directive 2010/63/EU on the other side with a view to making the earliest possible intervention to avoid unnecessary animal testing and minimising any potential duplication of effort or inconsistencies that might arise due to the difference in expertise.
- Encourage data sharing and publication across legislative frameworks, such as between the pharmaceutical, food, industrial chemical frameworks, to ensure data sharing where feasible and avoid duplication of animal studies.

6.5. Encouraging funding

- Include validation and qualification of NAMs into funding plans with a clear end-goal and context of use from early stages of a project.
- Liaise with the funding bodies to tailor EU funding calls.

7. Methodology

7.1. Bibliometric Network Analysis

For each trend analysis, PubMed literature searches were conducted using the rentrez package in Rstudio (in Annex) in five time-bins spanning back 15 years:

(1) March 01 2021 to March 01 2024

(2) March 01 2018 to March 01 2021

(3) March 01 2015 to March 01 2018

(4) March 01 2012 to March 01 2015

(5) March 01 2009 to March 01 2012

Search terms used:

NAMs:

1. Constrain to papers **presenting a model** as the topic in title, not solely use within the paper

(model[Title] OR models[Title] OR technology[Title] OR technologies[Title] OR method[Title] OR methodology[Title] OR methodologies[Title] OR technique[Title] OR techniques[Title] OR assays[Title] OR assay[Title] OR approach[Title] OR approaches[Title] OR strategy[Title] OR strategies[Title] OR test[Title] OR testing[Title] OR assessment[Title] OR 3Rs[Title] OR 'alternative to animal'[Title] OR 'alternatives to animal'[Title] OR 'Three Rs'[Title])

AND

2. Constrain to be a **NAM or non-animal model**

AND ('new approach methodology' OR 'new approach methodologies' OR 'novel approach methodology' OR 'novel approach methodologies' OR 'in vitro' OR 'in silico' OR 'ex vivo' OR 'in chemico' OR '3Rs' OR 'Three Rs' OR 'alternative model' OR 'alternative to animal' OR 'reduce animal' OR 'replace animal' OR 'refine animal' OR 'replacement of animal' OR 'refinement of animal' OR 'reduction of animal')

AND

3. Be related to **medicines development or regulatory use**

AND ('drug development' OR 'pharmaceutical regulation' OR 'drug testing' OR 'medicinal development' OR 'medicines development' OR 'safety pharmacology' OR 'pharmacodynamics' OR 'efficacy testing' OR 'ADME' OR 'pharmacokinetics' OR 'drug metabolism' OR 'bioavailability' OR 'biodistribution' OR 'general toxicity' OR 'single dose toxicity' OR 'repeated dose toxicity' OR 'No Adverse Effect Level' OR 'reproductive toxicity' OR 'juvenile toxicity' OR 'genotoxicity' OR 'carcinogenicity' OR 'local tolerance' OR 'phototoxicity' OR 'immunotoxicity' OR 'batch release testing')

NOT

4. **Exclusion criteria** of animal models and disease management/diagnosis

NOT ('mouse model' OR 'rat model' OR 'murine model' OR 'rabbit model' OR 'in mice' OR 'in a rodent' OR 'in a rabbit' OR 'in an NHP' OR 'in non-human primate' OR management OR prognosis OR diagnosis)

The resulting records were downloaded and records containing author keywords were subset using R. A bibliometric network analysis in Vosviewer was conducted for each time-bin using the co-occurrence of author keywords option. A thesaurus was applied to merge synonymous terms and merge plural and

singular words. A cut-off of a minimum of 5 occurrences for a single keyword was applied for the NAM search. Vosviewer is an open-source tool developed at the University of Leiden that can be used for exploratory bibliometrics to analyse the morphology of nascent fields, visualising scientific landscapes (van Eck & Waltman, 2009; Kirby, 2023). As noted by Moral-Munoz *et al*. "it is one of the best options for performing a science mapping analysis" (Moral-Munoz *et al*., 2019). The resulting author keywords were compared to allow for the identification of trends in each field. A visual bibliometric network analysis for NAM R&D was generated for the top 120 most occurring keywords from the records from all 5 time-bins (from 2009 – 2024).

OoCs:

('organ-on-a-chip' OR 'organ on chip' OR 'organ-on-chip' OR 'organ on a chip' OR 'microphysiological system' OR 'microphysiological systems')

The resulting records were downloaded and records containing author keywords were subset using R. A bibliometric network analysis in Vosviewer was conducted for each time-bin using the co-occurrence of author keywords option. A thesaurus was applied to merge synonymous terms and merge plural and singular words. A cut-off of a minimum of 3 occurrences for a single keyword was applied for OoCs. The resulting author keywords were compared to allow for the identification of trends in each field. This resulted in multiple parameters as calculated by Vosviewer used for subsequent analyses:

(1) Number of occurrences of a keyword

(2) Total link strength of a keyword - *this attribute is defined as an "indication of the total strength of the co-occurrence links of a given keyword with other keywords"*

(3) Average publication year of publications with a given keyword

These were used to compute two trend indicators:

(4) Δ Total Link Strength

$$\Delta\ Total\ Link\ Strength\ = \frac{tot.link\ strength\ (2021\ 2024) - tot.link\ strength\ (2018\ 2021)}{tot.link\ strength\ (2018\ 2021)}$$

As a measure of how integral a keyword is to OoC research and development.

(5) Δ Occurrence

$$\Delta\ Occurrence\ = \frac{number\ occurrence\ (2021\ 2024) - number\ of\ occurrences\ (2018\ 2021)}{number\ of\ occurrences\ (2018\ 2021)}$$

As a measure of the importance put on this keyword in OoC research and development.

7.2. EMA stakekholder interactions

To identify 3Rs-related topics in EMA stakeholder interactions, a search of internal EMA databases was performed on ITF briefing meeting requests, PTMs, SA and QoNM from March 01, 2019, to January 01, 2024.

7.3. Consultation

Experts from the EU-IN and 3Rs Working Party (3RsWP) commented on all aspects of the report.

8. Annexes

8.1. Information Sources other than references

- Internal 3RsWP meetings
- Innovation Task Force Briefing Meetings
- Scientific Advice Procedures
- Technology and Portfolio Meetings

8.2. Clusters identified by the bibliometric network analysis

The 12 most pertinent clusters are briefly presented and contextualised in the table below with keywords in bold.

Cluster 1: Complex in vitro models (red, left)
The *'complex in vitro models'* research cluster within NAM R&D represents the use of human cells, including **stem cells**, to construct three-dimensional (3D) structures, such as **organoids** and **spheroids**, which simulate organ and tissue structures. These **3D cultures** provide a more physiologically accurate platform for safety and efficacy testing compared to two-dimensional (2D) models. They can be cultivated in bioreactors, and **tissue engineering** principles can be applied to construct models using hydrogels, chitosan, and other biomaterials. These materials create scaffolds to support growth and mimic the **extracellular matrix**, creating an environment conducive to cell growth and differentiation [38–40]. The introduction of **3D printing** and **bioprinting** technologies has transformed the field, enabling the creation of intricate **scaffolds** and the layer-by-layer printing of **bioinks** containing live cells. This results in complex, tissue-like structures that closely resemble *in vivo* conditions [41–43]. **Microfluidic** technology is employed in **organ-on-chip** systems, which simulate human physiological responses on a miniature scale in a controlled environment (discussed further in *Section 5.2.*). These models represent a significant area of NAM research with the potential to *replace* and *reduce* animal testing. For instance, by replicating the pathophysiology of **diabetes**, these models facilitate efficacy testing of **wound healing** therapeutics, thereby reducing reliance on animal testing to support efficacy testing of a medicinal product[44,45].
Cluster 2: Drug delivery system testing (green, middle-left)
This cluster presents a research cluster of NAM R&D focusing on medicine delivery testing. The importance of medicine delivery systems is rising in pharmaceutical development, especially with the advent of RNA therapies and other targeted therapies employing **nanotechnology** delivery systems, including **nanoparticles** and **liposomes**[46,47]**.** These systems aim to improve medicine **bioavailability**, **permeability** and allow for specific tissue targeting. However, the validation of these innovative systems often necessitates animal testing[48–51]. To circumvent the use of animal models, *in vitro* models that simulate biological barriers, such as the **blood-brain barrier**, are under development[51,52]. This can be achieved through the **co-culture** of distinct cell types and the incorporation of **mathematical modelling** to predict the performance of medicine delivery through these complex barriers[52,53].
Cluster 3: Toxicology applications of NAMs (dark blue, middle)
This cluster shows the most prevalent applications of NAMs across diverse toxicology domains identified in our bibliometric search. It includes keywords **cytotoxicity**, **genotoxicity**, **neurotoxicity**, and the effects of **endocrine disruptors**, using tools such as **apoptosis** studies, **DNA damage** analysis, and **transcriptomics**. **Zebrafish** models serve as a key *in vivo* system

spanning the breadth of toxicology assessment, to note their use in developmental toxicity assays[54,55]. This represents a *refinement* of animal use, as a less complex organism reduces the use of more complex organisms. All these elements contribute to **risk assessment** and **medicines development**, embodying the multifaceted toxicology applications of NAMs.

Cluster 4: Gut Microbiome (yellow, top left)

A distinct cluster including the **gut microbiome**, **probiotics** and **microbiome** shows the emergence of a niche in NAM R&D. It is highly connected to keywords outside the cluster **organoids**, **antimicrobial resistance,** and **metabolomics**.

Cluster 5: Precision Medicine (purple, bottom-centre)

This cluster represents the application of NAMs in the realm of **precision medicine**. The techniques incorporated within this cluster include **metabolomics**, **proteomics**, **mass spectrometry**, and **high-performance liquid chromatography** which can be integrated into NAMs to allow for comprehensive analysis of substance impacts on cells or tissues in toxicology assays, thus reducing reliance on animal testing. **Breast cancer** also appears in this cluster; given the heterogeneity of breast cancer, the application of such precision medicine techniques is particularly pertinent[56].

Cluster 6: Neurodegenerative disorders (light blue, top-centre)

This cluster outlines NAMs for **neurodegenerative disorders** (most cited being **Alzheimer's Disease** and **Parkinson's disease**) which are research areas often seen as requiring animal models due to brain and behaviour complexity[57–59]. It includes the keywords **oxidative stress, reactive oxygen species** and **mitochondrial** damage, key factors in **neurodegeneration,** and shows movement towards refinement by using less complex organisms as models such as ***Drosophila*** and ***Caenorhabditis elegans***. This highlights progress in developing NAMs even for highly complex disorders.

Cluster 7: Pharmacokinetics (orange, centre)

This cluster appears to be focused on the development and validation of NAMs modeling medicine **pharmacokinetics** and **metabolism**. The keywords suggest a focus on **modeling** techniques, including **physiologically based pharmacokinetic (PBPK) modeling**, to predict how a substance is absorbed, distributed, metabolized, and excreted in the body. To this end, for example, liquid chromatography-mass spectrometry (**LC-MS**), a technique used for the analysis of medicine metabolites, can be used. The presence of "**validation**" and "**prediction model**" indicates the development and testing of these models to ensure their accuracy and reliability. Keyword 'pharmacokinetics' is highly linked to many keywords throughout the network, most notably: machine learning, molecular docking and medicine discovery.

Cluster 8: Antimicrobial resistance (brown, top-left)

This cluster focuses on NAMs under development to study **antimicrobial resistance**[60,61]. The cluster studies the efficacy of **antimicrobia**l and **antibacterial** agents against resistant bacteria, particularly ***Pseudomonas aeruginosa***, a bacterium known for its resistance. This includes modelling **biofilms**, communities of microorganisms embedded in the extracellular matrix that contribute to resistance by protecting bacteria from antibiotics[62,63]. Keywords in this cluster are highly linked to machine learning, molecular docking and tissue engineering, showing an inter-disciplinary effort to develop models to understand resistance mechanisms and test efficacy of antimicrobials without animal testing.

Cluster 9: Oncology (pink, middle)

This cluster represents NAMs in development for oncology medicine development. The keyword **cancer** is highly linked throughout the network, showing NAM development from all angles applied to cancer. Other keywords include **glioblastoma**, **colorectal cancer**, and **prostate cancer** representing the most prominent and linked types of cancer in our search results (breast cancer appears in Cluster 5. Precision Medicine). The **tumor microenvironment** and **immunotherapy** are highly linked to cluster 1 (complex *in vitro* model) whereas **medicine resistance** is linked to molecular docking and bioinformatics. Furthermore, precision medicine and cancer are highly linked keywords.

Cluster 10: Machine Learning/ Artificial Intelligence (light red, middle)

This cluster represents the integration of advanced computational methodologies, such as **artificial intelligence** (AI), **machine learning**, and **deep learning** in NAM R&D. While these techniques are often employed in the **medicine discovery** phase, they are also used more broadly in NAM R&D mirrored by the high-level of connectedness of the terms throughout the network. The cluster also includes **network pharmacology** and **bioinformatics**, which are further *in silico* techniques unravelling complex biological interactions, disease mechanisms and can predict efficacy and safety end points aiding risk assessment of medicines.

Cluster 11: Computational Drug Discovery, Efficacy and Safety Assessment (light orange, right)

This cluster represents computational techniques currently used in medicine discovery stages, but which are being developed for more robust assessment of efficacy and safety endpoints[64]. **Molecular Docking** techniques predict the binding affinity of potential medicine molecules to their target proteins, providing insights into the therapeutic effect and thus, the efficacy of new medicines. **Molecular Dynamics Simulation** offers a dynamic view of molecular interactions, revealing potential off-target effects or toxicities, contributing to safety assessments. **ADMET** (Absorption, Distribution, Metabolism, Excretion, Toxicity) studies, conducted *in silico,* predict pharmacokinetic properties crucial to the safety profile of new medicines. Lastly, **Density Functional Theory** provides detailed insights into the electronic structure of medicine molecules and their interactions with target proteins at a quantum mechanical level, which is used, for example, in pharmacokinetic modelling. Collectively, these techniques enable in-depth *in silico* prediction and analysis, that potentially could offer valuable insights into the efficacy and safety of new medicines.

Cluster 12: Computational Structure-based Drug Discovery, Efficacy and Safety Assessment (light blue, right)

This cluster represents further **molecular modelling** techniques which are most used for **virtual screening** of compound libraries for predictions of efficacy and/or safety profiles of molecules. Utilizing statistical models, quantitative structure-activity relationship (**QSAR**) models link a compound's chemical structure to its biological activity, allowing for the prediction of characteristics and toxicity of a substance [59–61]. Similarly, **pharmacophore modelling** identifies key features of a molecule necessary for biological activity, which can be used for virtual screening, ADMET prediction, side effects modeling, off-target prediction, and target identifications[65]. These techniques are now mostly integrated in early stages of medicine discovery, however, if the predictive value of these models is validated, this information could be used to inform, *replace* or *reduce* animal use.

8.3. List of recent IHI funding calls relating to 3Rs and NAMs

Funding Call	Budget (EUR)	No. projects	Deadline	Title	No. of proposals
HORIZON-JU-IHI-2023-04-02-two-stage	8,5 million	1 project	8 Nov 2023 (1st stage) 23 Apr 2024 (2nd stage)	Minipigs : a path to reduce and replace non-human primates in non-clinical safety assessment	3
HORIZON-JU-IHI-2023-05	30 million	2-3 projects	16-Jan-24	Accelerate implementation of NAMs for development, testing & production of health technologies	3
HORIZON-HLTH-2024-TOOL-05-06	25 million	3-6 projects	19 Sep 2023 (1st Stage) 11 Apr 2024 (2nd Stage)	Innovative non-animal human-based tools and strategies for biomedical research	190
HORIZON-HLTH-2024-IND-06-09	2 million	1 project	11-Apr-24	Gaining experience and confidence in NAMs for regulatory safety and efficacy testing	4
HORIZON-INFRA-2024-DEV-01-01	12 million	4-12 projects	12-Mar-24	Research infrastructure concept development	NA
HORIZON-HLTH-2024-TOOL-11-02	25 million	3-6 projects	11-Apr-24	Bio-printing of living cells for regenerative medicine	81

Regulatory Science Research Needs:

Number	Title	Research Topic	Objectives	Expected Impact
H2.1.1	Driving collaborative evidence generation - improving the scientific quality of evaluations	Conduct research to identify the best approaches to stimulate developers to use novel pre-clinical models, including those adhering to the 3Rs	Review results of 3R research from previous EU funded pre-clinical studies and existing literature. Compare and contrast these studies with those submitted non-clinical data in SA and recently approved files. Define the underutilised 3R approaches and best approach to incentivising their use. Identify obstacles to moving from animal models to non-animal alternatives	Encourage the use of 3R methods and reduce animal testing in medicines development

Number	Title	Research Topic	Objectives	Expected Impact
V1.2.2	Catalysing the integration of science and technology in medicines development	Conduct research to identify the best approaches to stimulate developers to use novel pre-clinical models, including those adhering to the 3Rs	Review results of 3R research from previous EU funded pre-clinical studies and existing literature. Compare and contrast these studies with those submitted non-clinical data in SA and recently approved files. Define the underutilised 3R approaches and best approach to incentivising their use. Identify obstacles to moving from animal models to non-animal alternatives	Wider use of 3Rs methods and reduced animal testing in medicines development
V3.4.3	Addressing emerging health threats and availability/therapeutic challenges	Research how the detection of nucleic acids can be applied for the quality control of veterinary vaccines, e.g. in the detection of extraneous agents and vaccine strain characterisation	Facilitate the development, standardisation and regulatory acceptance of new technologies (like Next generation sequencing) for the detection of extraneous agents in biological veterinary medicinal products	New technologies may offer an alternative to replace or complement current tests for detection of extraneous agents, offering a quicker, cheaper, possibly more sensitive, simultaneous, in vitro approach for extraneous agent (EA) testing. This will reduce risks at early stages of product development when seeds need to be proven free from EAs. It also offers opportunities for implementation of 3Rs

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