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Preclinical Drug Development: Application of Advanced Models and Predictive Methods in Safety Assessment

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Abstract

Preclinical drug development faces inherent limitations associated with conventional models, such as two-dimensional cell cultures and animal models, which exhibit limited physiological relevance and contribute to high attrition rates in clinical phases. NAMs, including human organoids, organ-on-chip systems, micro-physiological platforms, physiologically based pharmacokinetic modeling, omics technologies, and artificial intelligence, provide an integrated and predictive framework for the assessment of organ-specific toxicities, mechanistic characterization, and human extrapolation. These tools enable dose optimization, improved risk monitoring, and reduction in animal use. Nevertheless, challenges remain regarding functional maturation, experimental reproducibility, regulatory validation, and population representativeness. The strategic and combined application of NAMs constitutes an advanced preclinical ecosystem that complements traditional approaches and holds significant potential to enhance the safety and efficacy of drug candidates in development.

Keywords: New Approach Methodologies, Predictive toxicology, Preclinical, Safety assessment, Human organoids, Organ-on-chip, Two-dimensional cell cultures.

1. Introduction: Innovative Models and New Approach Methodologies

The Preclinical development constitutes the first systematic barrier for evaluating safety and therapeutic feasibility prior to human exposure to a new medicinal product, characterizing the benefit-risk profile through in vitro and in vivo studies that assess toxicity, including the pharmacokinetics (PK), pharmacodynamics (PD), and the mechanisms of action in validated experimental models [1,2]. The data obtained support regulatory decisions regarding the initiation of clinical trials, determine starting doses and dose-escalation schemes, and inform safety monitoring strategies during clinical development. Historically, international regulatory frameworks have progressively reinforced the need for well-structured preclinical studies [3],

incorporating concepts such as the no observed adverse effect level (NOAEL), maximum tolerated dose (MTD), minimum anticipated biological effect level (MABEL), and the conduct of specific studies on genotoxicity, carcinogenicity, or reproductive toxicity according to the type of drug and its therapeutic indication [4,5].

Traditional methodologies based on animal models and two-dimensional cell cultures present significant limitations in predicting human responses. Animal models allow the evaluation of systemic toxicity and target organ effects; however, substantial differences exist in drug metabolism, transporter expression, immune responses, and susceptibility to specific toxicities, contributing to the fact that approximately 90% of drug candidates that pass the preclinical phase fail in clinical trials due to unforeseen adverse effects [6].

Two-dimensional cell cultures lack three-dimensional tissue architecture, appropriate cellular polarization, and heterogeneity of cell types, resulting in altered phenotypes and pharmacological responses that do not reflect human physiology, thereby limiting their utility for assessing metabolism-dependent toxicities, as occurs with primary hepatocytes [7,8].

Growing awareness of these limitations, together with ethical considerations regarding animal use and the need to accelerate the development of safe and effective medicines, has driven the development of New Approach Methodologies (NAMs). These technologies integrate advances in cell biology, tissue engineering, microfluidics, computational modeling, and omics analyses, offering greater translational relevance to humans, mechanistic information on toxicity pathways, and superior predictive capacity compared to traditional methods. Furthermore, they contribute to reducing or replacing animal studies, optimizing resources through early compound screening, and evaluating exposures in special populations such as pediatric, geriatric, or pregnant individuals [9,10].

The present work aims to critically analyze the role of these innovative preclinical methodologies in drug safety assessment. It examines how human organoids, organ-on-chip systems, computational pharmacokinetic modeling, toxicogenomic tools, and machine learning enable the prediction of toxicities, identification of target organs, understanding of mechanisms of action, and extrapolation of findings to clinical scenarios, thereby improving the translation of preclinical data to humans. It also considers the advantages and limitations of each approach, challenges of standardization and validation, ethical implications, and regulatory perspectives. This analysis provides a comprehensive framework for evaluating how these innovations may optimize dose selection, improve risk monitoring, and strengthen pharmacological safety, laying the foundations for more efficient, predictive, and human-physiology-aligned preclinical development [9,10].

2. Human Organoids: In Vitro Tissue Complexity

Human organoids represent one of the most promising innovations in predictive toxicology, offering three-dimensional stem cell-derived systems that recapitulate key aspects of tissue architecture, cellular heterogeneity, and

physiological function of human organs [11,12]. Unlike traditional two-dimensional cell cultures, organoids self-organize into three-dimensional structures containing multiple differentiated cell types, exhibit appropriate cellular polarization, and can maintain tissue-specific functions over prolonged periods. These can be obtained through two main strategies: derivation from human induced pluripotent stem cells (hiPSC) through directed differentiation protocols that reproduce stages of embryonic development, or culture of tissue-specific progenitor cells isolated from human biopsies [13].

2.1. Cerebral Organoids: Assessment of Developmental Neurotoxicity

Cerebral organoids, also referred to as "mini-brains" derived from hiPSC, represented in Figure 1, have emerged as particularly valuable tools for the assessment of developmental neurotoxicity [12,14].

These three-dimensional models reproduce the key early stages of human neurogenesis, including the formation of ventricular zones, neuronal migration, differentiation into specific neuronal subtypes, and the establishment of rudimentary synaptic circuits.

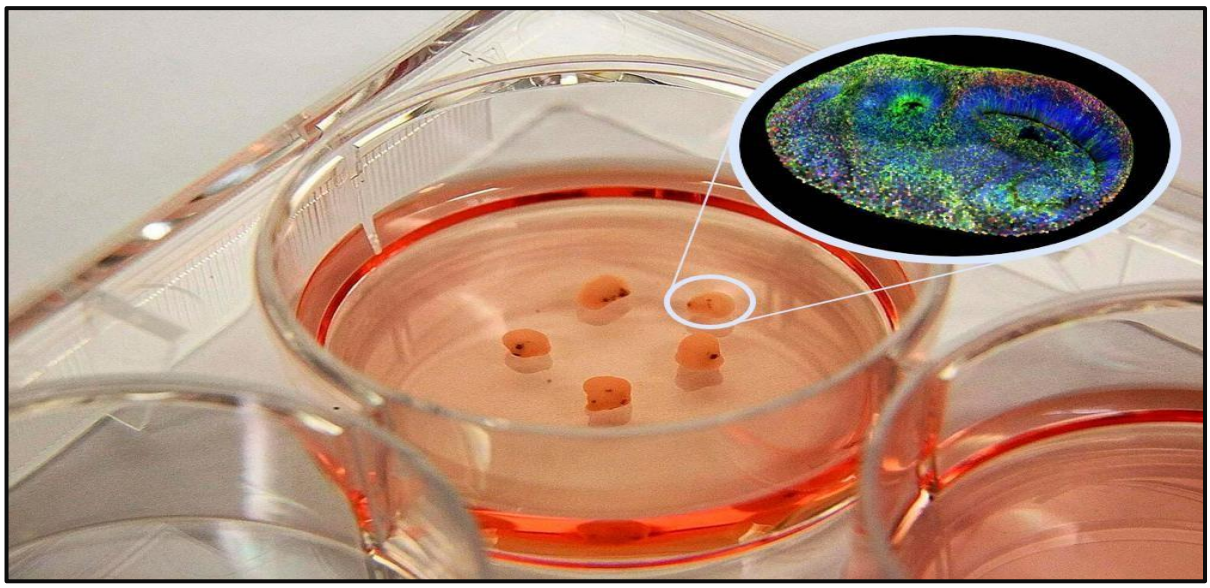

Figure 1. *Human hiPSC-derived cerebral organoids cultured in vitro, displaying the characteristic spherical morphology of three-dimensional models at early stages of neurodevelopment. The fluorescence microscopy inset demonstrates tissue organization and cellular differentiation compatible with the formation of neuroepithelial regions resembling primitive brain structures [15,16].*

Several studies have demonstrated their capacity to detect developmental neurotoxicity induced by well-characterized compounds such as valproic acid, methylmercury, and ethanol, while also providing mechanistic information on alterations in signaling pathways involved in neurological development [12,14].

In this context, cerebral organoids allow the evaluation of effects on critical processes of fetal neurodevelopment, such as proliferation of neuronal progenitors, neuronal differentiation and migration, synaptogenesis, and apoptosis. Their utility is particularly significant considering the substantial limitations of traditional animal models in predicting effects on human brain

development. These limitations primarily derive from interspecies differences in developmental timing, cortical complexity, and susceptibility to neurotoxic agents. In contrast, human cerebral organoids offer the advantage of using human cells at biologically relevant developmental stages, potentially contributing to improved prediction of risks to fetal and neonatal neurological development [17].

2.2. Hepatic Organoids: Metabolism and Hepatotoxicity

Hepatic organoids derived from hiPSC or hepatic progenitor cells reproduce key metabolic liver functions, including expression of cytochrome P450 enzymes (CYP1A2, CYP2C9, CYP2D6, CYP3A4), phase II conjugation (glucuronidation, sulfation, glutathione-S-transferase), and synthesis of plasma proteins (albumin, coagulation factors) [8,18]. These systems offer substantial advantages over conventional hepatic models. Primary human hepatocytes rapidly lose metabolic functionality in two-dimensional culture, with a 50-90% decrease in cytochrome P450 activity within 48-72 hours, whereas immortalized hepatic cell lines such as HepG2 or HepaRG exhibit limited metabolic capacity compared with primary hepatocytes. However, hepatic organoids maintain stable metabolic function for weeks or even months in three-dimensional cultures, making them particularly suitable for the assessment of chronic and cumulative toxicity [19].

These organoids have demonstrated the ability to detect hepatotoxicity induced by well-characterized compounds such as acetaminophen, diclofenac, and troglitazone, and to provide mechanistic insight into different types of liver injury, including steatosis, cholestasis, direct cytotoxicity, and activation of cellular stress responses [20,21]. Furthermore, integration of these models with transcriptomic analyses has enabled identification of molecular pathways associated with drug-induced hepatotoxicity, as recently evidenced in studies conducted with diclofenac [21].

2.3. Renal Organoids: Prediction of Nephrotoxicity

Renal organoids, representing miniature human nephrons, reproduce nephron segmentation in an organized manner, including glomerulus-like structures, proximal tubules, and distal tubules, exhibiting filtration, reabsorption, and secretion functions [22]. These systems are particularly valuable for the assessment of nephrotoxicity, one of the most frequent adverse effects associated with drug use, which is often inadequately predicted in traditional preclinical studies due to interspecies differences in renal physiology, transporter expression, and susceptibility to toxicants [22,23]. They have demonstrated the capacity to detect nephrotoxicity induced by cisplatin, gentamicin, and cyclosporine, recapitulating clinically observed mechanisms [22,23]. Expression of drug transporters in renal organoids enables evaluation of toxicities dependent on intracellular accumulation mediated by active transport.

2.4. Reproductive Organoids: Assessment of Reproductive Toxicity

Reproductive organoids, including testicular and ovarian models, are currently under development as tools for evaluating reproductive and fetal developmental toxicity [24,25]. Testicular organoids, derived from germ or somatic cells, reproduce key aspects of spermatogenesis and the testicular microenvironment. Ovarian organoids allow investigation of fundamental

processes such as folliculogenesis and ovarian endocrine function. These systems hold promise for providing human-relevant models to evaluate effects on gametogenesis, gonadal endocrine function, and early embryonic development, complementing or potentially replacing animal studies for certain reproductive toxicity endpoints [24,25]. Assessment of reproductive toxicity is particularly challenging due to the complexity of the processes involved and significant interspecies differences in reproductive physiology.

2.5 Challenges for Validation and Regulatory Application of Human Organoids

Despite their high potential as advanced in vitro models, human organoids present relevant limitations that must be addressed before large-scale regulatory adoption. One of the main limitations is their degree of functional maturity, as most organoids reproduce fetal or neonatal stages of tissue development and do not fully achieve adult tissue characteristics [26]. For example, cerebral organoids exhibit gene expression profiles and electrophysiological properties similar to those of the second-trimester fetal brain, limiting their utility for assessing toxicities dependent on mature neuronal functions [26]. Likewise, hepatic organoids typically express cytochrome P450 enzymes at levels between 10% and 50% of those observed in adult human hepatocytes [27].

Another key limitation is the absence of functional vascularization in most organoids, restricting their size due to oxygen and nutrient diffusion limitations and hindering modeling of endothelial interactions and compound-induced vascular effects [27,29]. Organoids also display considerable variability between batches, cell lines, and laboratories, attributable to genetic and methodological differences, posing challenges for standardization and reproducibility required in regulatory contexts [28,29]. Additionally, their biological complexity is reduced compared with in vivo organs due to the absence of immune system interactions, innervation, and inter-organ communication, which may result in incomplete toxicological responses. Quantitative characterization of toxicological endpoints in three-dimensional systems also requires more complex analytical methodologies than those used in conventional two-dimensional cultures [29].

To overcome these limitations, strategies are being developed to promote functional maturation through prolonged protocols, biophysical cues, and cellular co-cultures, as well as approaches to induce vascularization through incorporation of endothelial cells, microfluidic systems, or in vivo transplantation [26,29]. In parallel, standardization initiatives are being promoted through international consortia, use of reference cell lines, and inter-laboratory studies, together with increased biological complexity through co-culture with immune cells and development of interconnected multi-organoid systems [29].

From a regulatory perspective, agencies such as the FDA, EMA, and PMDA have expressed interest in the use of organoids as complementary tools for drug safety assessment, conditioning their acceptance on demonstration of reproducibility, validation with reference compounds, clear definition of contexts of

use, and correlation with clinical data [30]. Ethically, although organoids raise fewer concerns than animal models, considerations remain regarding informed consent, protection of genetic privacy, and, in the case of advanced cerebral organoids, reflection on their potential functional status, although current models lack the connectivity necessary for complex cognitive functions [30,31]. Overall, human organoids constitute a promising tool to improve the physiological relevance of toxicity studies; however, at present they should be considered complementary to other preclinical methods. Integration of these models with technologies such as microfluidics, omics analyses, and computational modeling will be key to maximizing their predictive value and advancing toward broader regulatory acceptance.

3. Organ-on-Chip Systems and Microphysiological Platforms

Organ-on-chip (OoC) systems aremicrodevices designed to replicate the architecture and microenvironment of specific organs. These systems consist of microchannels fabricated from transparent and biocompatible materials that contain human cells cultured in three-dimensional configurations, with continuous perfusion of culture medium simulating blood flow [32]. In this manner, cellular function, drug response, and toxicity are evaluated under dynamic conditions.

Microphysiological systems (MPS) expand this concept by integrating various OoC devices or multiple cell types into interconnected compartments, using porous membranes that facilitate paracrine communication, perfusion that generates physiological shear stress, and mechanical stimuli that reproduce characteristic organ movements. This enables the simulation of inter-organ interactions, pharmacokinetic (ADME) modeling, and evaluation of metabolite-dependent toxicities within a systemic context in addition, the integration of sensors allows real-time monitoring, enhancing the models' ability to recreate in vivo conditions and dynamically assess cellular responses.

Collectively, OoC systems and MPS platforms constitute advanced in vitro models that combine tissue engineering, microfluidics, and cell biology to recreate fundamental aspects of human physiology [32].

3.1. Lung-on-Chip

The lung-on-chip, one of the most advanced OoC systems, recreates the alveolar–capillary interface by culturing human alveolar epithelial cells and microvascular endothelial cells on opposite sides of a porous and flexible membrane, incorporating cyclic stretching to simulate respiratory movements [33]. This model has demonstrated the ability to reproduce physiologically relevant pulmonary inflammatory responses, including cytokine release and neutrophil recruitment, as well as pulmonary edema characterized by increased vascular permeability, nanoparticle-induced toxicity, and the effects of cigarette smoke, overcoming the limitations of static cell cultures [33]. It evaluates both inhalation exposure effects, through application of compounds to the epithelial compartment exposed to air, and systemic effects via perfusion in the vascular compartment, offering a versatile platform for studies of pulmonary pathophysiology and toxicology.

3.2. Liver-on-Chip

The liver-on-chip integrates human hepatocytes, whether primary or stem cell-derived, in three-dimensional configurations with continuous perfusion, frequently co-cultured with non-parenchymal cells to recreate the hepatic functional unit [34]. Constant perfusion provides continuous oxygen and nutrient supply, metabolite removal, and physiological shear stress, enabling maintenance of key metabolic functions including CYP450 expression, metabolite conjugation, and albumin and urea synthesis for several weeks, far exceeding the functional duration of hepatocytes in static cultures [34,35]. These systems are capable of detecting various types of hepatotoxicity, such as metabolism-dependent toxicity, idiosyncratic hepatotoxicity, steatosis, cholestasis, and direct cytotoxicity [34,35].

3.3. Kidney-on-Chip

The kidney-on-chip recreates renal tubular architecture using human proximal tubular epithelial cells cultured under fluid flow in microchannels that simulate tubular geometry [36,37]. These dynamic conditions induce formation of the apical brush border, appropriate cellular polarization, and functional expression of transporters, allowing a more faithful representation of proximal tubular physiology. These systems reproduce key kidney functions, including transport processes such as glucose and amino acid reabsorption, active secretion of organic anionic and cationic compounds through transporters, as well as cellular stress responses via release of kidney injury biomarkers [37]. Fluid flow is a critical factor for model functionality, as proximal tubular cells cultured under these conditions exhibit transporter expression levels five to ten times higher than those observed in conventional static cultures [36,37]. Compared with static two-dimensional cultures, these models show increased sensitivity and allow detection of toxic effects at clinically relevant concentrations, reinforcing their utility for preclinical nephrotoxicity assessment.

3.4. Intestine-on-Chip

The intestine-on-chip incorporates human intestinal epithelial cells cultured under flow and simulated peristalsis through cyclic stretching, promoting differentiation into specialized cell types and formation of three-dimensional villi [38]. These systems enable analysis of drug absorption by evaluating intestinal permeability, active transport, intestinal metabolism, and potential interactions with the microbiota through co-culture with commensal or pathogenic bacteria. They detect gastrointestinal toxicity associated with epithelial barrier damage and inflammation, and allow analysis of the effects of different pharmaceutical formulations [38].

3.5. Heart-on-Chip

The heart-on-chip employs human hiPSC-derived cardiomyocytes cultured in configurations that allow simultaneous assessment of contractility, electrophysiology, and drug responses [39]. The most advanced systems incorporate controlled electrical stimulation to maintain physiological contraction rhythm and promote structural alignment of cardiomyocytes, thereby improving in vitro cardiac tissue functional maturation. These systems are highly useful for identifying different types of cardio-toxicity, including structural alterations such as cardiomyocyte damage and fibrosis, functional dysfunction characterized by reduced contractility or arrhythmias, and

electrophysiological effects such as QT interval prolongation or ion channel blockade. A recent study predicted, in a NAM-based cardiac model, the proarrhythmic potential of vanoxerine [39]. However, the study also highlighted technical limitations related to dosimetry due to substantial compound loss from adsorption to system materials.

3.6. Multi-Organ-on-Chip Systems

The MPS approach based on interconnection of multiple OoC systems through a common fluidic flow, illustrated in Figure 2, represents a particularly innovative strategy. This architecture enables integrated modeling of inter-organ pharmacokinetics, sequential metabolism, and metabolite-mediated toxicities, more faithfully reproducing the systemic dynamics observed in vivo [40]. The most commonly used configurations include intestine-liver-kidney connections to simulate oral absorption; liver-heart, liver-kidney, or liver-brain connections to evaluate toxicity of hepatic metabolites in target organs; and lung-liver-kidney systems to model inhalation exposure followed by systemic distribution, thereby addressing the complex metabolism-dependent toxicity scenarios.

Recent studies have shown that integration of intestine-liver-kidney MPS systems with PBPK modeling and transcriptomic analysis enables accurate prediction of human pharmacokinetics and reveals molecular mechanisms of gastrointestinal toxicity, as demonstrated with diclofenac, where the full sequence of absorption, metabolism, and exposure was captured, highlighting the utility of these integrated platforms for modeling complex pharmacokinetic and toxicological processes [40,42]. However, development of MPS platforms faces challenges such as physiological scaling of compartments and flows, media compatibility for different cell types, and complex dosimetry due to compound adsorption and sequential metabolism [41].

3.7. Challenges and Regulatory Perspectives of OoC Systems and MPS Platforms

OoC systems and MPS platforms represent advanced tools for predictive toxicology by recreating human physiological contexts not achievable in static cell cultures. These models use human cells under dynamic conditions including fluid flow, mechanical forces, and biochemical gradients, influencing function, differentiation, polarization, and xenobiotic response, and enabling continuous measurement of biomarkers, metabolites, and tissue functional parameters through integrated sensors or effluent sampling [40].

Despite their advantages, they face significant technical challenges limiting regulatory adoption. Their complexity requires specialized equipment, microfluidics expertise, and advanced dynamic culture protocols, while diversity in device designs, cell types, media, and analytical methods complicate standardization and reproducibility across laboratories [42,43]. Additionally, compound adsorption by system materials may alter effective concentrations and complicate dose-response interpretation. Formal validation of these models remains insufficient, and low throughput and analytical complexity restrict their use in large-scale compound screening [43,44].

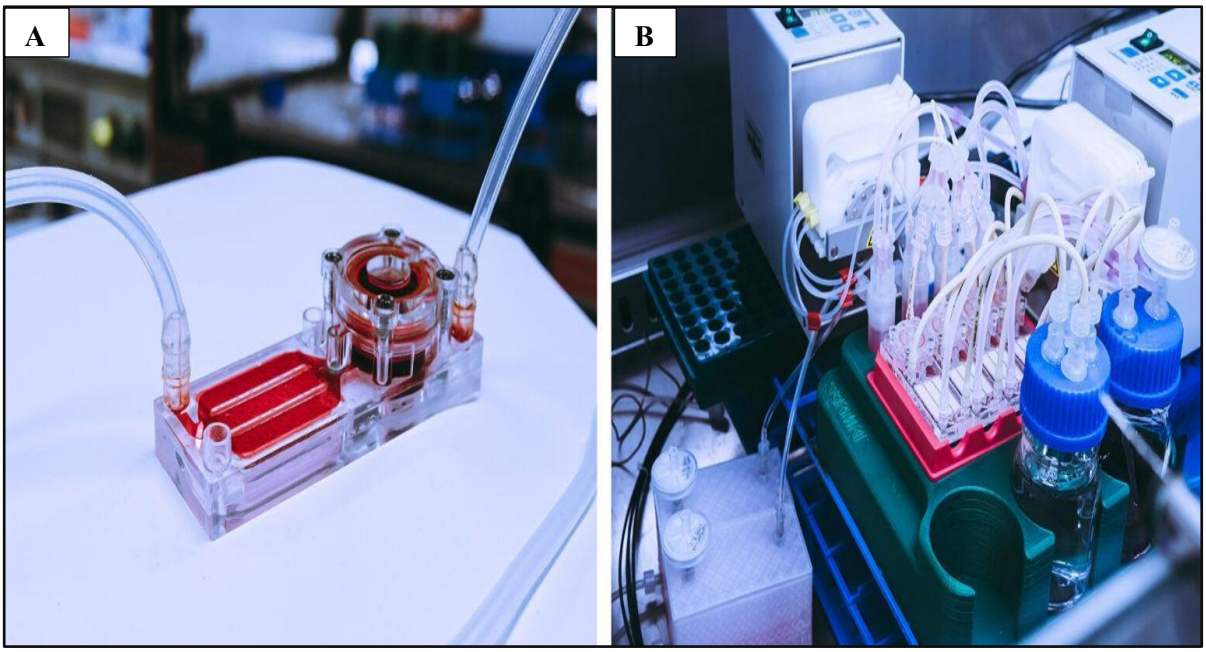

Figure 2. *OoC system: On the left, image* ***(A)*** *is an microdevice equipped with microfluidic channels designed to enable three-dimensionalcell culture, accurately recreating the physiological microenvironment of specific tissues. On the right, image* ***(B)*** *is an MPS platform integrating several OoC devices with automated perfusion, allowing controlled simulation of dynamic interactions between different organs and study of their collective behavior under physiological or pathological conditions. This comparison illustrates the evolution from individual devices replicating a single organ to more complex platforms capable of modeling interconnected organ networks [44].*

To overcome these limitations, strategies are being developed such as standardization of protocols and reference compounds by academic, industrial, and governmental consortia [45,46], use of optimized commercial platforms [42,43], alternative low-adsorption materials and surface treatments, as well as automation of liquid handling, media exchange, and sampling to increase throughput and reduce variability [46]. Inter-laboratory validation studies comparing OoC predictivity with clinical data and traditional preclinical methods are essential to demonstrate value [45,46].

Regulatory agencies recognize the potential of OoC systems as complementary safety assessment tools and actively participate in validation studies. Emphasis is placed on clearly defining context of use, demonstrating reproducibility and correlation with known human toxicities, and establishing acceptance criteria [42,45,46]. Regulatory adoption will likely be gradual, beginning with applications where these systems demonstrate added value, such as prediction of idiosyncratic hepatotoxicity, evaluation of metabolite toxicity, and modeling of drug-drug interactions. Overcoming these challenges is key to maximizing predictive value and facilitating future regulatory integration.

From an ethical perspective, they offer advantages over animal models by promoting the 3R principle (Replacement, Reduction, Refinement), reducing animal numbers and refining preclinical testing. However, implementation requires consideration of informed consent for cell

donation, protection of genetic information, and technology accessibility to avoid inequities among organizations. Overall, organ-on-chip and multi-organ-on-chip systems represent a significant advancement toward in vitro models capable of capturing dynamic and multicellular human organ physiology, with the capacity to predict specific toxicities and provide valuable mechanistic information. Their adoption will depend on standardization, automation, inter-laboratory validation, and demonstration of predictive value superior to conventional methods [43].

4. Emerging Trends in Preclinical Toxicology

4.1. PBPK Modeling and Computational Toxicokinetics

Physiologically based pharmacokinetic (PBPK) modeling constitutes an advanced computational tool to predict the distribution and internal concentration of chemical compounds across different tissues and species [47]. These models enable extrapolation of toxicological data from animals to humans and estimation of exposures in special populations (pediatric, geriatric, pregnant, or patients with hepatic or renal impairment), representing a mechanistic approach compared with traditional empirical methods [47,48]. PBPK models integrate information on anatomy, physiology, physicochemical properties of compounds, and biochemical processes, simulating the ADME of chemical substances [48]. They also allow comparison of doses and routes of exposure, evaluating internal effects derived from oral, intravenous, dermal, or inhalation administration, and are useful in occupational or environmental risk assessment. Integration with advanced in vitro systems such as organoids or OoC enables parameterization of models with relevant human data, improving prediction accuracy and mechanistic interpretation of molecular responses [47,48].

PBPK modeling allows integration of data from different sources, prediction of experimentally untested scenarios, identification of critical parameters through sensitivity analysis, anticipation of potential drug–drug interactions, reduction of animal studies, and performance of adjusted pharmacokinetic predictions without the need for extensive clinical trials [48]. However, PBPK model reliability depends on input data quality, simplification of biological processes, limited validation for novel compounds, and difficulties in predicting metabolism and population variability [49].

Systematic sensitivity and uncertainty analyses are recommended, along with validation using clinical data, use of specialized software, transparent documentation of assumptions and parameters, and integration of artificial intelligence to accelerate parameterization and PBPK model generation for multiple compounds [49]. Regulatory agencies increasingly recognize PBPK value, incorporating it into FDA and EMA guidelines for first-in-human dose selection, drug-drug interaction assessment, extrapolation to pediatric populations, and evaluation of hepatic or renal impairment impact. From an ethical perspective, it contributes to reducing animal and human studies provided transparency, equity, and appropriate representation of population variability are ensured [49].

4.2. Omics Technologies

Toxicogenomics studies genome response to xenobiotics through transcriptomics, proteomics, metabolomics, epigenomics, and genomics, enabling characterization of molecular perturbations, identification of biomarkers, and anticipation of adverse effects [47]. Genomics analyzes deoxyribonucleic acid (DNA) sequence and genetic variants influencing susceptibility to toxicity, while epigenomics studies heritable DNA and chromatin modifications regulating gene expression without altering sequence [50,51]. Transcriptomics measures changes in expression levels of thousands of genes simultaneously, identifying affected pathways and molecular networks. Proteomics examines proteins and post-translational modifications, detecting biomarkers and complexes formed by reactive metabolites [51]. Finally, metabolomics analyzes endogenous metabolites and xenobiotics, providing insight into functional and phenotypic cellular and tissue states. Integration of these technologies transforms toxicology toward a mechanistic and predictive approach, facilitating risk assessment interpretation [50,51].

Omics approaches allow detailed understanding of how compounds affect biological systems and potential adverse effects. However, they face significant challenges including data complexity, technical and biological variability, result interpretation, and extrapolation to humans [50]. Overcoming these challenges requires protocol standardization, creation of reference databases, integration of different omics sciences, and application of machine learning to detect patterns and generate predictive models, always accompanied by rigorous validation to meet regulatory criteria. Agencies value these data for their ability to clarify mechanisms, identify biomarkers, and assess clinical relevance, although full acceptance depends on validation, clear context of use definition, and correlation with clinical outcomes. Combination with in vitro systems and computational modeling enhances utility, consolidating it as a key tool for generating robust and reliable predictions [50,51].

4.3. Artificial Intelligence and Computational Models in Predictive Toxicology

Artificial intelligence (AI) and machine learning (ML), a branch of artificial intelligence that enables computers to learn patterns and relationships from data without being explicitly programmed for each specific task, facilitate toxicity prediction based on molecular structure, physicochemical properties, in vitro data, and biological data, accelerating safety screening, reducing animal use, and providing evidence for regulatory decision-making [52,53].

Quantitative structure-activity relationship (QSAR) models are computational tools that relate chemical structure to biological activity or toxicity through mathematical and statistical analyses [54]. The central concept is that molecular structure determines chemical and biological properties, so by knowing certain molecular characteristics, effects can be predicted without exhaustive experimental testing. Deep learning, a sub-branch, uses artificial neural networks to learn complex data representations and is particularly useful for images, DNA sequences, and chemical molecules

[54,55]. These techniques allow prediction of hepatotoxicity, cardiotoxicity, nephrotoxicity, and neurotoxicity with high precision.

These tools have revolutionized predictive toxicology by enabling virtual screening of large compound libraries, facilitating chemical structure optimization, metabolite prediction, multi-modal data integration, read-across, and prioritization of compounds for experimental testing [52,53,55]. They provide clear advantages including speed and scalability in compound evaluation, cost reduction and decreased animal use, identification of underlying molecular mechanisms, and optimization of molecules with favorable pharmacological and toxicological properties.

Despite their advantages, implementation of these tools constitutes a new paradigm in modern toxicology characterized by issues related to data quality and potential biases, need for rigorous validation, complexity of toxicity influenced by biological and environmental factors, limitations of applicability domains, and difficulty interpreting complex models [53,54]. These aspects underscore the importance of an integrative and carefully validated approach to ensure reliable and reproducible predictions.

To overcome these limitations, strategies such as explainable AI (XAI) have been implemented to make complex model predictions understandable by identifying factors influencing each outcome [54]. High-quality curated databases ensure accurate, representative, and error-free information, reducing bias. Ensemble and consensus methods combine multiple models to improve predictive accuracy. Transfer learning and multi-task learning approaches allow models to leverage prior knowledge or related data to improve performance in new tasks with limited data [52-55]. Integration of mechanistic knowledge incorporates known biological and toxicological information, strengthening interpretation and prediction relevance.

Finally, standardized validation protocols ensure consistent and rigorous model evaluation, guaranteeing reliability and reproducibility in regulatory and experimental contexts. Regulatory agencies are beginning to recognize the potential of in silico models, particularly when applied to well-defined contexts of use. Their combination with omics data, advanced in vitro systems, and PBPK modeling promises to maximize toxicity prediction and mechanistic understanding [52,53,55]. However, these tools complement rather than replace experimental testing, and their implementation requires methodological standardization, development of representative databases, and interdisciplinary collaboration among scientists, toxicologists, and regulators.

5. Discussion

Preclinical drug development is undergoing significant transformation with incorporation of NAMs, notably human organoids and OoC systems, which reproduce key aspects of human physiology not captured by animal models or static cell cultures. These platforms enable study of organ-specific toxicities with greater biological relevance, offering predictive and mechanistic value for risk assessment, although their application still faces technical challenges related to maturity, scalability, and validation. PBPK modeling provides a robust quantitative framework for human

extrapolation and exposure prediction, with growing regulatory acceptance when high-quality data are available. Omics and AI approaches complement these methodologies by enabling biomarker identification, mechanistic understanding of toxicity, and screening of large chemical libraries. However, challenges remain regarding data integration, model interpretability, and validation of results. Strategic combination of these tools allows overcoming individual limitations and building an integrated ecosystem for preclinical safety assessment.

The predictive value of NAMs has been supported by specific studies. In vitro genotoxicity assays show high concordance with human carcinogenicity [10]. Hepatotoxicity models based on human hepatocytes and transcriptomic analyses achieve accuracies above 80%, while cardiac systems derived from hiPSC cardiomyocytes present sensitivities greater than 85% for cardiotoxicity detection [56]. These findings demonstrate that certain NAMs can predict human toxicities with accuracy comparable to or greater than animal studies, improving early risk identification, optimizing dose selection, and contributing to the 3Rs principle through replacement, reduction, and refinement of animal use. Collectively, NAMs represent a substantial advancement over traditional animal-based models, whose ability to predict human toxicities is limited by interspecies differences in metabolism, physiology, and susceptibility to adverse effects. These differences generate false negatives and false positives, significantly contributing to high clinical failure rates due to safety issues. Integration of NAMs thus offers a promising pathway to mitigate these limitations and significantly improve predictive value in preclinical assessment. Currently, NAMs cannot fully replace animal studies for comprehensive toxicological evaluation due to the complexity of systemic toxicities, absence of functional immune systems in most in vitro systems, limitations in reproducing chronic exposures and cumulative effects, and lack of formal validation for many endpoints [57]. Therefore, their most realistic implementation consists of gradual introduction in specific contexts where they provide clear added value, complementing animal studies.

Effective implementation of NAMs depends on overcoming technical and scientific challenges, with reproducibility being one of the main obstacles. Inter-laboratory studies have reported variability in organoids, OoC systems, and omics analyses due to differences in protocols, reagents, equipment, and expertise. Furthermore, limited functional maturity of many models, typically recapitulating fetal or neonatal stages, restricts assessment of adult tissue-dependent toxicities [36,37]. AI models, although powerful, often lack internal transparency, limiting understanding of how predictions are generated and hindering mechanistic hypothesis formulation. Risks associated with data bias and extrapolation also exist, such as over-representation of certain chemical classes, publication bias, or limited population representativeness, potentially affecting prediction in underrepresented groups such as pediatric, geriatric, or diverse ethnic populations.

Ethical and equity considerations are also associated with NAM use. It is essential to ensure informed consent from cell donors, protect genomic privacy, and address issues of ownership and

commercialization of cell lines. Evolution of cerebral organoids toward models with advanced neuronal connectivity and higher functional capacities raises questions regarding their moral status [31]. In terms of equity and accessibility, advanced technologies require costly equipment, specialized expertise, and computational resources not always available, while models derived from limited populations may not reflect human genetic diversity, compromise representativeness and perpetuating regulatory gaps. Effective implementation requires an integrated approach combining standardization, formal validation, strategic integration of multiple methodologies, infrastructure and capacity development, clear regulatory frameworks, integrated databases, and multi-sector collaboration. In summary, although NAMs present limitations, their strategic and validated use can complement animal studies, improve prediction of human toxicities, reduce animal use, and support safer and more ethical regulatory decisions.

6. Conclusion

New Approach Methodologies (NAMs) constitute a transformative advancement in preclinical toxicology by providing more accurate predictions of human toxicity and offering detailed mechanistic information on compound effects in specific organs and systems. Their strategic integration under standardized and validated protocols improve dose selection, optimizes risk assessment, and significantly reduces dependence on animal models, contributing to implementation of the 3Rs principle. Moreover, these methodologies facilitate extrapolation of preclinical findings to clinical scenarios, strengthening reliability and biological relevance of generated information and supporting more robust, ethical, and evidence-based regulatory decisions.

Conflict of Interest Statement

Victoria Montes Gimeno declares no conflicts of interest. The work was conducted independently, ensuring objectivity and scientific integrity.

Data Availability Statement

All data referenced in this review are derived from previously published sources cited herein. No original data were generated or analyzed as part of this study.

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