Transparency in Non-Technical Summaries to Sustain the 3Rs in Respiratory Diseases Research

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Abstract

Non-Technical Summaries (NTS) are legal documents first introduced by the Directive 2010/63/EU[1] to enhance transparency within scientific animal experimentation. Researchers intending to conduct biological research on animal models must fulfil the NTS requirements by outlining their use of animals and how they would implement the 3Rs (Replacement, Reduction, Refinement of animals in research[2]). This study outlines a new systematic methodology approach which enables the assessment of NTS transparency based on the accurate reporting of the 3Rs-specific criteria. This made-to-measure strategy will advance the development of the practical guidelines for the Animal Welfare and Ethical Review Bodies (AWERBs) of establishments conducting animal research, in the process of scrutinising NTS. This would contribute towards the identification of gaps in the reporting of the 3Rs regarding experimental animal procedures: a remarkable hurdle for the achievement of openness in scientific communication.

Additionally, this study supports the development of transparent NTS models as tools, for the advancement of new technologies in replacement-demanding research fields such as respiratory diseases (RD). Although NTS were originally conceived as informative tools for the lay audience, we concluded that data contained in NTS provides the basis for systematic analysis, and identification of limitations regarding the use of Replacement strategies. Reviewing NTS highlighted relevant information regarding the experimental limitations of replacement techniques adopted by researchers, which can be addressed in future studies.

Introduction

Promoting transparency in scientific writing represents the first step towards achieving reliable and accurate data[3]. Failure in reporting detailed methodology and truthful outcomes of scientific experimentations has long been known as a common pitfall in different scientific fields. In particular, studies concerning the use of animals in medical procedures have reported an alarming lack of transparency[4]. Applying the 3Rs (Replacement, Reduction, Refinement of animals in research[2]) promotes transparency and drives high quality research improving data accountability and reproducibility[5].

Lack of transparency is an important issue around the potential for unethical practice in animal procedures that fail to translate into effective therapies[6]. Although concern around animal research is often seen to stem from concerns regarding animal welfare, funding bodies and pharmaceutical businesses experience major economic losses due to unsuccessful end results of clinical trials[6,7]. Attrition of drug candidates at clinical trials is most likely due to over-reliance on data from animal models and poor study design[8,9]. Additionally, insufficient quality reporting of scientific information carries a human cost which cannot be ignored. Often, patients in clinical trials do not respond adequately or suffer severe, occasionally fatal, adverse effects from therapeutics developed solely on animals[10]. The level of trust the public has in animal scientific procedures has a significant impact on how funds are distributed in medical research[11,12]. Transparency in scientific communication is a critical component in ensuring the development of safe and effective medicine.

To promote transparency, in 2010, the EU Commission introduced Directive 2010/63/EU which implemented the use of NTS (Non-Technical Summaries) as documents intended for the public outlining procedures carried out on laboratory animals[1]. Subsequently, this directive was amended by the Implementing Decision (EU) 2020/569, which incorporated further measures aimed at “moving transparency to the next level”[13]. In addition to implementing an open access central EU database for NTS and member states annual statistics, the new regulation defines novel and more stringent requirements for the reporting of NTS information.

Other instruments are aimed at improving the laboratory animal research quality and welfare by promoting transparency and accuracy in experimental design and data reproducibility. These are the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines[14,15], the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines[16], the Experimental Design Assistant (EDA)[17], and the Animal Study Registry (ASR) for pre-registration of animal studies[18]. Additionally, in Germany,

systematic and quantitative analysis of the information contained in the NTS was enabled through the database AnimalTestInfo[19].

The benefits derived from publishing accurate and transparent NTS are not only increasing general openness around research involving animals but also providing insight into how the 3Rs are being applied. NTS are valuable tools that report the planned use of animals, as well as the reasons why no suitable non-animal method could be used: a robust identification of these issues will contribute towards the development of novel techniques aimed at the further replacement of animals in medical and scientific research.

The aim of this study was to investigate variation in the transparency of NTS, applying a systematic methodology designed around the reporting of accurate information. Our case study focused on NTS discussing animal experiments and 3Rs application in the field of Respiratory Diseases (RD) research. In 2019, RD accounted for the second leading cause of death in the World after cardiovascular diseases[20]. Furthermore, the deleterious consequences of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) outbreak has highlighted the need for fast and effective solutions[21]. The COVID-19 pandemic also demonstrated the importance of transparency and sharing of data within the field of RD by promoting availability of funding and accountable scientific data[22,23].

Our data present a substantial variation in transparency across NTS published from 2013 to 2020. However, requirements for transparent reporting of NTS increased in 2020 with the adoption of the new EU NTS template with the 2019/1010 EU amendment. For this reason, we reviewed separately the transparency of information provided in 2013-2019 NTS from that provided under the new guidelines in the first volume of 2020 NTS. We created a systematic methodology intended as guidance for AWERB lay members during the review of proposed license applications.

Additionally, this study demonstrates how information relevant to 3Rs practice could be systematically extracted from the NTS and further explored. We examined the issues reported by researchers in the 2013-2019 NTS that limited their application of the Replacement strategies, thus highlighting aspects of alternative strategies that could be improved.

Materials & Methods

1. NTS Selection

NTS are publicly available documents that have been accessible on the UK Home Office Website since 2013[24], when the Directive 2010/63/EU was introduced[1]. Each year, the Animals in Science Regulation Unit of the Home Office grants licenses under Animals (Scientific Procedures) Act 1986 for scientific procedures using animals and publishes the NTS from those applications, categorising them based on either subject area (e.g., "projects on the respiratory system", "projects on the immune system", etc.) or a defined time frame (e.g., volumes comprising of 6 months each: January-June/July-December). This study is intended as a comprehensive, chronological meta-analysis of all UK NTS concerning RD research from January 2013 to June 2020. With RD, this study refers to "a type of disease that affects the lungs and other parts of the respiratory system"[25]. For the search strategy to identify NTS specific to RD in the UK, the Medical Subject Headings 2021 (MeSH) browser was used to create a list of "Respiratory Diseases Research" and "Animal Testing" related Keywords[26]. Additionally, the word search browser Power Thesaurus was used to identify less jargonised synonyms of "Respiratory Disease", "Lungs", "Animal Testing"[27]. Subsequently, NTS were selected based on their title and Keywords matching the lists of words created with MeSH and Power Thesaurus.

2. NTS Classification: Time & Research Areas

To provide the basis for a comprehensive analysis and exploration of trends in transparency, NTS were classified based on both thematic and year of publication. Five RD research areas (Infectious; Chronic; Acute; Circulatory; Other) were identified as representative of all diseases affecting the respiratory system in humans. The selected research areas are the equivalents of the following sections of the International Classification of Diseases and Related Health Problems (ICD-10, Chapter IX - X)[28]. Infectious RD included pathologies and disorders described as in: Chapter X - J00-J06; J09-J18; J85-J86. Chronic RD: Chapter X - J40-J47. Acute RD: Chapter X - J20-J22; J30-J39; J80-J84. Cardiovascular RD: Chapter IX I27. Other or Unable to classify: Chapter X - J90-J94; J95-J99; RD J60-J70. NTS were allocated to a singular RD research area based on the information reported within their title and Keywords. The year of publication (2013-2020) was extracted from the document files available on the UK Home Office website[24].

3. Systematic Analysis of NTS

3.1 EU NTS Templates: 2013-2019 & 2020

The systematic selection of NTS highlighted two different templates: one adopted for 2013-2019 NTS, in compliance with the Directive 2010/63/EU (**Figure S1**) and one for 2020 NTS with additions implemented by EU/2019/2020 Regulation (**Figure S2**). NTS templates were compared, and two datasets created based on the different NTS requirements. The identification of comparable variables as well as additional sections of information was a critical step for further analysis. A schematic summary of the differences in the NTS writing requirements is provided in **Table 1**.

The reporting of **General** information in the NTS supports wide scientific accessibility and the basic classification necessary for the contextualisation of the research undertaken. The **Aims & Objectives** of the NTS were identified as pivotal information for understanding the research aims and target fields. Information extracted from these sections indicated the importance of transparency when explaining the proposed projects and their likely impact on society. The NTS also provide information relating to the **Severity Levels** of harm and pain that animals are expected to experience: this section contains information on the range of severity (mild; moderate; severe; non-recovery) experienced by each animal species being used, the period of animal use, as well as the planned fate of animals after experiments were completed. The NTS also demonstrate researchers' compliance with the 3Rs by reporting sections related to: Replacement highlighting limitations of alternative techniques and justification of animal use, Reduction**,** and Refinement, providing the rationale for the chosen animal models and the relative welfare costs.

The 2020 dataset differed from the 2013-2019 dataset by including additional sections which provided more detail around the use of animals. Information regarding the life stages of the animals used as well as an indication of whether a Retrospective Assessment was needed were added to the list of requirements. Additionally, within the NTS **Aims & Objectives**, a section stating the short, middle, and long-term impacts of the research and its outputs was included. Finally, detail regarding the experimental procedures and duration of animal suffering was added to provide a more comprehensive understanding of the **Severity Levels** of the research undertaken.

The 2020 template of the NTS also demanded more information with respect to the application of the 3Rs. For Replacement, researchers had to provide background on their search for

alternative methods and why they could or could not be applied to their type of research. The research's experimental design including calculations with statistical power models determining the appropriate number of animals strengthened Reduction in the 2020 NTS template. In addition, for Refinement, a justification of the animals used according to their life stage and an indication of the chosen best practice guidance were included.

Table 1. Summary of NTS Requirements based on differences between 2013/2019 and 2020 EU templates.

3.2 Selection of Metrics for Assessment of Transparency

From the NTS requirements in the 2013-2019 dataset, 12 metrics - as referred in **Table 2** - were selected in this study for the assessment of transparency. These were chosen based on the guidelines reported in Directive 2010/63/EU and previous analysis of the structure of the NTS[31]. The assessment criteria and weights applied are reported in **Table 2**. In addition to the metrics assessed in the 2013-2019 NTS dataset, 17 more were introduced in the 2020 NTS dataset following the implementation of EU/2019/1010 (**Table S2**). Due to unavailability of raw data at the time this study was conducted, no year-on-year trends could be calculated that included the 2020 dataset.

Table 2. Transparency metrics used for assessment of 2013-2019 NTS dataset. Assessment criteria represent the rationale behind awarding a specific score (yes; no; partially) to each metric, representing different aspects of transparency in the NTS. Weighting values are also reported relative to each metric. Spearman's Rank Coefficients of Correlation between individual metrics and the combined Transparency Scores represent the contribution towards the overall Transparency Score. A higher coefficient indicates a higher correlation of the metric with the Transparency Score.

In the weighting the metrics which were awarded the highest contribution towards the overall Transparency Score were those which were explicitly cited by Article n.43 in Directive 2010/63/EU[1]. Accuracy Score and contributed towards the NTS transparency and better reception, accounting for half weight. However, those awarded the least weight, such as Project Duration and Period of Animal Use, did not report essential information for NTS transparency assessment, yet they are specific requirement that must be met by the researchers.

To demonstrate the relative variation in each metric in each respective reporting period (2013-19 and 2020, respectively), each metric was rescaled to values from 0 to 1. The full range of raw scores, their mean and standard errors were calculated for each. Additionally, the Spearman's Rank Coefficients of Correlation between values of each metric and the overall Transparency Scores calculated for the 2013-2019 period were used to indicate the relative contribution of each metric to the overall Transparency Score. Metrics which had coefficients greater than 0.5 were the most influential on the overall level of transparency, regardless of weighting applied in the creation of the overall score.

Given that the reporting requirements for NTS were consistent up to 2019, Transparency Scores were applied to NTS from 2013 to 2019 and each submission assigned to a single, primary research area. Transparency data were sufficiently normally distributed and homogeneous within groups to support examination of the variance among year groups and research types using a Gaussian generalised linear model with an identity link function. The weighted average of the suite of metrics was calculated and then rescaled from 1 to 100 so that the Transparency Score represented a % compliance with assessment criteria, presented as a percentage. Data were not treated as 'bounded' in this case given that the interquartile range was 61-82%, the lowest score was 32%, well above the minima, and only three cases achieved the maximum possible score of 100%. Data were not sufficient to support examination of interaction between years and research types because not all research types were represented in each year. The model was refined using stepwise deletions of insignificant terms (at $p>0.05$). All analyses were conducted in the 'stats' package of the R programme v 4.0.4[32].

3.3 Accuracy Score

Based on the official regulatory reporting guidelines, we previously devised an Accuracy Score aimed to evaluate the clarity of NTS in communicating animal science information to the lay public[33-35]. Three categories were included: Grammar & Syntax; Lay Terminology; Statements Specificity.

Depending on whether the NTS fulfilled the marking criteria described for each of the three Accuracy Score metrics, they were awarded a score from 0-3, which was subsequently re-scaled from 0-1 to allow comparison with the other metrics. If *Grammar & Syntax* was always correct and the sentences were generally short (15-20 words), the NTS were awarded with 1 for *Grammar & Syntax*. However, if *Grammar & Syntax* was generally incorrect and sentences exceeded 25 words length[36], the NTS were awarded 0. The use of *Lay Terminology* in the

NTS was also identified as an important indicator of NTS language openness and clarity[31]. Therefore, if the terminology and writing style of NTS was generally accessible to the lay reader and meanings of abbreviations were explained, the NTS were awarded 1. Conversely, if the terminology and writing style was not accessible or abbreviations were not explained, the NTS received the score of 0 for *Lay Terminology*.

A third factor contributing towards the NTS overall Accuracy Score was *Statements Specificity*: if the statements were consistently true and specific and the summary did not overstate the potential benefits; AND the aims & objectives were clearly outlined, then the summary was given a score of 1. When the statements were generally vague and not supported by secondary literature; OR the summary overstated the potential benefits; OR the aims & objectives were not clearly outlined, the NTS was awarded a 0.

4. Assessment of Replacement Limitations

Replacement strategies include all laboratory techniques aimed at either partial or full replacement of animals in scientific experiments[37]. As presented in **Table 1**, the 2013-2019 NTS template provided researchers with a section specifically intended for the reporting of relevant information regarding Replacement. By reporting the reasoning why animal models were essential for their case study (Animal Use Justification in **Table 1**), researchers must discuss the limitations of the available Replacement alternatives and why they could not be adopted in their research study (Tools Limitations in **Table 1**).

A comprehensive list of limitations (**Table S3**) concerning Replacement tools was created through an extensive literature and database search[38-44]. Eighteen categories (Issues A-R in **Table S3**) were highlighted spanning from Replacement limitations related to reproducing living variables such as oxygen levels, blood supply, tissue stiffness (i.e., Cancer Biology; Issue O) to more ethical and traditionalist ones (Tradition & Ethics; Issue Q & R). Based on information contained in the Animal Use Justification and Tools Limitations sections, NTS were assigned to the corresponding category. We identified the most and least reported issues by examining the number of projects reporting issues in different years and areas of research**.** Issues reported by >10% of NTS were selected as the most frequent and considered for further evaluation and contextualisation.

Results

1. NTS – what do they contain?

In the UK, from January 2013 to December 2019, a total of 174 NTS (**Table S1**) were identified on the Home Office website which were associated with applications to use animals for the advancement of RD research. On average, 24 NTS were published each year. The highest number of RD research based NTS were granted in 2013 and 2018 (31 and 34, respectively). Fewer (10) RD NTS were submitted in 2016 compared to other years.

Infectious RD research was the most represented in 2013-2019 NTS (48%). Chronic and Circulatory RD also accounted for a relatively higher frequency (relatively 27% and 14%, respectively) compared to Other (6%) and Acute (5%). The year 2016 was the only exception, whereby NTS reporting chronic RD research was more frequent than the infectious counterparts. Nevertheless, the number of NTS granted that year was also relatively lower compared to other years (i.e., only 6% of total NTS from 2013-2019).

From **Figure 1**, it is evident that NTS focused on acute RD research became present starting from 2015 although their frequency remained the lowest (5%). Additionally, 2015 and 2017 were the only years that reported NTS included all five different types of RD.

Figure 1. Numbers of NTS by year (2013 – 2019) and RD research area (Acute; Chronic; Circulatory; Infectious; Other).

It is an essential requirement in NTS to provide the species of animal selected for the experimental studies. From the analysis of the 2013-2019 NTS datasets, 20 animal types were selected by the researchers for the purpose of RD research (**Figure 2**). Mice were the most frequently cited animal model accounting for 50% of the total animal models frequency. After this, rats comprised 19% and guinea pigs 10%. The least frequently used animal models were badgers, horses, macaques, partridges, pheasants, pigeons, and adult zebrafish. In total, 10 NTS (4%) failed to provide the type of animals used.

Figure 2. Frequency of animal models used reported in NTS from 2013 to 2019.

2. Exploration of Transparency in NTS

2.1. Transparency in 2013-2019 NTS dataset

Across the 2013-2019 dataset, the Transparency Scores for the NTS published each year and within research areas ranged widely, typically between 40% and 90% transparency (**Figure 3**). As a result of this high variability in scores, there was no significant variation at all among Transparency Scores between different research areas. A weakly significant effect of the year of publication on transparency (F=2.908, year df=6, residual df=167, p-value: 0.010, **Figure 3**) reflected higher score estimates in 2016 (80%), 2017 (75%) and 2018 (77%) compared to those of 2013 (66%). There was no temporal trend towards either increased or decreased transparency throughout the reporting period (**Figure 3**). The score estimates for 2019 (66%) were similar to those for 2013.

Figure 3. Variation in raw and mean (+/- SE) Transparency Scores of NTS submissions between 2013 and 2019 presented **a)** by Year and **b)** across Research Areas.

In **Figure 4a,** ninety five percent of NTS were fully compliant with the requirement to provide the NTS Benefits (scoring 1). Period of Animal Use and Animal Model were fully compliant in 94% of NTS. Animal Use Justification was also a metric reported transparently for which 71% of NTS scored fully (1), only 15% were partially compliant (0.5) and 14% were non-compliant (0).

Figure 4. Raw and mean (+/- SE) values of metrics that describe the transparency of NTS in **a)** 2013-19 and **b)** 2020.

The 3Rs were reported the least well amongst all metrics with Replacement reporting a mean value of 0.48 (+/- 0.04), Reduction: 0.31 (+/- 0.04), Refinement: 0.44 (+/- 0.04). On average, Replacement was reported the best out of all the three Rs. Among the 3Rs, Reduction is reported with the least transparency among all metrics as 68% of the NTS scored 0. Replacement and Refinement metrics yielded zero values in 50 and 56 percent of NTS, respectively.

The Accuracy Score, after rescaling from 0-1, was relatively well adhered to and there was little variation in this across the NTS (mean 0.60, +/- 0.02 standard error). On the raw scale of compliance from 0-3, Accuracy scores of 1 and 2 were the most frequently attained in 32%

and 41% of the NTS, respectively. A lack of compliance in Accuracy, with a score of 0, was demonstrated by 4% of NTS whilst 22% achieved full compliance, attaining the top score of 3.

Spearman's Rank Coefficients of Correlation between individual metrics and the combined Transparency Score in **Table 2** represent the contribution of these different aspects of transparency towards overall score of transparency. Accuracy Score and the 3Rs were more closely reflected in the overall Transparency Score (**Table 2**). Compliance with the reporting requirements for the justification of use of animal models mirrored Transparency Scores most closely of the remaining metrics, while the clarity of benefits of the research to scientific development and reporting of 'severity levels' had less bearing on the overall Transparency Score (**Table 2**).

2.2. Transparency in 2020 NTS Dataset

The Transparency Score of 2020 NTS was represented by just 13 projects in a 6-months' time frame (not a full year since the only 2020 NTS available on the U.K. Home Office website were from January and June 2020).

Overall, as shown in **Figure 4b**, the Transparency Score of the 2020 NTS metrics was close to 1. In 2020, 12 metrics scored highest (1): Experimental Design Reported, Guidance Reported, Harms Reported, Impact Clarity, Method Justification Reported, Procedures Reported, Retrospective Assessment, Species Reported, Stages Reported, Animal Type Reported, Project Duration Reported. However, in 2020 no NTS reported Keywords, compared to the 2013-2019 dataset where 80% of NTS did (2019 NTS did not report Keywords).

Concerning the 3Rs, they were all reported with high level of transparency compared to the 2013-2019 NTS. Refinement was the one reported with highest level of transparency compared to Replacement and Reduction also very well reported but with larger degree variation compared to 3R. Animal Number Calculation was the second least well reported metric indicating, there would still be aspects that could be advanced.

Considering that only half of the 2020 NTS were analysed for transparency, the main conclusion from **Figure 4** is that the metrics of transparency were more variable and generally lower in 2013-2019 than they were in 2020, when additional requirements for transparency were introduced.

3. *Evaluation of Replacement Limitations*

The screening of information cited in the 2013-2019 NTS about the limitations of Replacement tools, highlighted four categories which each represented more than 10% of the issues reported. These were: Issue A (Immune System Reproducibility 23%), C (Pathology Reproducibility 11%), D (Viral Models for Vaccine Studies 10%), and H (Systemic and Cellular Complexity 15%). Such issues were further explored based on research area: we found that Infectious RD reported 50% of the total 303 issues regarding availability and suitability of alternative models. Chronic RD also reported 27% of the total being the second most represented research area in issue counts. As reported in **Figure 5**, Issue A and Issue H included most RD areas across the years. Furthermore, Issue D and Issue C were specific to infectious RD particularly.

Figure 5. Number of times Issues (A, C, D, H) were cited in NTS by year and research type. Issue A: Immune System Reproducibility; Issue C: Pathologies Reproducibility; Issue D: Viral Models for Vaccine Studies; Issue H (Systemic and Cellular Complexity).

NTS reporting issues related to Immune System Reproducibility highlighted difficulties in reproducing the human immune system *in vitro* and recreating the complex multicellular interactions between different organs/systems using non-animal alternatives.

Those reporting limitations such as Pathology Reproducibility discussed the inability of current alternatives to reproduce infection scenarios which included interactions between immune cells, pathogens and different mediators providing quantifiable indices of disease. *In vitro* models were also defined as being unable to determine the sequential change in physiology from injury to inflammation and then repair. Viral Models for Vaccine Studies were also highlighted as a limitation hindering the full replacement of animals in RD research projects.

The NTS reported issues related to *in vitro* systems which researchers said did not provide information related to viral shedding or lung function changes due to respiratory viral infections. Additionally, Replacement systems were said to not provide information regarding vaccine efficacy, memory cells function and mechanisms of transmission from one living organism to the other.

Lastly, the most cited Replacement limitation concerned Systemic and Cellular Complexity, in fact most NTS highlighted a lack of systemic and cellular complexity when utilising *in vitro* strategies. This poses limitations regarding the development of 3D multicellular structures in

lung cell cultures. The challenges that remain include the assessment of how the nature of a drug/chemical is altered and alters different communicating organs in Replacement alternatives remains challenging[45]. The use of *ex vivo* immortalised lung tissue cultures also represents a challenge for Replacement strategies as these behave differently to lungs *in vivo*[46].

Discussion

Since the beginning of the 21st century, ethical dilemmas have been raised about a lack of transparency in the reporting of scientific procedures on animals[47]. The consequences of poorly reported animal research results in data which is neither robust nor reproducible, often causing significant economic and efficiency losses, as well as being unethical[48,49].

Therefore, to improve reporting transparency of high-quality scientific research, EU governments and scientific organisations such as the National Centre for the Replacement, Reduction and Refinement of Animals in Research (NC3Rs) joined forces and enacted several initiatives. They formulated guidelines aimed at improving the reporting of animal welfare procedures and experimental methodology. The most well-known guidelines are ARRIVE, originally published in 2010 and recently updated as ARRIVE 2.0[14]. These have been adopted by over 1,000 journals Worldwide, including for example Nature, and Cell and Science Translational Medicine[50].

It is important to note that it is not just through the engagement of the scientific community that research quality is improved, in fact, the lay public holds some power to influence funding availability. Previously, Pound and Blaug[51] argued that openness can be achieved through public involvement and that this can bring improvements in animal research. EU member states have implemented directives like EU/2010/63 & regulations like EU/2019/1010 which are aimed at increasing the level of transparency in animal science reporting[52]. Among the different measures, the adoption of NTS as transparent tools written specifically for the public was significant progress (**Figure 6**).

Figure 6. Comprehensive representation of NTS communication targets. Being in the public domain, NTS can inform different target audiences and be used for different purposes: **1) Regulations**: Governments and regulatory bodies make use of the NTS to monitor laboratory work using animals; **2) Lay Community**: NTS must be written in a transparent, accurate and universal manner so that anybody older than 12 may be able to understand the basics of the research undertaken[35]; **3) 3Rs Advancement**: Researchers justifying with reasons why partial, full or no Replacement was applied provides useful information for the identification of limitations related to alternative strategies; **4) Scientists and Funding Agencies**: Data drawn from NTS can provide a basis for the development, validation, and implementation of directed 3R strategies as well as guidance for rethinking the role of animal research models[53]. This figure was created with BioRender.com

In this study, we demonstrate the effectiveness of using the NTS as transparency tools for the development of the 3Rs by providing an evaluation of the levels of transparency itself. Systematic analysis of all NTS related to RD research published since 2013 up to 2019 has revealed no consistency in terms of NTS transparency levels (**Figure 3**). Closer scrutiny of these documents could improve the quality of information about animals in science available to the public.

However, interestingly after the introduction of the 2019/1010 EU regulation, the transparency level increased significantly (**Figure 4**) due to the adoption of a new template, in line with the suggestions previously given by Taylor et al.[34]. We found that the 2020 NTS template guides researchers to describe and justify the use of animals more explicitly. This shows that cooperation between policy makers and researchers can positively impact the communication of what happens in laboratories.

Similarly, as in Bert et al.[53], we identified NTS as useful tools for the exploration of trends and patterns related to animal use in the field of RD research. For example, infectious respiratory diseases accounted for a substantial proportion of projects using animals (**Figure 1**) and we screened for the factors that may have made researchers look to using animal models by analysing what was being reported as justification of animal use in the NTS (**Figure 5**).

This analysis also identified elements contributing to a faulty system. We identified reports of a lack of Replacement methods able to recreate the complex interactions between lungs, heart, and brain, as well as the immune system reaction to pathogens infection as being the major limiting steps towards the adoption of replacement (**Figure 5**). However, although the scientific community recognise such limitations, a growing number of attempts have been made by scientists led by the 3Rs ethos to overcome those. Of current relevance for the recapitulation of the complex interactions between organs are organs-on-a-chip with microfluidic devices[54,55], and *ex vivo* cultured organoids (e.g., Human Lung Stem Cell-Based Alveolospheres)[56]. *In silico* tools enable accurate compound-target simulations, for toxicity and pharmacological studies (e.g., Quantitative Structure Activity Relationship)[57].

A continued focus on improvement in the quality and detail of the NTS will improve transparency and help share good practice. This would help address the scientific gap that currently exists where animal research projects are not published and allow other researchers to assess the value of methodologies using animals and their outcomes. This would also allow more scrutiny of the value of animal research which in turn impacts the ethical case for future research in the same area. All improvements in transparency shed light on where animal

research is working or failing and help identify gaps to be filled to ensure researchers have the tools to implement the 3Rs more robustly in the future.

Conclusions

NTS are useful tools that, if written in a transparent and accurate manner, can be employed for regulatory reasons as well as to identify the limitations of currently existing tools enabling scientists and funding agencies to validate directed 3R strategies. This study supports how transparent NTS sustain the use of animal models within respiratory diseases research by providing a rationale behind the researcher's decision to use animal models and openness regarding laboratory procedures.

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Declaration of Conflicting Interests

The Authors declare no conflicting interests.

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Ethical Approval

Not applicable

Informed Consent

Not applicable

Others

Raw data can be made available upon request to the corresponding author at the following email (bonasseram@cardiff.ac.uk).

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Table 1. Summary of NTS Requirements based on differences between 2013/2019 and 2020 EU templates.

NTS Requirements	2013/2019	2020 (Jan-Jun)	Notes
General	✓	✓	Generic information reporting essential information for the identification of the project.
Project ID Number	✓	✓	Numerical order.
Project Volume	✓	✓	NTS volumes categories: 2013 – numerical; 2014 – field of research; 2015 – field of research; 2016 – field of research; 2017 – grant period (i.e. Jan-Jun/Jul-Dec); 2018 – grant period (i.e. Jan-Jun/Jul-Dec); 2019 – alphabetical; 2020 – grant period (i.e. Jan-Jun/Jul-Dec).
Project Year	✓	✓	Year in which the NTS were granted by ASRU.
Project Title	✓	✓	Title of the project.
Project Duration	✓	✓	Duration of the project (years).
5C(3)ASPA Purpose	✓	✓	NTS Project Purpose categories as in **Animal Scientific Procedures Act (ASRU) 1986** section *Determining an application: further provision* 5C(3);(a)-(g)[29].
Keywords	✓	✓	Reporting of "Keywords" related to the NTS research field.
Retrospective Assessment	✗	✓	In 2020, a new section on Retrospective Assessment was introduced requiring competent authorities to state whether researchers must submit additional documents to support their project license.
Aims & Objectives	✓	✓	Clarification of NTS relevance towards scientific development.
NTS Aims	✓	✗	Description of the project's Aims & Objectives (e.g. the scientific unknowns or scientific/clinical needs being addressed).
NTS Benefits	✓	✓	Description of the potential benefits likely to derive from the project's completion.
NTS Impact Field	✓	✓	Description of the proposed project impact on humans, animals and/or the environment.
NTS Temporal Impact	✗	✓	Description of the short, middle, and long-term impact of the project on the above stated fields.

NTS Outputs	✗	✓	Description of the project's outputs.
Animal Model	✓	✓	In 2020 NTS, researchers must determine both "Animal Species" & "Animal Model". Although these fields may implicate similar information, at the same time "Animal Species" (i.e. *mus musculus*) demands more specificity than "Animal Model" (i.e.mice). Therefore, in the 2020 NTS format, additional detail is required.
Life Stage	✗	✓	Life stage at which the animal is undergoing experimentations. This field is of major important considering that experimental outcomes may vary greatly in accordance to the life stage of animals[30]. Additionally, with the introduction of the **Directive 2010/63/EU - Article1.3,(a),(ii):** *"foetal forms of mammals as from the last third of their normal development"* are included in the protected list of experimental organisms[1].
Animal Species	✓	✓	Chosen species of animal suitable for the project's experimentations.
Animal No.	✓	✓	In the 2020 NTS pro-forma, the word "approximate" referred to Animal No. is removed.
Severity Level	✓	✓	Indication of the severity levels of harm and pains that animals are expected to experience.
Severity Level by Animal Species	✓	✓	Indication of Severity Level and the proportion and animal species in each category.
Description of Procedures	✗	✓	General description of the procedures which animals will undergo, e.g. number of injections, frequency of surgical operations.
Animal Suffering Duration	✗	✓	Indication of the likely duration of suffering for the animals.
Period of Animal Use	✓	✗	In 2020 NTS, the field "Period" referring more specifically to the "Period of Animal Use" is replaced by the 2020 NTS field "Animal Suffering Duration". Often in 2013/2019 NTS, the period of animal use was intended as the project's duration time.
Animal Harms	✗	✓	Description of the harms likely to be inferred to the animals during experimental procedures.
Animals Fate	✓	✓	Indication of what occurs to the animals post – experimentations.
Replacement	✓	✓	Demonstration of compliance to Replacement.

Alternatives Search	✗	✓	Elucidation of experimental strategies aimed at the search of non-animal alternative models.
Tools Limitations	✓	✓	Explanation of why alternative models could not be adopted.
Animal Use Justification	✓	✓	Explanation of why the use of animals is necessary for the project's outcomes.
Reduction	✓	✓	Demonstration of compliance to Reduction.
Experimental Design	✗	✓	Description of the experimental design strategies applied. This section may include calculations or graphical representations of pilot studies, computer modelling, examples of sharing of tissue and reuse.
Animal No. Calculations	✗	✓	Description of the steps taken to estimate the number of animals required for the study.
Refinement	✓	✓	Demonstration of compliance to Refinement
Animal Model Justification	✓	✓	Explanation of animal models suitability to the type of study conducted.
Explanation of Methodology	✗	✓	Explanation of why the experimental method adopted is the most appropriate considered the NTS objectives.
Justification of Life Stage	✗	✓	Explanation of the choice of species and their life stages.
Best Practice Guidance	✗	✓	Indication of which published best practice guidance was followed to guarantee the application of refined methods.
Welfare Costs	✓	✓	Description of strategies adopted to minimize animal welfare costs.

Table 2. Transparency metrics used for assessment of 2013-2019 NTS dataset. Assessment criteria represent the rationale behind awarding a specific score (yes; no; partially) to each metric, representing different aspects of transparency in the NTS. Weighting values are also reported relative to each metric. Spearman's Rank Coefficients of Correlation between individual metrics and the combined Transparency Scores represent the contribution towards the overall Transparency Score. A higher coefficient indicates a higher correlation of the metric with the Transparency Score.

Metric	Assessment Criteria / Score	Weight	Spearman's Correlation Coefficient
Keywords	Were keywords reported? Yes: 1; No: 0.	0.5	0.27
NTS Benefit	As a lay reader, did the NTS report the aims and objectives, providing a clear rationale of the reason why animal research is relevant and beneficial to society? Yes: 1; No: 0.	1	0.23
Animal Model	Was the animal model reported? Yes: 1; No: 0.	1	0.25
Animal No.	Was the number of animals used reported? Yes: 1; No: 0; Partially: 0.3;0.5;0.6. *Example of partial reporting of Animal No. (0.3;0.5;0.6): ID.9.10.2014 "Mathematically the total number of animals used (24,000) does not match the number of total animals used when adding up each species individually (25,000)."* An NTS was awarded 0.3 if the Animal No. was partially reported, and the Animal Model was not. An NTS was awarded 0.6 if the Animal No. was fully reported, but the Animal Model was not.	1	0.29
Severity Level by Animal Species	Were the severity level and predicted harms reported? Yes: 1; No: 0; Partially: 0.5. *Example of partial reporting of Severity Level by Animal Species (0.5): ID.51.24.2014: Severity levels were reported however the harms inflicted to the animals were not elucidated.*	1	0.21
Replacement	Was Replacement addressed? As a lay reader, is it clear why animals are necessary and non-animal alternatives cannot be used? Yes: 1; No: 0.	1	**0.61**

Reduction	Was Reduction addressed? is it clear how the minimal use of animals is assured? Yes: 1; No: 0.	1	**0.56**
Refinement	Was Refinement addressed? As a lay reader, is the animal model justified? What are general measures to minimize welfare costs? Yes: 1; No: 0.	1	**0.56**
Accuracy Score	A compound score representing Grammar & Syntax, Lay Terminology, and Statement Specificity in accordance with NTS marking criteria. See ***3.3 Accuracy Score***.	0.5	**0.7**
Animal Use Justification	As a lay reader, was the use of animals justified by the NTS with solid basis? Yes: 1; Partially: 0.5; No: 1. *Example of partial reporting of Animal Use Justification (0.5): ID.1.5.2013: Important factors regarding the reproducibility and validity of experiments were stated. However, these did not address any limitations of currently existing non-animal alternatives*.	1	0.42
Project Duration	Was the duration of the project specified? Yes: 1; No: 0.	0	0.21
Period of Animal Use	Was the period of animal use reported? Yes: 1; No: 0.	0	0.28

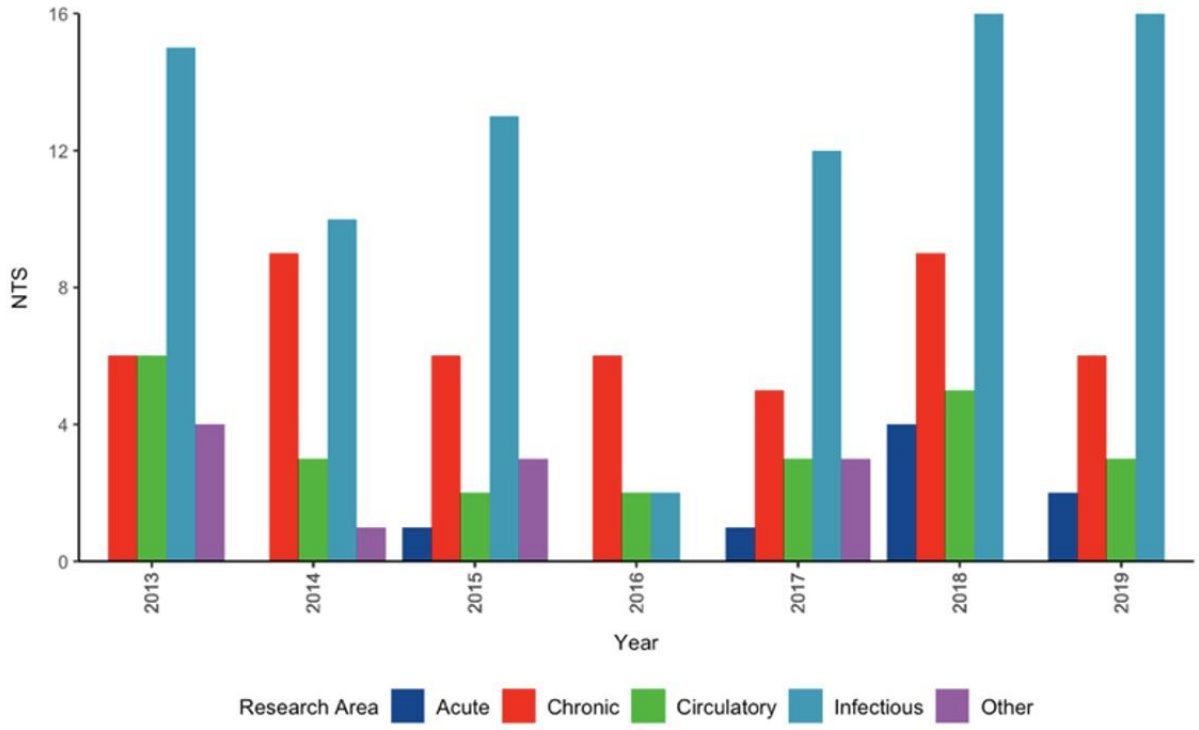

Figure 1. Numbers of NTS by year (2013 – 2019) and RD research area (Acute; Chronic; Circulatory; Infectious; Other).

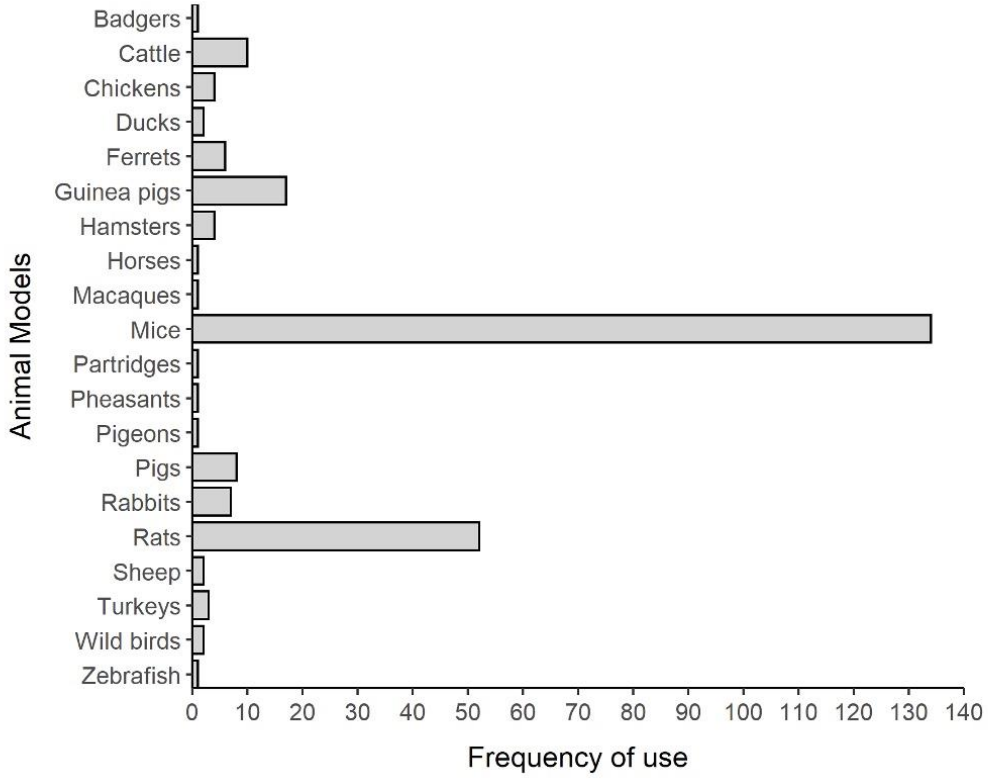

Figure 2. Frequency of animal models used reported in NTS from 2013 to 2019

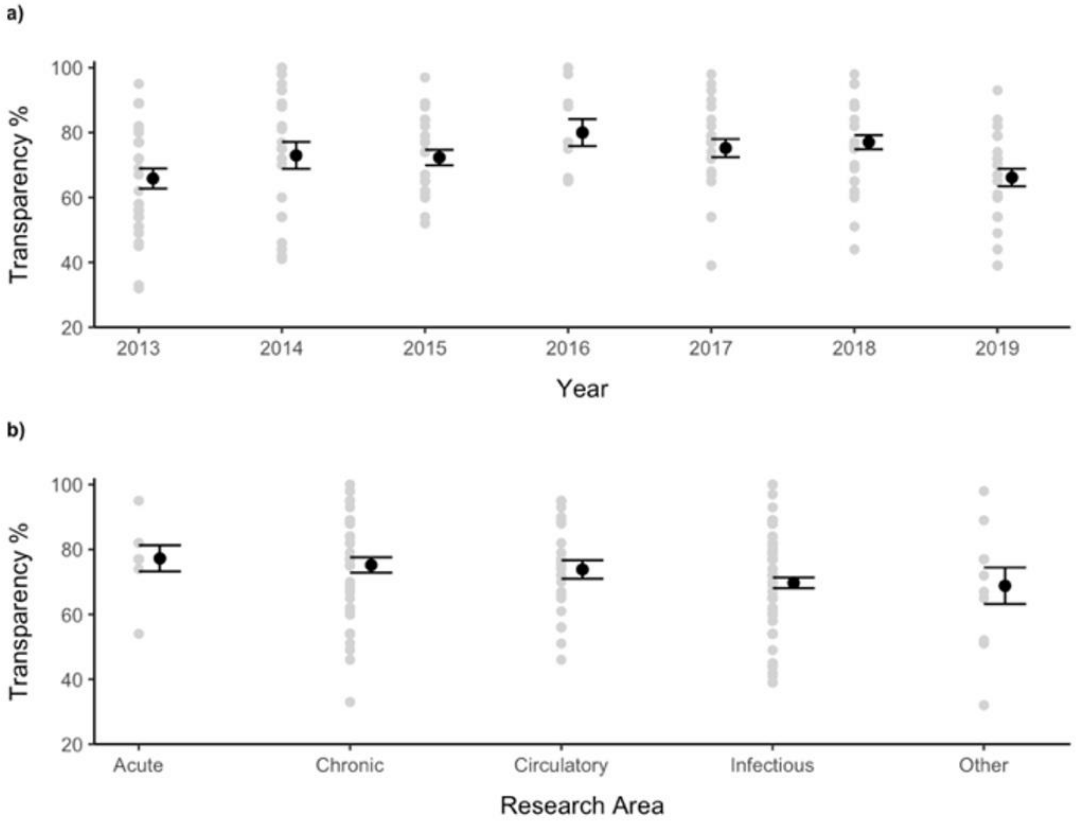

Figure 3. Variation in raw and mean (+/- SE) Transparency Scores of NTS submissions between 2013 and 2019 presented **a)** by Year and **b)** across Research Areas.

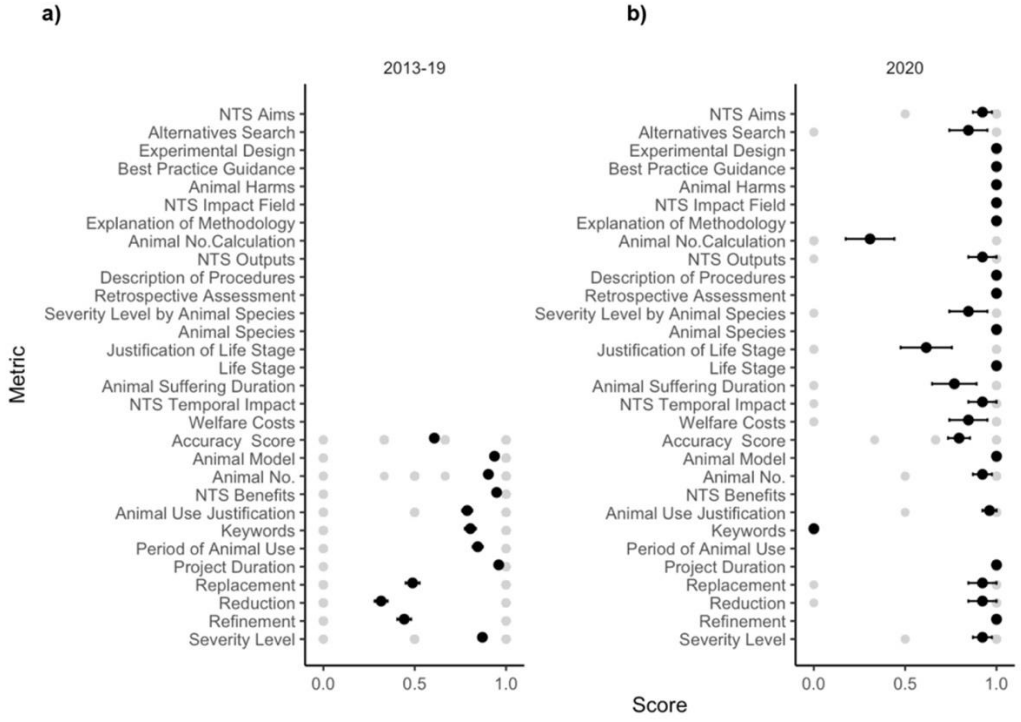

Figure 4. Raw and mean (+/- SE) values of metrics that describe the transparency of NTS in **a)** 2013-19 and **b)** 2020.

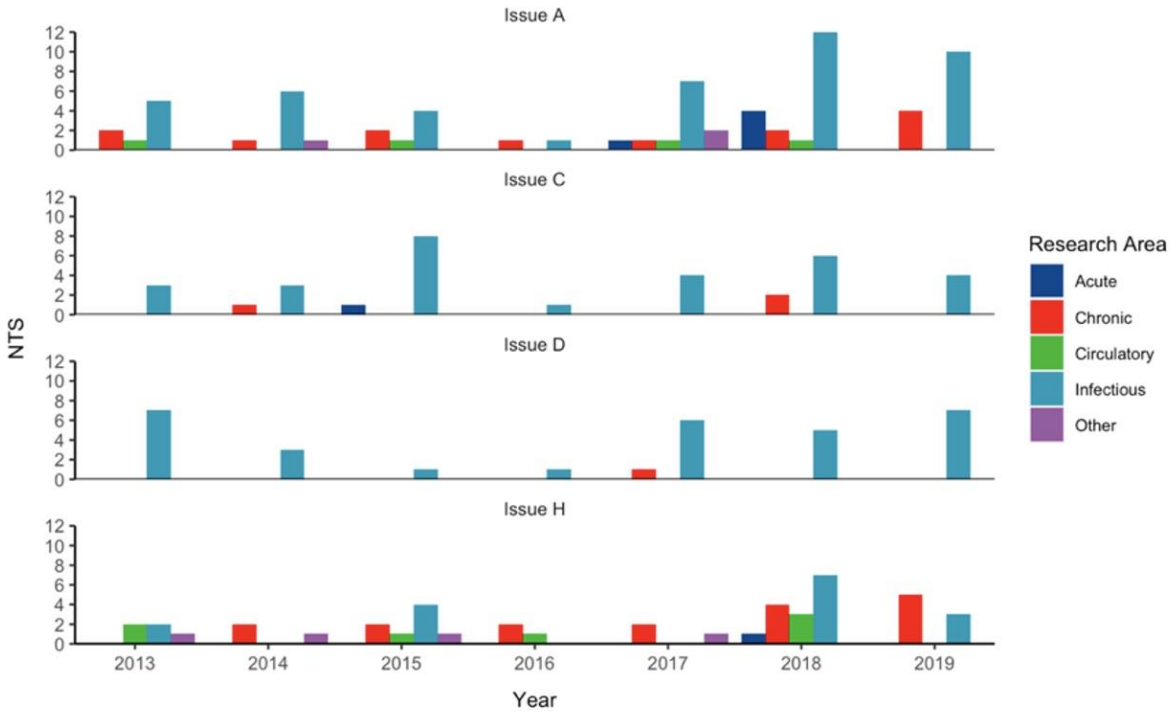

Figure 5. Number of times Issues (A, C, D, H) were cited in NTS by year and research type. Issue A: Immune System Reproducibility; Issue C: Pathologies Reproducibility; Issue D: Viral Models for Vaccine Studies; Issue H (Systemic and Cellular Complexity).

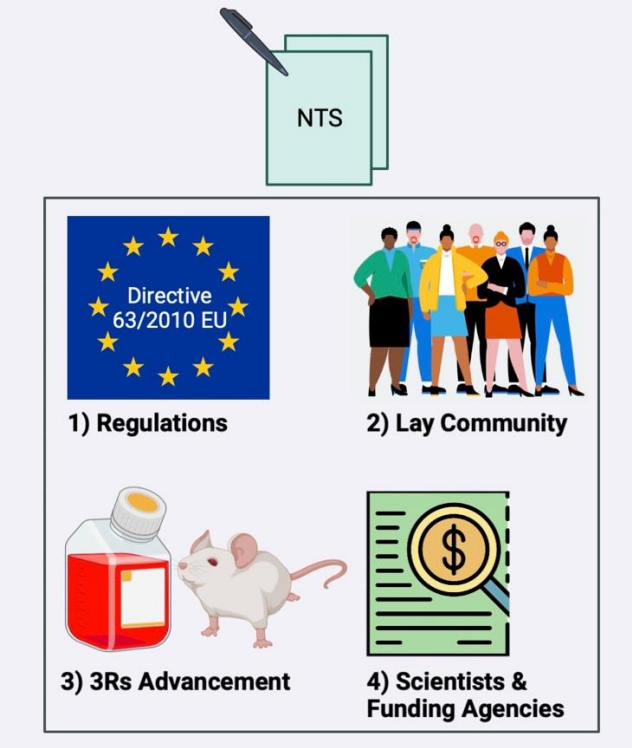

Figure 6. Comprehensive representation of NTS communication targets. Being in the public domain, NTS can inform different target audiences and be used for different purposes: **1)**

Regulations: Governments and regulatory bodies make use of the NTS to monitor laboratory work using animals; **2) Lay Community**: NTS must be written in a transparent, accurate and universal manner so that anybody older than 12 may be able to understand the basics of the research undertaken[35]; **3) 3Rs Advancement**: Researchers justifying with reasons why partial, full or no Replacement was applied provides useful information for the identification of limitations related to alternative strategies; **4) Scientists and Funding Agencies**: Data drawn from NTS can provide a basis for the development, validation, and implementation of directed 3R strategies as well as guidance for rethinking the role of animal research models[53]. This figure was created with BioRender.com

Figure S1: 2013-2019 EU NTS template. Reproduced from[26].

Template/Headings for a Non-Technical Summary

Project Title			
Duration of project			
Key Words (maximum of 5)			
Purpose of Project (as in Article 5)	Basic research	Yes	No
	Translational and applied research	Yes	No
	Regulatory use and routine production	Yes	No
	Protection of the natural environment in the interests of the health or welfare of human beings or animals	Yes	No
	Preservation of species	Yes	No
	Higher education or training	Yes	No
	Forensic enquiries	Yes	No
	Maintenance of colonies of genetically altered animals, not used in other procedures	Yes	No
Describe the Objectives of the Project (e.g the scientific unknowns or scientific or, clinical needs being addressed)			
What are the potential benefits likely to derive from this Project (how science could be advanced or humans or animals could benefit from the project)			
What species and approximate numbers of animals are expected to be used			
In the context of what is being done to the animals, what are the expected adverse effects on the animals, the likely/expected level of severity and the fate of the animals?			
Application of the Three Rs			
1. Replacement State why animals need to be used and why non-animal alternatives cannot be used			
2. Reduction Explain how the use of minimum numbers can be assured			
3. Refinement Explain the choice of species and why the animal model(s) used are the most refined, having regard for the scientific objectives Explain the general measures to be taken to minimise welfare costs (harms) to the animals.			

Figure S2: 2020 NTS EU NTS template. Adapted from[17].

Project Title	
Project Duration	
Project Purpose	
Keywords	
Animal types and Life Stages	
Retrospective Assessment	
Objectives and Benefits	
What is the aim of this project?	Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.
Why is it important to undertake this work?	Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
What outputs do you think you will see at the end of this project?	
What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?	
How will you maximize the outputs of your work?	
Species and numbers of animals expected to be used	
Predicted harms	
Describe, in general terms, the procedures animals will undergo, e.g. injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.	Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behavior - and how long those effects are expected to last.	
What are the expected severities and the proportion of animals in each category (per species)?	Expected severity categories and the proportion of animals in each category, per species.
What will happen to the animals at the end of the study?	
Replacement	
Why do you need to use animals to achieve the aim of your project?	State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
What was your strategy for searching for non-animal alternatives?	
Why were they not suitable?	
Reduction	
How have you estimated the numbers of animals you will use?	Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?	numbers, and principles used to design studies. Describe practices that are used throughout the project to minimize numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
What other measures apart from good experimental design will you use to minimize numbers?	
Refinement	
Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?	Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimize welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.
Why can't you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetized animals)?	
What are you going to do to refine the procedures (for example increased monitoring, post- operative care, pain management, training of animals) to minimize the welfare costs (harms) to the animals?	
How will you ensure you continue to use the most refined methods during the lifetime of this project?	
Explain the choice of species and the related life stages	

Table S1: List of RD related 2013-2019 NTS. The table indicates a unique identifier Project ID for each NTS, allowing systematic search of the documents via the UK Home Office website. The Project ID was devised by the first author Martina Bonassera. Note that the first number represents the NTS Project Number relative the Volume (second number) of the year in which it was granted (third number).

Project ID	Project No.	Volume	Year
2.3.2013	2	3	2013
2.4.2013	2	4	2013
9.4.2013	9	4	2013
1.5.2013	1	5	2013
6.7.2013	6	7	2013
1.10.2013	1	10	2013
2.11.2013	2	11	2013
7.11.2013	7	11	2013
4.13.2013	4	13	2013
2.20.2013	2	20	2013
4.22.2013	4	22	2013
9.23.2013	9	23	2013
10.24.2013	10	24	2013
3.25.2013	3	25	2013
8.28.2013	8	28	2013
1.29.2013	1	29	2013
4.29.2013	4	29	2013
5.31.2013	5	31	2013
3.32.2013	3	32	2013
2.33.2013	2	33	2013
1.34.2013	1	34	2013
6.34.2013	6	34	2013
8.37.2013	8	37	2013
8.39.2013	8	39	2013
1.40.2013	1	40	2013
8.40.2013	8	40	2013
3.41.2013	3	41	2013
3.43.2013	3	43	2013
8.46.2013	8	46	2013
9.47.2013	9	47	2013
11.52.2013	11	52	2013
6.3.2014	6	3	2014
1.10.2014	1	10	2014
5.10.2014	5	10	2014

9.10.2014	9	10	2014
20.11.2014	20	11	2014
16.13.2014	16	13	2014
17.14.2014	17	14	2014
25.14.2014	25	14	2014
8.16.2014	8	16	2014
1.21.2014	1	21	2014
2.21.2014	2	21	2014
3.21.2014	3	21	2014
4.21.2014	4	21	2014
5.21.2014	5	21	2014
6.21.2014	6	21	2014
7.24.2014	7	24	2014
15.24.2014	15	24	2014
24.24.2014	24	24	2014
25.24.2014	25	24	2014
30.24.2014	30	24	2014
51.24.2014	51	24	2014
1.26.2014	1	26	2014
2.26.2014	2	26	2014
1.5.2015	1	5	2015
2.5.2015	2	5	2015
3.5.2015	3	5	2015
4.5.2015	4	5	2015
5.5.2015	5	5	2015
6.5.2015	6	5	2015
7.5.2015	7	5	2015
8.5.2015	8	5	2015
6.7.2015	6	7	2015
2.8.2015	2	8	2015
6.8.2015	6	8	2015
4.10.2015	4	10	2015
31.17.2015	31	17	2015
12.19.2015	12	19	2015
1.20.2015	1	20	2015
2.20.2015	2	20	2015
3.20.2015	3	20	2015
4.20.2015	4	20	2015
5.20.2015	5	20	2015
18.23.2015	18	23	2015
49.23.2015	49	23	2015
34.27.2015	34	27	2015

3.30.2015	3	30	2015
22.30.2015	22	30	2015
25.30.2015	25	30	2015
16.1.2016	16	1	2016
1.4.2016	1	4	2016
2.4.2016	2	4	2016
5.16.2016	5	16	2016
11.16.2016	11	16	2016
22.18.2016	22	18	2016
1.20.2016	1	20	2016
2.20.2016	2	20	2016
3.20.2016	3	20	2016
1.28.2016	1	28	2016
4.1.2017	4	1	2017
37.1.2017	37	1	2017
41.1.2017	41	1	2017
47.1.2017	47	1	2017
56.1.2017	56	1	2017
168.1.2017	168	1	2017
227.1.2017	227	1	2017
12.2.2017	12	2	2017
38.2.2017	38	2	2017
40.2.2017	40	2	2017
42.2.2017	42	2	2017
50.2.2017	50	2	2017
64.2.2017	64	2	2017
97.2.2017	97	2	2017
126.2.2017	126	2	2017
127.2.2017	127	2	2017
137.2.2017	137	2	2017
168.2.2017	168	2	2017
236.2.2017	236	2	2017
245.2.2017	245	2	2017
255.2.2017	255	2	2017
290.2.2017	290	2	2017
291.2.2017	291	2	2017
305.2.2017	305	2	2017
8.1.2018	8	1	2018
25.1.2018	25	1	2018
102.1.2018	102	1	2018
103.1.2018	103	1	2018
114.1.2018	114	1	2018

128.1.2018	128	1	2018
132.1.2018	132	1	2018
134.1.2018	134	1	2018
141.1.2018	141	1	2018
142.1.2018	142	1	2018
155.1.2018	155	1	2018
164.1.2018	164	1	2018
187.1.2018	187	1	2018
207.1.2018	207	1	2018
233.1.2018	233	1	2018
236.1.2018	236	1	2018
244.1.2018	244	1	2018
286.1.2018	286	1	2018
317.1.2018	317	1	2018
323.1.2018	323	1	2018
331.1.2018	331	1	2018
4.2.2018	4	2	2018
22.2.2018	22	2	2018
48.2.2018	48	2	2018
55.2.2018	55	2	2018
57.2.2018	57	2	2018
73.2.2018	73	2	2018
97.2.2018	97	2	2018
126.2.2018	126	2	2018
132.2.2018	132	2	2018
140.2.2018	140	2	2018
149.2.2018	149	2	2018
169.2.2018	169	2	2018
174.2.2018	174	2	2018
6.1.2019	6	1	2019
25.1.2019	25	1	2019
33.1.2019	33	1	2019
59.1.2019	59	1	2019
61.1.2019	61	1	2019
99.1.2019	99	1	2019
142.1.2019	142	1	2019
231.1.2019	231	1	2019
407.1.2019	407	1	2019
408.1.2019	408	1	2019
413.1.2019	413	1	2019
459.1.2019	459	1	2019
506.1.2019	506	1	2019

528.1.2019	528	1	2019
572.1.2019	572	1	2019
36.2.2019	36	2	2019
78.2.2019	78	2	2019
80.2.2019	80	2	2019
128.2.2019	128	2	2019
150.2.2019	150	2	2019
167.2.2019	167	2	2019
215.2.2019	215	2	2019
216.2.2019	216	2	2019
273.2.2019	273	2	2019
284.2.2019	284	2	2019
343.2.2019	343	2	2019
367.2.2019	367	2	2019

Table S2: 2020 NTS Transparency Metrics with relative Description and Weight

Metric	Description	Weight
Keywords	Were any keywords reported?	0
Project Duration	Was the duration of the project reported?	0
Retrospective Assessment	Based on the type of animal procedures planned, this NTS section indicates whether it has been reported the necessity of a retrospective assessment on behalf of the applicants	0.5
NTS Aims	Score indicative of the clarity of the NTS project aims	1
NTS Project Impact Field	Score indicative of the clarity regarding the section describing the potential areas deriving benefits from the NTS project	1
NTS Project Outputs	Score indicative of the clarity of the NTS section describing methods through which the project outputs will be maximized.	1
NTS Temporal Impact	Score indicative of the clarity of the NTS temporal impacts.	1
Animal Species	Was the species of animal used reported?	1
Animal Model	Was the animal model used reported?	1
Animal No.	Mean score for animal numbers reported for animal models used in the project.	1
Life Stage	Were the life stages relative to each animal model used reported?	1
Severity Level	Were the severity level and predicted harms reported?	1
Animal Harms	Description of the harms likely to be inferred to the animals during experimental procedures.	1
Severity Level by Animal Species	Indication of whether the severity level was reported for each animal species included in the project.	1
Description of Procedures	Indication of whether the NTS researchers included a description of the animal experimental procedures.	1
Animal Suffering Duration	Indication of whether a duration of animal suffering was reported.	1

Replacement	Section showing compliance with Replacement strategies including an explanation of animal models necessity, raising any issues related to the application of supplementary non-animal alternatives aimed at the progress of the NTS project.	1
Reduction	Section showing compliance with Reduction strategies adopted by the NTS project holders to ensure that minimal numbers of animals will be used in experiments.	1
Refinement	Section showing compliance with Refinement strategies to supervise animal welfare. In this section, a justification specific to the animal models chosen should be reported showing relevance to the study proposed by the NTS.	1
Accuracy Score	Summary system as in[28]. The accuracy score number is the sum of Grammar & Syntax, Lay Terminology and Statements Specificity individual scores which can be either 0 or 1. Thus, the total accuracy score could range from 0 to 3.	0
Animal Use Justification	Indication of whether a justification for animal use in the NTS was reported	0.5
Alternatives Search	Indication of whether researchers explained how they searched for replacement alternatives	0.5
Experimental Design	Indication of whether details regarding NTS experimental design procedure was reported	0.5
Animal No. Calculation	The researchers must provide an explanation of how they calculated the numbers of animal used, showing figures and numbers.	0.5
Explanation Methodology	Indication of whether a justification relative to the project's experimental procedure was reported	0.5
Justification of Life Stage	Indication of whether a justification of the animals' life stages was reported	0.5
Best Practice Guidance	Indication of whether a 3Rs experimental guidance material was reported in the NTS	0.5
Welfare Costs	Indication of whether the NTS reported strategies aimed at the minimization of animal welfare costs	0.5

Table S3: Replacement Limitations Categories

Issue	Limitation
A	Immune system reproducibility
B	Legal requirements
C	Pathogenicity & pathophysiology reproducibility
D	Virus infection models & vaccines
E	Species-specific experimentations
F	Gene regulation & function
G	System toxicity & sensitization
H	Lack of systemic and cellular complexity in in vitro models
I	TB in vitro models
J	Ex vivo limitations
K	The use of "alternative" species
L	Inability to reproduce cardiovascular system features in vitro
M	Inability to replicate the physiology & anatomy of the lungs
N	Limitations in recreating a nervous system in vitro
O	Limitations in reproducing tumor & cancer models in vitro
P	Educational purposes
Q	Tradition
R	Ethical issues