

**Identification of biomarkers
of the retinoic acid signaling pathway
in the zebrafish embryo model to predict
human developmental toxicants**



Laura Samrani

**Identification of biomarkers of the
retinoic acid signaling pathway in the
zebrafish embryo model to predict human
developmental toxicants**

Laura Samrani

Identification of biomarkers of the retinoic acid signaling pathway in the zebrafish embryo model to predict human developmental toxicants

ISBN: 978-94-6483-475-8

DOI: <https://doi.org/10.33540/1979>

Cover design: Eric Gremmer

Layout design: Iliana Boshoven-Gkini | AgileColor.com

Print: Ridderprint | Ridderprint.nl

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, by photocopying, recording, or otherwise, without the prior written permission of the author.

Identification of biomarkers of the retinoic acid signaling pathway in the zebrafish embryo model to predict human developmental toxicants

Identificatie van biomarkers van het retinolzuur metabolisme in het zebravis embryo voor de voorspelling van schadelijkheid van stoffen voor de prenatale ontwikkeling
(met een samenvatting in het Nederlands)

Identification de biomarqueurs de la voie de signalisation de l'acide rétinoïque dans le modèle de l'embryon de poisson zèbre pour prédire les substances toxiques pour le développement humain
(avec un résumé en français)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op vrijdag 17 november 2023 des ochtends te 10.15 uur

door

Laura Marie-Elise Mathilde Samrani

geboren op 23 juli 1991 te Clermont-Ferrand, Frankrijk

Promotor:

Prof. dr. A.H. Piersma

Prof. dr. Pallardy

Copromotor:

Dr. R. Bars

Beoordelingscommissie:

Prof. dr. M. van den Berg

Prof. dr. A. Guillouzo

Dr. A. Kienhuis

Prof. dr. ir. J. Legler

Prof. dr. H.P. Spaink

The degree is awarded as part of a Joint Doctorate with Université Paris-Saclay.

Dit proefschrift werd (mede) mogelijk gemaakt met financiële steun van CIFRE vanuit l'Association Nationale de la Recherche et de la Technologie (ANRT) grant no. 2017/1154.

This work is dedicated to the original Dr. Samrani, my father.

I wish you could have read this.

À la mémoire de mon père, le véritable Dr. Samrani

J'aurais tant aimé que tu puisses lire ceci.

Contents

Abbreviations	12
Chapter 1 General introduction	15
Birth defects	16
Birth defects in history	16
Birth defect today	16
Examples from the past: BD caused by chemical exposures	17
Developmental toxicology	18
Regulatory framework of DART	19
Regulatory testing guidelines for developmental toxicity	22
Advantages and limitations of current guidelines	22
Beyond the 3Rs: pushing towards alternatives to animal testing	24
Integrative systems increase predictivity: Adverse Outcome Pathways	25
Example of in vitro models available for hazard identification	27
The Zebrafish embryo model in DART	28
Advantages of the zebrafish embryo model	29
Readouts of the zebrafish embryo model	31
Vertebrate development and its regulation	37
Vertebrate development	38
Morphogens and developmental signaling pathway	38
The Retinoic acid signaling pathway	39
ATRA and RA-SP role in development and teratology	41
Outline of Thesis	46
References	48
Chapter 2 Dynamic regulation of gene expression and morphogenesis in the zebrafish embryo test after exposure to all-trans retinoic acid	59
Chapter 3 Nervous system development related gene expression regulation in the zebrafish embryo after exposure to valproic acid and retinoic acid: a genome wide approach	85
Chapter 4 Retinoic acid signaling pathway perturbation impacts mesodermal-tissue development in the zebrafish embryo: biomarker candidate identification using transcriptomics	127

Chapter 5 Summary and General discussion	151
Summary of main findings	152
The zebrafish embryo model for transcriptomic analysis	153
Optimization for the zebrafish embryo model for transcriptomic analysis	154
Gene expression predictive biomarkers of RA-SP mediated toxicity	155
Human relevance and zebrafish gene expression	156
Model limitations	157
Future perspectives	158
References	160
 Appendix	 165
Résumé Vulgarisé (Francais)	166
Résumé Scientifique (Francais)	167
Laymans Summary (English)	169
Scientific Summary (English)	170
Laymans Samenvatting (Nederlands)	172
Wetenschappelijke Samenvatting (Nederlands)	173
List of publications	175
Acknowledgements	177
Curriculum vitae	183

Abbreviations

3Rs	Replacement, Reduction and Refinement
ADH	Alcohol dehydrogenase
ADME	Absorption, distribution, metabolism, and excretion
AI	Artificial intelligence
AO	Adverse outcome
AOP	Adverse outcome pathway
ATRA	All-trans retinoic acid
BD	Birth defects
CYP26	Cytochrome P450 26
DART	Developmental and reproductive toxicology
DEG	Differentially expressed gene
DT	Developmental toxicology
ECHA	European Chemical Agency
ECVAM	EU Reference Laboratory for alternatives to animal testing
EFD	Embryo-fetal development
ESC	Embryonic stem cell
FA	Folic acid
FASD	Fetal alcohol spectrum disorder
FDA	Food and Drugs Administration
FEED	Fertility and early embryonic development
FGF	Fibroblast growth factors
GE	Gene expression
HC	Hazard characterization
HI	Hazard identification
HPF	Hours post fertilization
ICH	International Council for Harmonization
KE	Key events
KER	Key event relationship
LC50	Lethal concentration 50%
MIE	Molecular initiating event
MOA	Mode of action
NAMs	New approach methodologies
Ncad	National Committee on animal testing
NCAO1	Nuclear receptor co-activator 1
NCC	Neural crest cell
NGRA	Next generation risk assessment
OECD	Organization for Economic Co-operation and Development
PA	Pharyngeal arches

PBK	Physiologically based pharmacokinetic modeling
PPND	Pre-and postnatal development
qAOP	Quantitative adverse outcome pathway
QVIVE	Quantitative <i>in vitro</i> - <i>in vivo</i> extrapolation
RA	Retinoic acid
RALDH	Aldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RA-SP	RA signaling pathway
RBP	Retinoic-binding protein
RDHs	Retinol dehydrogenases
REACH	Registration, Evaluation, Authorization, and Restriction of Chemicals
RNAseq	RNA-sequencing
RXR	Retinoid X receptor
SC	Spinal cord
TF	Transcription factor
TG	Test guideline
VA	Vitamin A
VPA	Valproic acid
WEC	Whole embryo culture
WHO	World Health Organization
WNT	Wingless
ZE	Zebrafish embryo
ZET	Zebrafish embryotoxicity test

Chapter 1

General introduction



Birth defects

Birth defects in history

The World Health Organization estimated that 8 million children are born with birth defects (BD) yearly [1]. These anomalies have been recognized throughout history; the earliest written record of BD dates back to at least 2000 BC, on clay tablets in Babylon [2]. In this civilization, birth defects were considered a prophecy [2-4]. The Chaldean tablet consisted of 62 ear, nose, mouth, reproductive organ, and limb malformations associated with their respective significance in predicting future events [5, 6]. For example, an infant born with the ear of a lion predicted the coming of a powerful king. In 400 BC, Plato challenged the accepted idea that BD were of a supernatural cause with the idea that BD were caused by problems in reproduction called *maternal impression* [6]. He believed that maternal emotions and experiences during pregnancy affected fetal development. This theory remained the common belief until the 19th century when anatomist Johann Friedrich Meckel proposed it is actually the deviation from a normal developmental process that causes malformation, founding the first teratology journal "*Teratologia: Antenatale pathology*" [3].

Birth defect today

Teratology, the study of BD, has evolved since then, with 7000 kinds of BD recorded and increased knowledge of their causes discovered [7]. These known causes include genetic anomalies (e.g., trisomy 21), infection (e.g., Zika virus, syphilis, toxoplasma), and exposure to certain chemicals [8]. However, this knowledge remains insufficient, as the causes of 70% of the BD happening worldwide are still not understood [7, 9]. Environmental factors are generally believed to play a more significant role in these BD numbers than is currently known [7]. Thus, additional work remains to be done both to understand the perturbation of the developmental mechanisms leading to BD and to understand the implication of chemicals in this perturbation. These points are explored in chapters 3 and 4 of the current work.

Studying the teratogenicity potential of chemicals is crucial, as BD are currently estimated to lead to the death of 240 000 children before the age of five every year worldwide and contribute to long-term disability of children, which takes a significant toll on individuals, families, health

care systems and societies [8]. These numbers are expected to be higher in reality because they do not take into consideration spontaneous and elective abortions as well as stillborn.

Examples from the past: BD caused by chemical exposures

The cause of chemical exposure can be multiple, including voluntary ingestion such as alcohol or medication and involuntary such as contamination of the food chain or the environment.

Ethanol is one of the first chemicals to have been linked with BD. In 1904, Ballanthyne associated alcohol intake during pregnancy with an increase in the risk of adverse outcomes at birth, such as spontaneous abortion, premature labor and structural dysmorphia [10]. Fetal alcohol spectrum disorder (FASD) was defined in later years, consisting of structural, cognitive and emotional deficiencies in newborns [10-12]. To this day, FASD remains a societal concern, being the leading cause of preventable mental disabilities in major developed countries [13].

Periodic outbreaks of birth defects are typically due to a manmade or environmental disaster. In the late 1950s, Minamata in Japan saw a rise in developmental defects with neurological symptoms similar to those in cerebral palsy such as severe mental retardation, tremor, epilepsy, abnormal reflexes, and sensory disturbances [14-16]. This was due to ingestion of seafood contaminated with an abnormally high level of methylmercury during pregnancy. Even though the mother exhibited only mild symptoms of methylmercury poisoning, it significantly affected the normal neurodevelopment of the fetus, thus demonstrating the exquisite sensitivity of the developing fetus to chemicals. This outbreak, sometimes known as Minamata disease, gave scientists the first evidence that chemical poisoning can be transmitted to the fetus.

Other examples in history are related to pharmaceutical compounds. Diethylstilbestrol, a synthetic estrogen, marketed as a drug to avoid miscarriage and premature labor, was prescribed in early pregnancy from about 1940 through the early 1970s [17-20]. Later epidemiology studies found the drug caused multigenerational birth defects in both sexes, including tissue and structural anomalies of the reproductive tract in women, genitourinary anomalies in men, and multigenerational reproductive difficulties such as lower birth weight and preterm birth recorded in the second generation [17-20].

1 Valproic acid, an antiepileptic drug, is a recent example of a chemical causing BD. In 2009, the FDA and other regulatory agencies issued a warning of use during pregnancy due to post-approval drug safety surveillance and epidemiological studies showing an increased risk of spina bifida, cleft palate and altered neurodevelopment including lower cognitive test scores [21-25].

From 1957 to 1962, the drug thalidomide was prescribed for morning sickness in early pregnancies but led to BD such as heart defects, amelia or phocomelia (absence of or poor development of extremities), and anotia (absence of development of the ears) [26-28]. Since no BD were detected in the rodent-based safety studies during its development, the thalidomide case is often cited as THE example of BD caused by drug exposure in which species-specificity is crucial [29, 30]. In the aftermath of this disaster, significant changes in legislation were implemented. This resulted in mandatory conduct of developmental toxicity testing in rodents and non-rodents, and the development of post-marketing drug surveillance systems, leading to a more effective regulation of these drugs. As Paul A. L. Lancaster wrote in his paper *"Causes of birth defects: Lessons from history"*, the thalidomide disaster *"was a new impetus to the fledgling science of teratology"* [26].

Given the increased sensitivity during prenatal development and the existence of species differences illustrated by these tragedies, there is a crucial need to improve safety assessment for prenatal chemical exposure.

Developmental toxicology

In order to protect humans, the field of toxicology has developed methods to study and measure chemical toxicity to help predict their potential perturbing effects on human physiology. A particular branch of toxicology focuses on studying toxic effects during the developmental stages from zygote to adulthood and considers the unique sensitivities of embryos, fetuses and children: this is known as developmental toxicology (DT). In an established regulatory framework, this field is often evaluated alongside reproductive toxicology, then called developmental and reproductive toxicology (DART).

Regulatory framework of DART

The regulatory framework for chemical safety testing and registration is controlled by national and international laws (e.g., pan-European laws). In Europe, ECHA (European Chemical Agency) oversees the implementation of chemical legislation. Since 2007, ECHA requires that chemicals be registered through the REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) system before being put on the market if their production level amounts to over one ton per year [31]. This principle places the responsibility on manufacturers to assess the risks from their chemicals and to provide safety information on substances, which is done via a thorough risk assessment process submitted to ECHA for regulatory decision-making. This formalized risk assessment process includes four steps (Figure 1) [32-34]. In short:

- The hazard identification (HI) step, which identifies adverse effects caused by chemical exposures,
- The hazard characterization (HC) step, which evaluates these adverse effects in a qualitative and quantitative way (e.g., dose-response relationships),
- The exposure assessment step, which investigates relevant exposure scenarios in the population,
- The risk characterization step, which estimates the probability of occurrence/severity of the adverse effects, based on the previous steps.

The two first steps (HI and HC) together are defined in this work as the regulatory testing process. Major international organizations help codify and standardize this regulatory testing process for human health and the environment e.g., International Council for Harmonization (ICH) for pharmaceuticals and Organization for Economic Co-operation and Development (OECD) for non-pharmaceuticals such as pesticides and industrial chemicals. Over time, these organizations have developed specific testing and assessment frameworks.

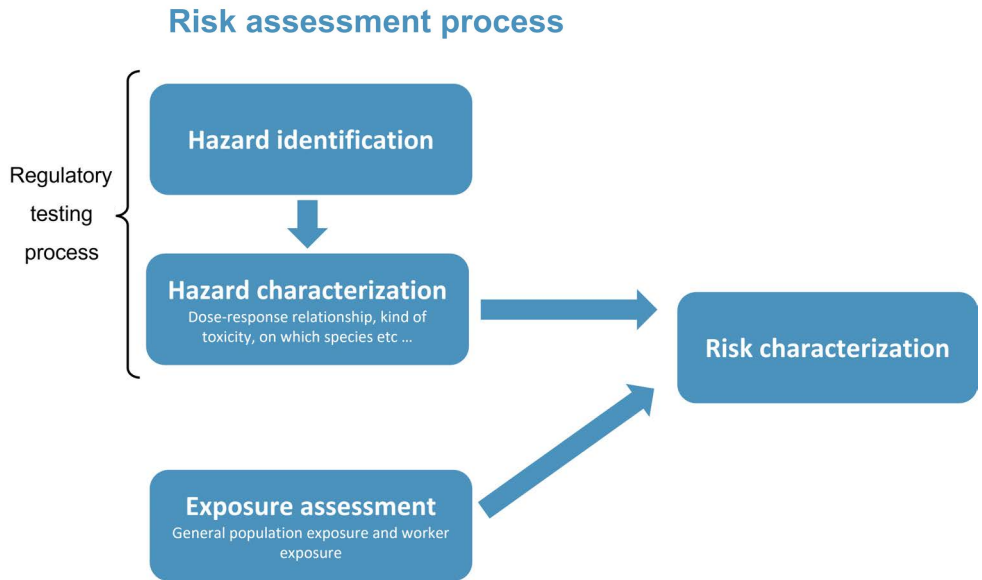


Figure 1 | Risk assessment process for regulatory decision-making [33].

Table 1 | Overview of OECD guidelines for DART-testing. (Obtained from: OECD, Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment).

Test	Stage of the reproductive process studied	Exposure period
Generation studies	-fertility and early embryonic development -embryo-fetal development -pre-and postnatal development	Continuously over one, two or several generations From 2 weeks prior to mating to adulthood
Prenatal Developmental Toxicity Study (Teratology study)	- fertility and early embryonic development -embryo-fetal development	From implantation to the day before birth
Developmental Neurotoxicity study	-embryo-fetal development -pre-and postnatal development	During pregnancy and lactation
Reproduction/ Developmental toxicity screening test	-fertility and early embryonic development - embryo-fetal development -pre-and postnatal development	From 2 weeks prior to mating until day 4 post-natal

Regulatory testing guidelines for developmental toxicity

The fundamental principle of DART regulatory testing currently relies on the observation of adverse outcomes in animal models at different life stages. As stipulated in the ICH guidelines, their DART preclinical studies (ICH S5 R3 [35]) are organized so as to “*leave no gaps between stages and allow for evaluation of all stages of the reproductive process.*” The first test guideline focuses on fertility and early embryonic development (FEED), whereas the second focuses on embryo-fetal development (EFD) and the third on pre-and postnatal development (PPND). The six studies from the OECD guidelines (Table 1) include the prenatal developmental toxicity study (OECD TG 414 [36]), the two-generation reproduction toxicity study (OECD TG 416 [37]), the reproduction/developmental toxicity screening test (OECD TG 421 [38]), the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD TG 422 [39]), the developmental neurotoxicity study (OECD TG 426 [40]), and the extended one-generation study (OECD TG 443 [41]).

Endpoints in parental and/or offspring	Guideline(s)
<ul style="list-style-type: none"> – Growth, development, and viability. Pregnancy length and birth outcome – Histopathology of sex organs and target organs – Oestrus cyclicity and sperm quality in TG 416 	TG 416: Two-generation study
<ul style="list-style-type: none"> – Reproductive/developmental – Developing immune system – Neurodevelopmental effect 	TG 443: Extended one-generation reproductive toxicology study
<ul style="list-style-type: none"> – Litter composition (e.g., resorptions, live, dead fetuses) – Embryonic development – Fetal growth – Morphological variations and malformations 	TG 414: Prenatal Developmental Study
<ul style="list-style-type: none"> – Pregnancy length and birth outcome – Physical and functional maturation – Behavioral changes due to CNS and PNS effects – Brain weights and neuropathology 	TG 426: Developmental Neurotoxicity Study
<ul style="list-style-type: none"> – Fertility – Pregnancy length and birth outcome – Histopathology of sex organs and target organs (and brain in TG 422) – Fetal and pup growth and survival until day 3 	TG 421: the reproduction/developmental toxicity screening test TG 422: the combined repeated dose toxicity study with the reproduction/developmental toxicity screening test

Advantages and limitations of current guidelines

As exemplified in Table 1, the chemical risk assessment process currently depends on animal data for hazard identification and characterization. This dependency on animal testing for regulatory decision-making is highly criticized among the general population and the scientific community. In the general population, animal testing is viewed as unethical; and push to move away from it [42, 43]. In the scientific community, it is generally believed that new approach methodologies (NAMs) are more reliable and relevant to the human situation [44]. However, additional knowledge is needed before further implementation of NAMs in regulatory decision-making.

Even though the historical approach is criticized nowadays, it is nevertheless difficult to replace animals with non-animal approaches while maintaining the same level of safety [45-47]. This is because, for example, animal testing uses a complete biological system; therefore, relevant routes of exposure can be tested (e.g., inhalation and ingestion), and comparable apical endpoints can be tested (e.g., malformation, fetal growth). Furthermore, the fate of a chemical is mimicked in the maternal organism; more specifically the pharmacokinetic and pharmacodynamic properties of the test substance as well as any metabolites are included in the *in vivo* testing, which are crucial when researching predictive impairment to development.

These characteristics make human extrapolation relatively easy and straightforward. Other non-negligible factors of animal testing include the high level of historical data available and the high level of standardization of testing procedures, which currently remain unmatched compared to NAMs.

Nevertheless, the use of animals in regulatory toxicology testing (and specifically in DART) has critical limitations. One of the major limitations is that animal physiology, even though similar to humans, is not an exact replicate [42, 48, 49]. So, focusing on adverse outcomes in animal models as endpoints to evaluate human toxicity comes with a certain degree of uncertainty. This has been highlighted by several studies in recent decades. For example, Tamaki et al. looked at drug toxicity, comparing animal studies with human adverse drug reactions [50]. They found that while most toxicities had around 75 to 80% correlation, some (musculoskeletal, respiratory, and neurological) had less than 30%. This poor correlation between animal studies with human adverse outcomes has also been found

in DART, demonstrated by the fact that 45% of all known teratogens were first identified in humans prior to being identified in animal models [42]. The thalidomide tragedy is also a good example of this limitation, illustrated by the absence of specific adverse outcomes due to its treatment in rodents compared to humans [30].

This interspecies difference makes selecting the right test species for specific toxicity testing a crucial but limiting factor for human safety. The current regulatory guidelines for developmental toxicity answer this uncertainty limitation by using at least two test species (usually rat and rabbit) for developmental toxicity testing. These guidelines lead to time-consuming, labor-intensive, expensive studies needing highly trained personnel without fully eliminating the uncertainty for human safety. They also require a high number of animals as around 25% of the total laboratory animals used in Europe every year is for regulatory purposes, which represent approximately two million laboratory animals every year [51]. Given the significant animal usage in these studies, there is clearly a need to consider how to improve the uncertainty limitation factor, while considering the ethical issues of animal testing.

Beyond the 3Rs: pushing towards alternatives to animal testing

In 1959, the first article on the 3Rs (Replacement, Reduction and Refinement) was published, creating the three major principles on which ethics towards animal testing are based nowadays [52]. These ethical principles are, in short: replacing animal use with non-animal methods wherever possible, reducing the number of animals used, and refining the use to reduce pain, suffering, and distress. In the past 60+ years, scientists and other stakeholders have found efficient methods to reduce and refine animal testing while staying statistically relevant, but the ethical concerns have remained [43, 53, 54]. Today, governments are pushing toward the exclusive use of alternatives to animal testing for regulatory testing, as illustrated by the ban in the EU of safety testing on animals for cosmetic products in 2004 [55].

In 2011 the EU also created ECVAM (EU Reference Laboratory for alternatives to animal testing), an organization in charge of *"covering the entire life cycle of alternative methods, i.e., from development and validation to regulatory acceptance, international recognition and proper scientific use"* [56, 57]. More recently, the Dutch government went further by setting the

1 goal to become world leader in innovations without laboratory animals by 2025 [58]. To achieve this, in 2016, they commissioned a report from the National Committee on animal testing (NCad) for a transition to non-animal research [45]. Yet, the validation of alternatives to animal testing by regulatory authorities remains a challenge. This challenge results from the current dichotomy between theory and practice in regulatory safety testing [47]. In theory, animal testing should only be used as a last resort, but in practice the assessment of apical endpoints in mammalian *in vivo* models is still the gold standard for human safety testing. In fact, this practice delays and limits the validation of the alternative methods already available. To facilitate the transition toward using non-animal testing, major stakeholders including the NCad have highlighted the importance of making “a paradigm shift away from existing mindsets and practices.” [45, 59]

The vision and theory behind the development and implementation of this paradigm shift were introduced in 2007 in a U.S. National Academy of Sciences report, “*Toxicity Testing in the 21st Century: A Vision and a Strategy*” [44, 59, 60]. In this report, they set the building blocks of a mechanistic-based approach arguing that every adverse outcome is driven by a toxicity pathway [61].

Integrative systems increase predictivity: Adverse Outcome Pathways

The adverse outcome pathway framework helps incorporate the mechanistic-based approach into the hazard profiling component of the regulatory safety process. This conceptual construct organizes scientific knowledge from a molecular initiating event (MIE) to an adverse outcome (AO) by interconnecting them through intermediate biological steps or key events (KE) (Figure 2 A, B) [62, 63]. The AOP framework is much broader than other chemical interaction frameworks (e.g., mode of action) as the AO goes up the organism level or even beyond to the population/ community level [63–65]. However, linear AOPs, as presented in Figure 2B, are overly simplistic. An integrated network of several AOPs is considered a more accurate representation of the toxic mechanisms leading to an AO (Figure2C) [66].

To harmonize this approach, the OECD has published guidance on developing and accessing AOPs [66]. This handbook focuses on how to structure an AOP description and facilitates the entry of AOP information into the AOP database, for example, AOP-Wiki [68, 69]. At the time of writing (May 2023), 28 AOPs have been endorsed by the OECD, helping in

the prediction of diverse AO such as liver cancer, early life stage mortality, neurodevelopmental impairment or body weight loss [70].

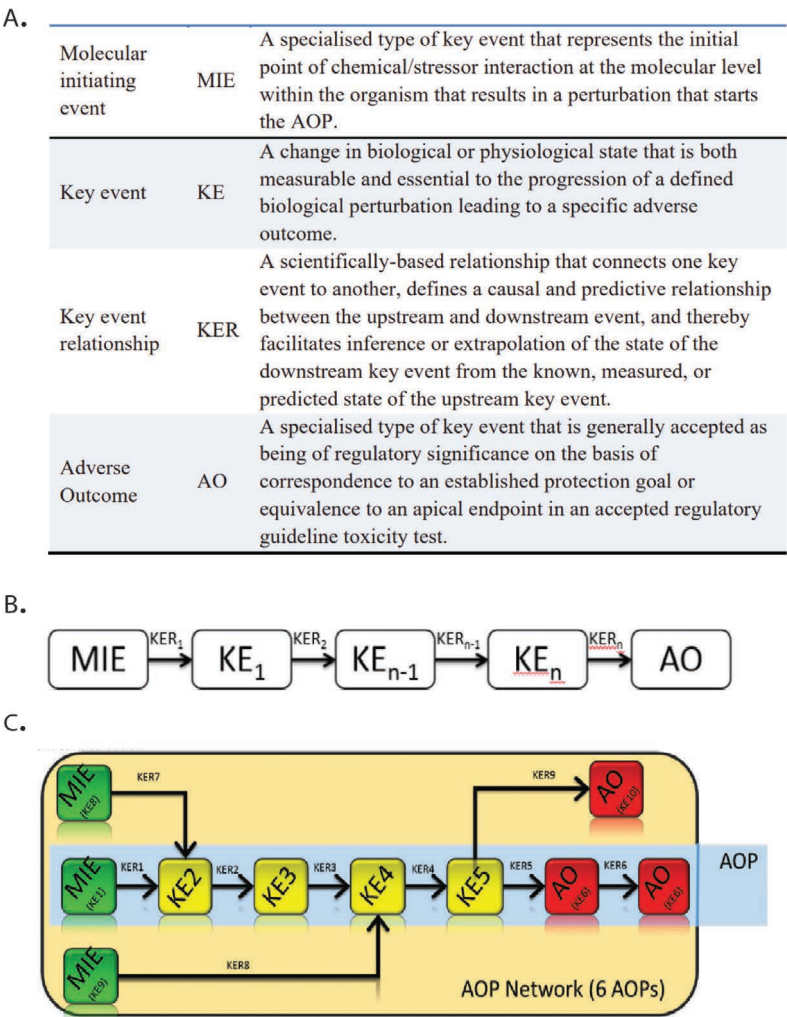


Figure 2 | A. OECD definition key terms used for AOP B. Example of generic AOP diagram from OECD: Users’ Handbook supplement to the Guidance Document for developing and accessing Adverse Outcome Pathways AOP. MIE = molecular initiating event, KE= Key event, KER= Key event relationship, AO = Adverse outcome [66] C. conceptual representation of AOP network author: Wittwehr and al. 2017 [67].

Substantially more draft AOPs already exist, but they are not yet validated, especially to predict developmental toxicity. For example, an AOP framework published in 2015 by Tonk *et al.* describes the mechanism of developmental toxicity due to an imbalance in the RA signaling pathway (RA-SP) [71]. This

framework includes AO such as neural tube and axial patterning defects, leading to craniofacial, heart, and limb defects (Figure 3). However, additional knowledge on the regulated genes caused by RA-SP imbalance is necessary to detail this AOP and to define gene expression biomarkers capable of predicting each AO. This is further explored in chapters 3 and 4 of this work.

By definition, KEs are measurable changes (Figure 2A) that can generally be studied by evaluating biomarkers in *in vitro* settings [66]. As DART represented the main area of animal uses for regulatory testing in Europe (240 000 animals in 2017), it could be a good starting point for the implementation of this new approach [72-74]. Additionally, a plethora of *in vitro* tests already exist and are already used as hazard identification screening tools [61].

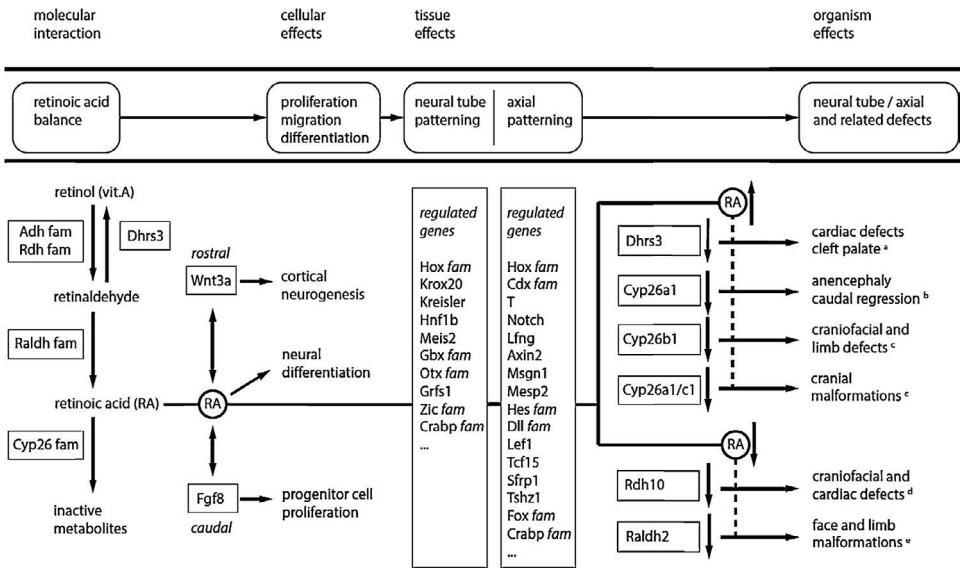


Figure 3 | Schematic representation of the retinoic acid–neural tube/axial patterning adverse outcome pathway (RA–NTA AOP) framework proposed by Tonk et al. in 2015 [71].

Example of *in vitro* models available for hazard identification

Over the past few decades, a variety of *in vitro* models have been developed to study DT, including assays using cell-based approaches such as embryonic stem cell (ESC) tests and assays using entire organisms such as whole embryo culture (WEC), as well as the zebrafish embryotoxicity test (ZET) [72, 75, 76]. Each of these models has a different applicability domain. For example,

1. The ESC test, which is based on the ability of stem cells (rodent or human) to differentiate into any specialized cell type, was validated by the EURL ECVAM as a tool for studying the effects of chemicals on differentiation processes.
2. The WEC test, based on the culture of rat embryos *in vitro* for the period when the embryo is still independent of the placenta, can be used to study several embryogenesis processes such as neurulation and early formation of major organs.
3. The ZET is based on the fertilized eggs of the tropical fresh water minnow *Danio rerio*, known as the zebrafish, that are followed up to 120 hours post fertilization (hpf); during this time, they will develop into complete, hatched, and swimming larvae [77].

The ZET and WEC hold a significant advantage compared to a cell-based model, in that they allow the study of potential toxic properties of a chemical in a complete developing vertebrate organism. Another advantage of the zebrafish over, for example, the WEC model, is the wider developmental window of the assay [72, 78]. With the culture of rat embryos, there is a limited embryogenesis period available for study, which is between gestation days 10 and 12 [75]. In contrast, the zebrafish allows for the study of the entire embryogenesis period, which includes the gastrulation, neurulation, the development of major organs, and the establishment of movement [77]. The zebrafish embryo thus holds great promise for developmental toxicology application, and as Hermesen says [78] : *"A broader developmental period may provide a larger window for detecting developmental effects."*

Although these *in vitro* tests are useful as alternative testing methods, expanding their use by establishing predictive molecular readouts is crucial to implement them in a mechanistic approach to risk assessment. The current work aims to advance this knowledge for the zebrafish embryo (ZE) model.

The Zebrafish embryo model in DART

The field of developmental biology was the first to use ZE as a model [79, 80]. In the late 1960s, George Streisinger searched for a genetic vertebrate model to study the embryogenesis of the vertebrate nervous system using mutant strains [81]. After considering numerous fish species, he selected the zebrafish, possibly because, compared to other species, this one is well suited for standard genetic manipulation. Adults breed prodigiously in

the laboratory, can be maintained in breeding conditions on a year-round basis, and individual females can give rise to hundreds of progenies. In the following decades, the ZE gained popularity in laboratories all over the world, leading to the discovery of thousands of mutations responsible for the disruption of embryogenesis such as *cys^{b16}*, a deficiency in growth factor disturbing axis patterning [82-85].

Nowadays, the ZE is also considered a useful model in toxicology [77, 86-88]. In 2013, it was adopted as a model to assess the acute toxicity of chemicals, in the OECD test guidelines (OECD TG 236 – Figure 4-)[89]. The zebrafish embryo acute toxicity test (ZFET- Figure 4), consists in exposing the freshly fertilized eggs to a chemical, then assessing four mortality endpoints every 24h. At the end of the exposure period, acute toxicity is determined based on the four apical observations recorded (Figure 4), and the lethal concentration 50% (LC50) is calculated.

Currently, in DT, the ZE is mostly used as an apical observation screening tool to evaluate the teratogenic potential of candidate molecules by manufacturers. However, the validation of TG 236 creates a precedent for the use of the zebrafish model in the regulatory testing process, potentially opening the opportunity for a further role in DT, especially as the central references for this test validation were based on research in DT [89].

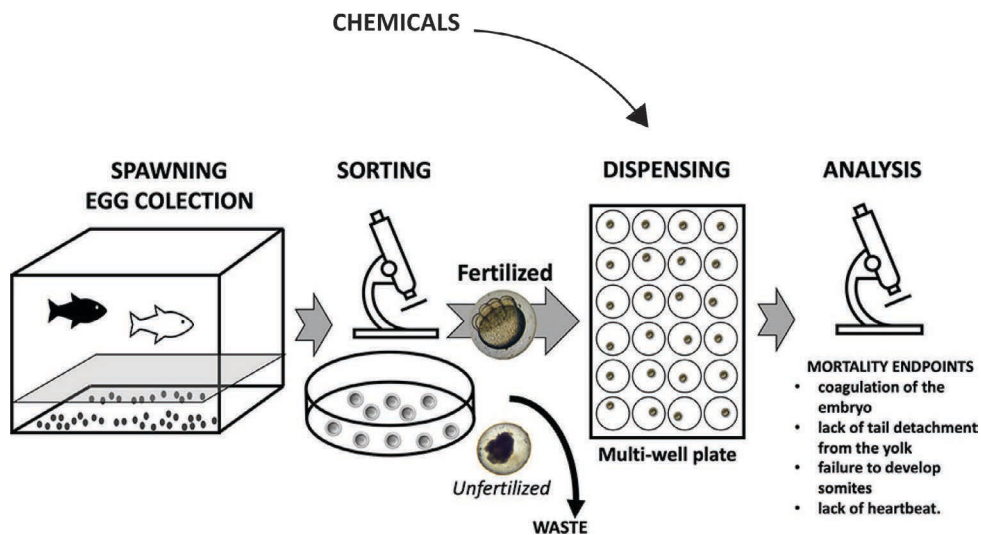


Figure 4 | Steps of the laboratory procedure for OECD TG 236, adapted with permission from Wlodkowic et al. 2021 [90] American Chemical Society."

Currently, the ZE is used as a research tool to study the mechanisms of chemically-induced toxicity in a broad variety of contexts [91-93]. For example, alternative tests using the ZE model are being developed as an inherent part of improving the hazard assessment approach in several major European projects (e.g. ERGO – improved endocrine disruptors chemicals assessment- [94, 95], PARC -improved Next generation risk assessment (NGRA)-[96], EUROMIX - improve the impact of mixture exposure assessment-[97]).

Advantages of the zebrafish embryo model

The zebrafish embryo model is a suitable DT alternative model to study chemically-induced mechanisms of developmental toxicity for several reasons [87, 88, 98-102]:

1. The mechanistic extrapolation based on the ZE, is relevant to the human situation. This is because the first steps of development of the body plan are highly conserved between vertebrate species. Not only that, but nearly all mammalian genes have a zebrafish counterpart, with a high conservation of key genes and developmental pathways between zebrafish and humans [101].
2. The ZE is a suitable model organism for a relatively high-throughput assay, given their year-round spawning, their small size, their fast development, the large number of eggs laid per breeding (200 per female on average), and their relatively cheap maintenance in the laboratory.
3. The assay is considered relatively simple. The exposure is generally performed in multi-well plates with several conditions on the same plate by simply putting the ZE in a well containing the chemical of interest mixed with the water.
4. Another advantage of this model is that the zebrafish is oviparous and so lays eggs which are hatched into offspring after they have been laid by the parent, in contrast to viviparous mammals which give birth to live young. Therefore, no euthanasia of the parent is necessary to obtain the fish embryo, effects of direct embryo exposure can be studied and differentiated from those secondary to maternal toxicity. Additionally, the transparency of the eggs allows stage-by-stage observation without termination of the embryo (Figure 5).
5. The ZE is legally not defined as an *in vivo* test during the developmental stage covered in the assay. Indeed, the ZE is not covered under the

European legislation for animal welfare before 120 hpf [103, 104]. According to the Directive EU Directive 2010/63/EU, larvae formally are experimental animals only after they have become independent from their yolk sac and free-feeding [104]. For ZE, it takes until 120 hpf before this crucial stage of the development is achieved. This means that sensitivity to chemically-induced toxicity can be studied in this window, during which time the zebrafish embryos already develop most of their cardiovascular, digestive, and nervous systems. These are similar to their mammal counterparts on a physiological and molecular level [87, 88].

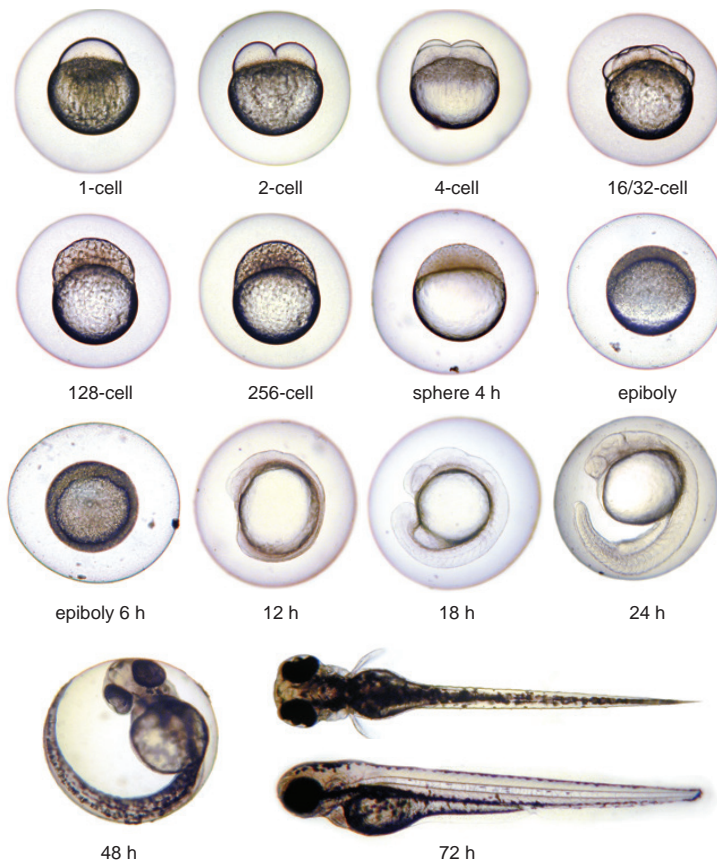


Figure 5 | Zebrafish embryo developmental stage from 1-cell to 72hpf. Picture from S. Hermesen thesis [78].

Readouts of the zebrafish embryo model

Thanks to all these advantages, the ZE is considered by many to be a bridge, filling a biological gap between simple, quite reductionist *in vitro* assays based on cells or tissue cultures and *in vivo* assays requiring whole animals (often adult ones) such as rodents [87, 105, 106]. Consequently, a plethora of ZE assays were developed to study developmental toxicity in this model, with different readouts for example: behavioral, morphological, and molecular parameters.

Morphological readout

The morphology of the ZE embryonic stages was characterized in depth early on; thus, the morphological readout was the first to be developed [107]. This readout measures observable morphological changes in the exposed ZE and quantifies them using scoring system based on visual observations, see an example of a scoring system in Figure 6. Several of these systems have been developed (Table 2), with each method trying to improve upon major practical limitations, e.g., the method is time-consuming while somewhat reductionist (only a small number of endpoints can be scored), requires trained scientists and their expert-judgment based scoring is somewhat subjective and technician-dependent which could impact experimental reproducibility. New, rather costly technology can automate the scoring, for example by coupling AI imaging technology (VAST BioImager™) and feature annotation AI (fishInspector). This could consequently solve these practical limitations, but at a high cost [90, 108].


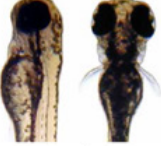

General Morphology Score				
Hpf	12	24	48	72
Detachment of tail	 0			
	 18hpf 1			
Somite formation	No = 0	Yes = 1	Yes = 1	Yes = 1
Eye development	 1	 2	 2 + 1 for pigment	 2 + 1 for pigment
Movement	No = 0	Yes = 1	Yes = 1	Yes = 1
Heartbeat	No = 0	Yes = 1	Yes = 1	Yes = 1
Blood circulation	No = 0	No = 0	Yes = 1	Yes = 1
Pigmentation head-body		 0	 1	 1
Pigmentation tail		 0	 1	 1
Pectoral fin			 0	 1
Protruding mouth			 0	 1
Hatching	0 No = 0	0 No = 0	0 No = 0	1 Yes = 1
GMS	1	7	12	15

Figure 6 | Example of the semi-quantitative scoring system based on normal development hallmark of a ZE up to 72hpf, used in this work. Published by Hermesen at al. 2011 [109].

Table 2 | Overview of example morphological scoring systems used when studying developmental toxicity of chemical in the zebrafish embryo model, summarizing the diversity of scoring methods available.

Authors	System principle	Reference
Herrmann et al., 1995	Semi-quantitative system scoring 8 specific malformation endpoints including oedemas in the yolk sac and/or the heart, deformities in different parts of the brain, and/or in the tail.	[110]
Nagel et al., 2002	Binomial system scoring 21 endpoints including. <div><div><u>Lethal endpoints</u> Coagulation Tail not detached. No somites No heartbeat <u>Sublethal/Development endpoints</u> Completion of gastrula Formation of somites Development of eyes Spontaneous movement Heartbeat/blood circulation Pigmentation Oedema</div><div><u>Teratogenic endpoints</u> Malformation of head sacculi/otoliths tail heart modified structure of the corda scoliosis rachischisis deformity of yolk growth-retardation Length of tail</div></div>	[105]
Panzica-Kelly et al., 2010	Quantitative morphological and functional system scoring 23 endpoints. <div><div>Body length Body shape Viability Head-trunk Otic vesicle Somite number Somite morphology Notochord morphology Cardiovascular function Facial structure morphology Brain morphology Jaw and pharyngeal arch morphology</div><div>Tail morphology Fin morphology Heart morphology Motility Pigmentation Swim bladder Stomach Intestine Liver Yolk Heart rate</div></div>	[111]
Hermesen et al., 2011	Semi-quantitative system scoring 11 endpoints. <div><div>Detachment of the tail Somite formation Eye development Movement Heartbeat Blood circulation</div><div>Pigmentation of head and body Pigmentation of tail Pectoral fin Protruding mouth Hatching</div></div>	[109]

Several studies have used the morphological readout to examine the general utility of ZE as a screening method for potential teratogens. The main strategy in the majority of cases is to test different chemicals with or without teratogenic properties and compare the ZE predictivity with a mammalian model (Table 3). The results of these studies overall show a variable accuracy ranging from 60% to 100% (Table 3). Several factors may contribute to this variability such as study design (e.g., start and end of exposure time, duration of exposure, morphology end points studied, and dechorionation of the embryo) as well as the chemical selection tested.

The factors described above illustrate major challenges facing toxicologists in implementing ZE assays for chemical safety assessment and show that additional work is needed for this model to be integrated into the regulatory testing process for DT.

For example, relying on using the ZE morphology readout as a stand-alone test to conclude on chemical safety is insufficient. Indeed, some chemicals cannot be assessed by morphological assessment as subtle non-visible effects may remain unnoticed [78]. Additionally, the morphological readout cannot discriminate between different chemical modes of action, making it impossible to determine if the toxicity or the absence of toxicity observed is relevant to the human situation. Thus, it is scientifically valuable to investigate further to reveal more important information about the chemical interaction with potential targets in developmental toxicity and improve the model predictivity by investigating different biological parameters, such as changes to the genes, proteins, and pathway targets induced by a chemical toxicant.

Table 3 | Selection of teratogenicity validation screening studies using morphology as readout with their accuracies. T=teratogen NT= non-teratogen CR=compared to rodent CH=compared to Human.

Reference	Number of compounds tested	% Accuracy CR	Time point tested.(hpf)	False positive Compare to mammals	False negative	Remarks
Song et al. 2021 [112]	Total = 85 T = 53 NT = 32	89% (76/85)	96	7	2 -	
Jarque et al. 2020 [113]	Total = 31 T = 16 NT = 15	87.10 % CR 74.19 % CH	96	6	1 -	
Yamashita et al. 2014 [114]	Total = 59 T = 32 NT = 27	90% CR (53/59)	144	2	4 -	
Sung Hak Lee et al. 2013 [115]	Total = 7 T = 7 NT = 0	100% CH (7/7)	72	0	0	Exposure starts at 5,25 hpf
Van den Bulck et al. 2011 [116]	Total = 15 T = 8 NT = 7	60% (9/15)	96	4	2 -	
Hermesen et al. 2011 [109]	Total = 14 T = 10 NT = 4	64%	72	-	-	Calculated by Nisha S. Sipes [117]
Brannen et al. 2010 [106]	Total = 31 T = 17 NT = 14	87% CR	120	2	2	Dechorionated egg

Molecular readouts

Understanding toxicity requires more than apical readouts such as changes in behavior, viability or morphology as different chemicals may cause a similar effect, yet their mechanisms of toxicity may differ [78]. Also, as discussed above chemically-induced morphological effects may not be the same between species. Thus, using a more integrative method, including molecular readouts of toxicity markers, could help distinguish the diverse chemical modes of action, increasing the test system sensitivity and predictivity.

Molecular readouts can be studied through many end-points, e.g., the level of gene expression, protein concentration, or epigenetic changes. The gene expression end-point is based on the observation that gene

1 expression changes precede virtually all toxic responses at the protein, cell, or organism level [118]. Since toxicants may alter gene expression, it is possible to link specific molecular changes to apical outcomes, through the use of AOPs [70, 119]. Multiple efforts have been made to develop this readout further; however, additional work to improve, for example, the harmonization of this readout is needed; this is explored in Chapter 2. Gene expression can be studied in different ways and the most extensive is to examine the expression at the scale of the whole genome. The genome-wide transcriptomic approach identifies transcriptome changes between experimental conditions e.g., comparing controlled and treated conditions, giving mechanistic insight into the molecular mode of action of the chemical. This approach is best suited for searching for predictive biomarkers of toxicological effects due to its comprehensive and unbiased properties (hypothesis generating). This approach has previously revealed critical effectors for signaling pathways and downstream targets for transcription factors, enabling the discovery of new biomarker candidates for some apical outcomes [120-123]. However, additional work is needed to cover more apical outcomes and this is explored in chapters 3 and 4.

Whole genome scale transcriptomic approaches have become more popular over the past decade due to the drastic decrease in cost per sample. The most popular technique of transcriptomics is RNA-sequencing (RNAseq). In contrast to previous microarray-based methods, it provides better coverage and greater resolution of the dynamic nature of the transcriptome, detects low abundance genes, and does not need prior knowledge of the genome to be used [124, 125]. By using deep-sequencing technologies, RNAseq can produce large-scale datasets [125]. The analysis of these datasets can be challenging, but a growing number of bioinformatics tools are becoming available to interpret these datasets and enable researchers to conclude at both the gene and pathway levels. For example, functional annotation tools like David [126, 127] and Gorilla [128] use public database information from Reactome [129], KEGG [130], Wikipathway [69], and Gene ontology to cluster the differentially expressed genes (DEGs). These clusters then help perform functional analysis, giving information on biological processes and pathways, protein-protein interaction and tissue expression in which the DEGs are involved. Other tools add a visualization layer to the analysis e.g., STITCH, one of the tools used in this work, creates a network of protein-protein and protein-chemical interactions [131]. One limitation of these

publicly available tools is that they are based on the DEG list and not on the gene expression level. Other more costly platforms, for example, Ingenuity Pathway Analysis, integrate the gene expression level in their analysis [132].

The study of molecular readouts, especially the transcriptomic endpoint, is a primordial building block for a new kind of safety assessment not based on *in vivo* testing called next generation risk assessment (NGRA). It enables a more precise definition of the molecular events and the selection of biomarkers relevant for assessing adverse effects and/or exposure [133]. As stated previously, these readouts are also used to construct and feed AOPs.

Vertebrate development and its regulation

The idea of developmental conservation between vertebrates has been around for centuries. For example, morphological comparative embryogenesis studies on human and other vertebrate species reveal a stage of similarity between vertebrates famously illustrated by Haeckel in 1892 (Figure 7A). This illustration nowadays contested started the funnel theory, which states that the beginning of embryology is the most conserved stage of development (phylotypic stage) [100]. However, the hourglass theory also exists (Figure 7B), based on mechanistic (gene expression) conservation, which places the phylotypic stage much later at the start of organogenesis [99, 100]. Nevertheless, both theories highlight the presence of analogous developmental mechanisms between the vertebrate species, making studying developmental toxicity mechanism in a non-human vertebrate species such as the zebrafish, a useful model for human predictivity [49, 99, 100, 134-137].

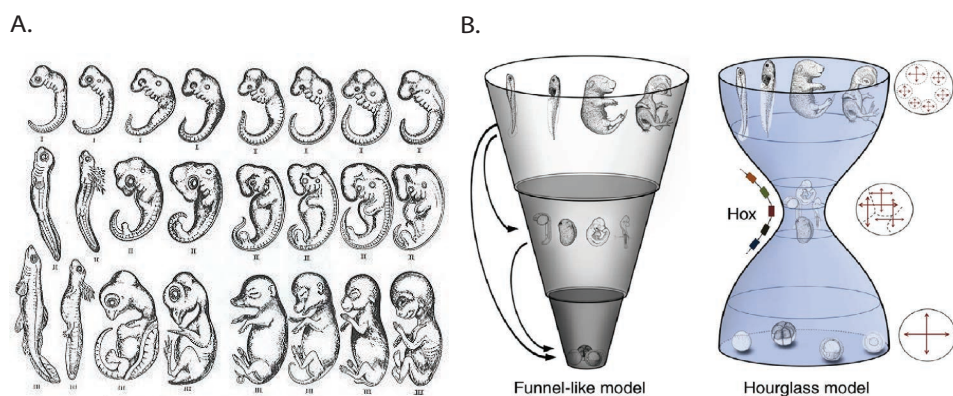


Figure 7 | A. Haeckel's illustration of vertebrate development B. two major hypotheses about developmental conservation between vertebrates [100].

Vertebrate development

Vertebrate development starts when an oocyte is fertilized by a sperm cell, creating a diploid cell or zygote. This zygote undergoes different steps of embryogenesis to become an organized multicellular embryo via a transformation that starts with several cell cleavages into the multi-cell morula, which re-organizes itself to become a blastocyst with two distinct cell types: the trophoblast and the inner cell mass. The inner cell mass undergoes gastrulation to transform into a 3-layered embryo [138]. This embryo will then undergo neurulation (the development of the future nervous system) that results in the formation of the neural tube and organogenesis (the development of the organs) to become a fetus in human and larvae in zebrafish. After this stage, the final maturation of the organs and a major growth in size occurs.

Vertebrate embryogenesis is described as the developmental process encompassing the zygote cell cleavage and specification, gastrulation, neurulation, and organogenesis stages. Although it happens at different timing and duration for each species, comparative embryology in the vertebrate taxon has suggested the idea of conserved mechanisms in embryogenesis (Figure 7) [137]. For example, gastrulation starts at week 3 in humans and at 10 hpf in zebrafish, but in both species, during this stage, the embryo will develop from totipotent inner mass cells to an embryo organized in three primary germ layers [139]. These layers will later engender specific tissues and organs similar in both species. For example, the nervous system, sensory organs, and epidermis originate from the external layer or ectoderm. The digestive system, including the gut, pancreas, and liver, will originate from the inner layer or endoderm. The circulatory, lymphatic, skeletal, muscular, urogenital, and reproductive systems and the notochord will originate from the middle layer or mesoderm.

Morphogens and developmental signaling pathway

Vertebrate development is a dynamic process involving cell proliferation and differentiation in combination with migration and apoptosis [140]. To succeed, this self-organization process must be tightly regulated by cell communication. A common communication method during development is the use of molecules that govern cell fate through their concentration or morphogens [141]. Generally, the morphogen concentration gradients are created by locally restricting the transcription of a protein [142]. Atypical

morphogens, such as all-trans retinoic acid (ATRA), are created by locally restricting the transcription of its synthesis enzyme [143-146]. The molecule is then excreted into the extracellular space. The specific concentration of this extracellular protein along the gradient determines the activation of intracellular signal transduction cascades, triggering dose-dependent activation or repression of target genes [147, 148]. It is important to note that one gene can be the target gene of several morphogens, thereby creating competition between morphogens. This mechanism is illustrated by the regulation of the Hox family, which can integrate and respond to opposing signaling gradients, such as those of ATRA, fibroblast growth factors (Fgfs), and wingless related integration sites (WNTs)[149-154].

As a key transcription factor for the regulation of axial patterning and the regional identity of cells, the Hox family has been studied in a multitude of models, enforcing the idea of a highly conserved regulatory network involving transcription factors (TFs) and signaling pathways in development [100, 135, 151, 155].

Due to this consistency of signaling pathways between vertebrate species, non-human vertebrates can be highly relevant in the context of toxicity testing for human predictivity.

The Retinoic acid signaling pathway

The importance of vitamin A (VA) for human health has been known in history for centuries; illustrated by the existence of treatment such as raw liver mix with honey in ancient times [156]. However, VA and its active form (the retinoids) were only characterized around the turn of the 20th century [157].

Retinoic acid metabolism: from vitamin A to inactive metabolite

VA or retinol is a critical micronutrient and can only come from dietary intake [148, 158]. Preforms of VA are present in plants such as carrots or sweet potatoes and in animal products such as fish-liver oils, eggs, and milk. These preforms of VA can be metabolized to active retinol in the body as needed [156]. This is an important mechanism in the body to help control the critical internal concentrations of VA because retinol plays a major role in vertebrate adult homeostasis and embryonic development [148]. In the developing embryo retinol is transferred through the placenta by binding to a retinol binding protein (RBP; Figure 8) [148].

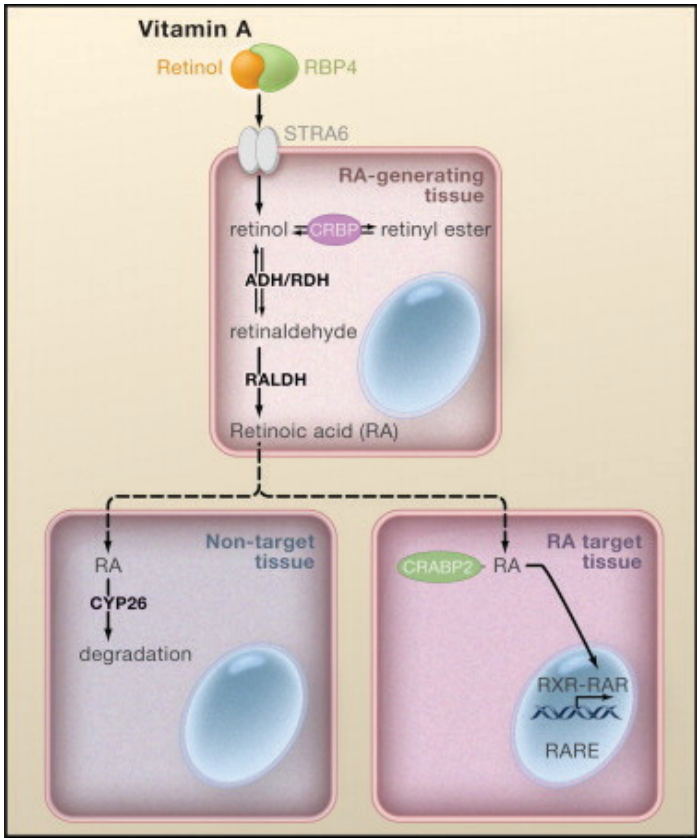


Figure 8 | RA metabolism in retinoic acid generated cells figure from Duester 2008 [148].

Figure 8 shows how retinoic acid sensitive cells (e.g., neural crest cells) can actively transport an extracellular RBP-retinol complex via a transmembrane cellular protein (*stra6*) [148, 159, 160]. Once in the cell, the retinol and RBP can separate, the retinol will then follow two sequential reactions to transform into retinaldehyde and then into retinoic acid (RA) (Figure 4). These reactions are catalyzed by three enzyme families. The cytosolic alcohol dehydrogenases (ADHs) and the retinol dehydrogenases (RDHs) are responsible for the first reaction; and the aldehyde dehydrogenases (RALDHs) for the second reaction (Figure 8). Retinoic acid is the active form in this signaling pathway and different isomers exist including all-trans retinoic acid (ATRA), 9-cis-retinoic acid and 13-cis-retinoic acid which have different roles in the organism. The work described herein focuses on ATRA, as it is the primary ligand implicated in developmental biology [160]. ATRA has a paracrine function which means it traverses the cell bound

to the Stra6. ATRA can act as a transcription factor by binding to nuclear receptors (RXRs and RARs) in hetero- or homodimers [161]. The complex translocates into the nucleus where cofactors are added (e.g., nuclear receptor co-activator 1 -NCOA1-), then it binds to retinoic acid response elements (RAREs) in the DNA to regulate target genes.

In the embryo, ATRA concentration must be tightly regulated in time and space; this is done by a spatially controlled balance between ATRA synthesis and degradation. The enzymes responsible for the ATRA degradation in vertebrates are members of the cytochrome P450 26 subfamily (CYP26) which convert ATRA into inactivated polar metabolites (Figure 8)[64, 162-165].

ATRA and RA-SP role in development and teratology

As VA cannot be synthesized *de novo* in vertebrates, research using depleted or enriched VA diet in animal models (e.g., chick, zebrafish, monkey, pig, guinea pig) has helped to understand the role of ATRA in embryonic development [106, 160, 164, 166-177]. These studies conclude that ATRA is generally promoting cell differentiation through its target genes, and it gives crucial positioning information through its concentration gradient and antagonist action on other morphogens. These effects are crucial for a plethora of developmental processes (Table 4) such as embryo body axis extension, somitogenesis, heart patterning, and early neural differentiation [151, 178].

Table 4 | Example of tissues and organs dependent of RA-SP balance for their development in zebrafish, table from Samarut et al. 2015 [179].

Hindbrain anteroposterior patterning	RA is required for proper patterning and exogenous RA exposure induces posteriorization.	[23], [30]
Spinal cord motor neuron differentiation	RA inhibition reduces branchiomotor neuron and axon development.	[23]
Somite symmetry	RA reduction causes a strong bias in left/right somite laterality	[39]
Heart development and patterning	Exogenous RA exposure early causes loss of cardiac precursors and posteriorization. RA is required for chamber formation.	[48], [49]
Hematopoiesis	RA inhibits differentiation of cells into hematopoietic fates.	[59]
Neural crest cells, pharyngeal arches (PAs)	Reduced RA during gastrulation causes NCC defects. RA is necessary for PA morphogenesis.	[18], [73], [74]
Pectoral fins	Blocking RA inhibits fin development.	[111]
Tooth	RA is required for tooth induction	[73]
Bone	Early increased RA exposure decreases mineralization, while later in development it increases mineralization.	[15],[17], [91]

Table 4 | *Continued.*

Lipid	RA inhibition decreases lipid content. Exogenous RA treatment increases adipocyte-related gene expression.	[91]
Kidney	Exogenous RA induces proximal pronephron segment fates.	[112]
Pancreas	RA is required for pancreas development	[113]
Liver	Blocking RA signaling inhibits liver development.	[113]
Intestine	RA is required for enterocyte differentiation.	[114]
Retina	Exogenous RA expands the optic stalk and gene expression associated with the ventral domain within the retina.	[115]

ATRA and body axis extension

Body axis extension is a dynamic process driven by several morphogen gradients such as ATRA, *fgf*, and *wnt* along the anterior-posterior axis [141, 159, 180]. The roles of these different morphogen gradients are complex and variable as such only the patterning of the spinal cord is detailed below.

The patterning of the spinal cord (SC) is driven by two synchronized but opposite morphogen gradients: the ATRA gradient with the highest concentration at the SC anterior section and the *fgf8* gradient with the highest concentration at the SC posterior section. Each of these morphogens promotes opposing effects: ATRA promotes cell differentiation and *fgf8* promotes cell division [141, 181]. Their effects are mediated through the regulation of their respective target genes, which is fine-tuned locally by the presence of a downregulation feedback loop between the morphogens (Figure 9). ATRA downregulates the transcription of *fgf8* directly and indirectly through inhibition of *wnt3a*, and *fgf8* downregulates the transcription of enzyme responsible for ATRA synthesis (*Raldh2*) [150, 182].

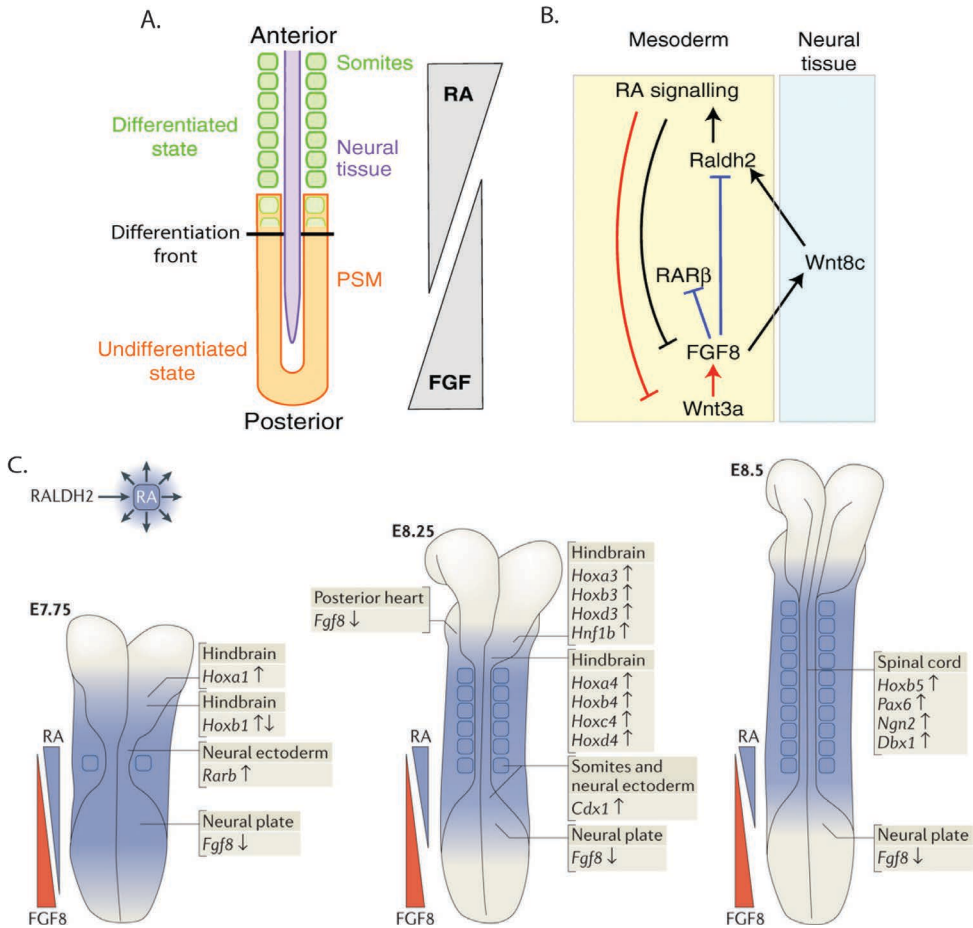


Figure 9 | ATRA (RA) and FGF8 antiparallel gradient during body axis extension. A schematic of body extension in mouse and chick embryos. B. Mechanism of gradient feedback loops. Black lines depict interactions shown in both chick and mouse, red lines are interactions shown in mouse only, and blue are interactions shown in chick only, figure from [182]. C. More detailed schematic of mouse body extension including timing and the genes up regulated (arrow up) and down regulated (arrow down) by ATRA figure from [159].

ATRA and neurodevelopment

Development of the vertebrate nervous system is controlled by distinct gene expression that results from the interaction between morphogen concentration gradients including ATRA, *Fgf*, *Wnt*, *Shh* and *Bmp* [183]. ATRA induces neural differentiation by directly up-regulating the expression of the pro-neural induction factor *Neurogenin 2* required for primary neuron specification and by down-regulating the signal from *fgf8* [184].

The RA-SP is also responsible for the hindbrain patterning, controlling the segmentation of the rhombomeres by directly regulating the collinear temporally and spatially defined *hox* family gene expression and more precisely the *Hoxb1* gene [152]. An AOP framework published by Chen et al. in 2020 considers an imbalance of RA-SP as the MIE for hindbrain, neural tube and nerve patterning defects (Figure 10)[185].

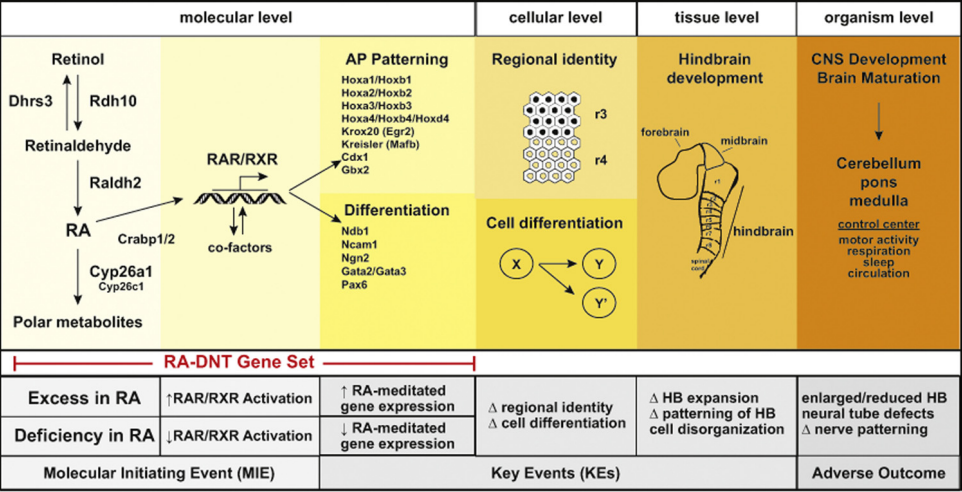


Figure 10 | AOP framework of hindbrain development related to RA-SP perturbation from Chen et al. 2020 [185].

ATRA in heart development

The RA-SP is essential in multiple steps of cardiac organogenesis such as anterior-posterior boundaries of cardiac mesoderm, specification of cardiomyocyte subtypes and primitive heart tube formation[186-190]. Defining the anterior-posterior boundaries of cardiac mesoderm, ATRA plays a crucial repressive role by inhibiting *fgf8* which limits the number of cardiac progenitor cells created. In the zebrafish, ATRA downregulation causes an increase in cardiac progenitor cell numbers resulting in the formation of anomalous large hearts [189].

ATRA excess in humans leads to congenital heart malformation such as transposition of the great vessels [191-193]. An AOP by Mennen et al. was recently created describing the decrease of ATRA due to inhibition of Raldh as a cellular KE for the transposition of the great arteries (<https://aopwiki.org/aops/436>) (Figure 11).

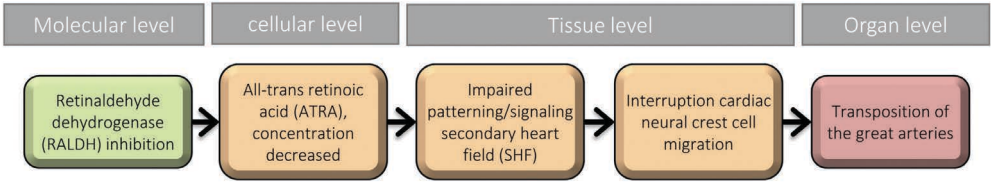


Figure 11 | Transposition of the great arteries AOP related to RA-SP perturbation published in AOPWIKI by R.H. Mennen and S. Mitchell-Ryan (<https://aopwiki.org/aops/436>).

ATRA and skeletal development

An ATRA gradient is essential in skeletal patterning morphogenesis [97, 194–200]. RA-SP perturbations have been shown to lead to skeletal defects in model species and humans [159, 174, 199, 201]. Inhibition of *cyp26b1* leads to severe limb defects and under-ossification of the maxilla, mandible, calvarial and sutural bones resulting in humans with hypoplasia of the cranium and craniosynostosis [174, 201]. Transgenic zebrafish that overexpressed *cyp26b1* exhibited over-ossification of the vertebral column [202]. Several AOPs have been created linking the imbalance in the RA-SP to skeletal defects (Figure 12).

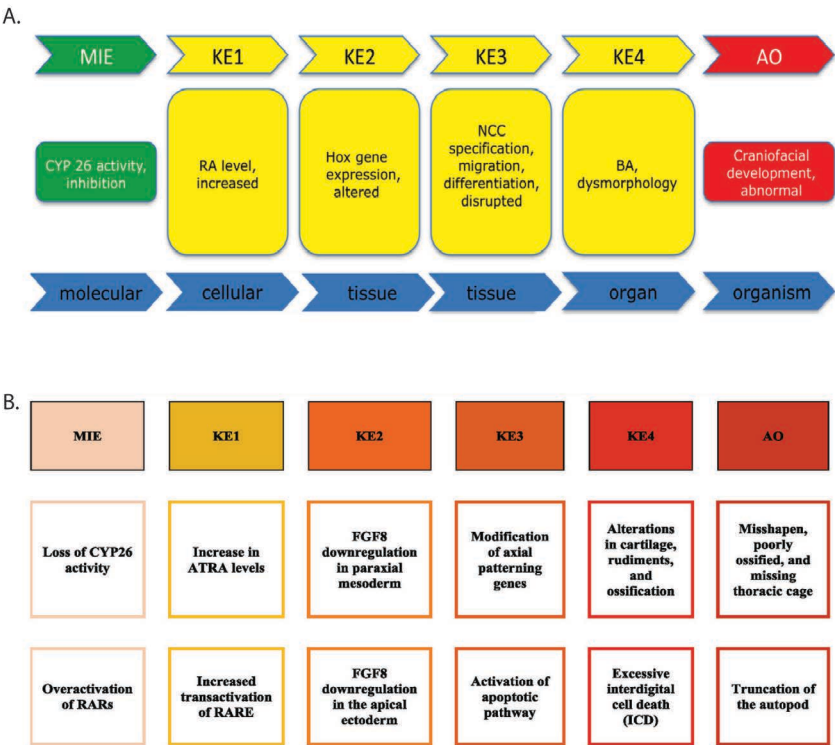


Figure 12 | AOP related to skeletal defect due to imbalance of RA-SP. A. [198] ; B. [203].

1 Although the role of the RA-SP in development is relatively well understood, the study of its toxicity mechanism leading to maldevelopment is still in its infancy. Therefore, this research project was developed to better understand these mechanisms, to identify potential predictive biomarkers related to these pathway toxicity mechanisms, and to optimize the ZE experimentation design for their study.

Outline of Thesis

The research described in this thesis aims to identify retinoic acid signaling pathway-related gene expression biomarkers of developmental toxicity in the zebrafish embryo model. This thesis consists of two parts.

The first part focuses on optimizing the experimental protocol for gene expression readout in the zebrafish embryo assay. In **chapter 2**, one often-forgotten dimension of an experimental protocol was explored, optimization of exposure duration. The exposure duration was assessed based on gene expression and morphological data collected after 2, 4, 6, 24, 48, 72, and 117 hours of exposure (Figure 13). This assessment was evaluated for RA-SP responses by using ATRA as a reference compound known to perturbate the RA-SP and by using a preselected gene set known to be related to the RA-SP. This gene set included ATRA metabolic genes, first effectors of ATRA, and a large spectrum of main developmental pathways and secondary actors in the neural tube closure process.

The second part of this thesis focuses on identifying biomarkers of teratogenicity related to the RA-SP in the zebrafish embryo model using the experimental conditions, particularly those concerning exposure duration defined in **chapter 2**.

In the second part of the thesis (**chapters 3 and 4**), the perturbation of the RA-SP was assessed based on genome-wide expression screening data after exposure to three reference compounds: two teratogenic compounds known to perturb the RA-SP, ATRA and valproic acid (VPA) and one non-teratogenic reference compound folic acid (FA; Figure 13). In **chapters 3 and 4**, potential biomarkers of teratogenicity were assessed based on a gene set commonly regulated by the two teratogens and not by the non-teratogenic compounds. Specifically, **chapter 3** describes the RA-SP perturbation common to the two teratogens and

potential neurodevelopmental biomarker exploration was based on the most significantly enriched gene ontology terms. In **chapter 4**, potential biomarkers specific for mesodermal-derived tissue maldevelopments were explored by selecting gene ontology terms related to these tissues.












	Exposure variables		RA-SP Toxicity	Readouts	
					
Chapter 2	hpf	ATRA	Overall		Morphological assessment
	3-5				Gene expression (PCR)
	3-7				
	3-9				
	3-24				
	3-48				
	3-72				
	3-120				
Chapter 3	3-7	ATRA VPA FA	Neurodevelopmental 		Gene expression (RNA-seq)
Chapter 4	3-7	ATRA VPA FA	Mesoderm-derived tissues    		Gene expression (RNA-seq)

Figure 13 | Schematic overview of the experiments described in this thesis.

References

1. Asia-, W.h.o.-S.-E. *World Birth Defects Day*. 2021 [cited 2023 05-06-2023]; Available from: <https://www.who.int/southeastasia/news/detail/02-03-2021-world-birth-defects-day-ms>.
2. Lipton, M., *The history and superstitions of birth defects. I*. J Sch Health, 1969. **39**(8): p. 579-82.
3. Beckwith, J.B., *Congenital malformations: from superstition to understanding*. Virchows Arch, 2012. **461**(6): p. 609-19.
4. Jastrow, M., *The Medicine of the Babylonians and Assyrians*. Proc R Soc Med, 1914. **7**(Sect Hist Med): p. 109-76.
5. DeSesso, J.M., *The arrogance of teratology: A brief chronology of attitudes throughout history*. Birth Defects Res, 2019. **111**(3): p. 123-141.
6. Mayer, A.M., *THE MONSTROUS BIRTH: THE THEORY OF MATERNAL IMPRESSION AND CONGENITAL MALFORMATIONS in History of medicine days*. 2001: Faculty of medicine, university of calgary.
7. Weinhold, B., *Environmental Factors in Birth Defects: What We Need to Know*. Environmental Health Perspectives, 2009. **117**(10): p. A440-A447.
8. organisation, W.h. *congenital disorders*. 2023 [cited 2023 06-2023]; Available from: <https://www.who.int/news-room/fact-sheets/detail/birth-defects>.
9. Willhite, C.C. and P.E. Mirkes, *Developmental Toxicology*, in *Encyclopedia of Toxicology (Third Edition)*, P. Wexler, Editor. 2014, Academic Press: Oxford. p. 14-44.
10. Brown, J.M., et al., *A Brief History of Awareness of the Link Between Alcohol and Fetal Alcohol Spectrum Disorder*. Can J Psychiatry, 2019. **64**(3): p. 164-168.
11. Popova, S., et al., *Fetal alcohol spectrum disorders*. Nat Rev Dis Primers, 2023. **9**(1): p. 11.
12. Hoyme, H.E., et al., *Updated Clinical Guidelines for Diagnosing Fetal Alcohol Spectrum Disorders*. Pediatrics, 2016. **138**(2).
13. Warren, K.R. and B.G. Hewitt, *Fetal alcohol spectrum disorders: when science, medicine, public policy, and laws collide*. Dev Disabil Res Rev, 2009. **15**(3): p. 170-5.
14. Harada, M., *Minamata Disease: Methylmercury Poisoning in Japan Caused by Environmental Pollution*. Critical Reviews in Toxicology, 1995. **25**(1): p. 1-24.
15. Hachiya, N., https://www.med.or.jp/english/pdf/2006_03/112_118.pdf. The history and the present of Minamata Disease -Entering second half a century-, 2006.
16. Yorifuji, T. and T. Tsuda, *Encyclopedia of Toxicology | Minamata*, in *Encyclopedia of Toxicology* Elsevier, Editor. 2014. p. 340-344.
17. Bibbo, M., et al., *Cytologic findings in female and male offspring of DES treated mothers*. Acta Cytol, 1975. **19**(6): p. 568-72.
18. Bibbo, M., et al., *Follow-up study of male and female offspring of DES-exposed mothers*. Obstet Gynecol, 1977. **49**(1): p. 1-8.
19. Yim, G., et al., *Grandmothers' endocrine disruption during pregnancy, low birth weight, and preterm birth in third generation*. International journal of epidemiology, 2022. **50**(6): p. 1886-1896.
20. Zamora-León, P. *Are the Effects of DES Over? A Tragic Lesson from the Past*. International Journal of Environmental Research and Public Health, 2021. **18**, DOI: 10.3390/ijerph181910309.
21. Administration, U.S.F.a.D. *FDA Drug Safety Communication: Children born to mothers who took Valproate products while pregnant may have impaired cognitive development*. 2018 [cited 2023; Safety Announcement]. Available from: <https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-children-born-mothers-who-took-valproate-products-while-pregnant-may>.

22. Gaily, E., et al., *Normal intelligence in children with prenatal exposure to carbamazepine*. Neurology, 2004. **62**(1): p. 28.
23. Meador, K.J., et al., *Cognitive Function at 3 Years of Age after Fetal Exposure to Antiepileptic Drugs*. New England Journal of Medicine, 2009. **360**(16): p. 1597-1605.
24. Adab, N., et al., *Additional educational needs in children born to mothers with epilepsy*. J Neurol Neurosurg Psychiatry, 2001. **70**(1): p. 15-21.
25. Adab, N., et al., *The longer term outcome of children born to mothers with epilepsy*. J Neurol Neurosurg Psychiatry, 2004. **75**(11): p. 1575-83.
26. Lancaster, P.A., *Causes of birth defects: lessons from history*. Congenit Anom (Kyoto), 2011. **51**(1): p. 2-5.
27. Lenz, W., *A short history of thalidomide embryopathy*. Teratology, 1988. **38**(3): p. 203-215.
28. Vargesson, N., *Thalidomide-induced teratogenesis: History and mechanisms*. Birth Defects Research Part C: Embryo Today: Reviews, 2015. **105**(2): p. 140-156.
29. Kazuki, Y., et al., *Thalidomide-induced limb abnormalities in a humanized CYP3A mouse model*. Scientific Reports, 2016. **6**(1): p. 21419.
30. Knobloch, J., D. Jungck, and A. Koch, *The Molecular Mechanisms of Thalidomide Teratogenicity and Implications for Modern Medicine*. Curr Mol Med, 2017. **17**(2): p. 108-117.
31. ECHA, E.c.a.-. *Understanding REACH*. [cited 2023 06-2023]; Available from: <https://echa.europa.eu/regulations/reach/understanding-reach>.
32. Younes, M. and N. Georgiadis, *Toxicological Risk Assessment*, in *Regulatory Toxicology*, F.-X. Reichl and M. Schwenk, Editors. 2020, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 1-12.
33. EFSA, E.f.s.a. *risk assessment*. Available from: <https://www.efsa.europa.eu/en/glossary/risk-assessment>.
34. Hopper, L.D. and F.W. Oehme, *Chemical risk assessment: a review*. Vet Hum Toxicol, 1989. **31**(6): p. 543-54.
35. ICH, I.C.f.H.-. *ICH S5 (R3) Guideline on detection of reproductive and developmental toxicity for human pharmaceuticals- Revision 4*. 2023.
36. OECD, *Test No. 414: Prenatal Developmental Toxicity Study*. 2018.
37. OECD, *Test No. 416: Two-Generation Reproduction Toxicity*. 2001.
38. OECD, *Test No. 421: Reproduction/Developmental Toxicity Screening Test*. 1995.
39. OECD, *Test No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*. 2016.
40. OECD, *Test No. 426: Developmental Neurotoxicity Study*. 2007.
41. OECD, *Test No. 443: Extended One-Generation Reproductive Toxicity Study*. 2018.
42. Hansen, J.M., *In vivo models of developmental toxicology*. Methods Mol Biol, 2012. **889**: p. 7-13.
43. Leenaars, C.H.C., et al., *Animal to human translation: a systematic scoping review of reported concordance rates*. J Transl Med, 2019. **17**(1): p. 223.
44. Brescia, S., et al., *Risk assessment in the 21st century: where are we heading?* Toxicology Research, 2023. **12**(1): p. 1-11.
45. purposes, N.N.C.f.t.p.o.a.u.f.s., *Transition to non-animal research*. 2016.
46. Schiffelers, M.-J.W.A., et al., *Regulatory acceptance and use of 3R models for pharmaceuticals and chemicals: Expert opinions on the state of affairs and the way forward*. Regulatory Toxicology and Pharmacology, 2014. **69**(1): p. 41-48.
47. Fentem, J., et al., *Upholding the EU's Commitment to 'Animal Testing as a Last Resort' Under REACH Requires a Paradigm Shift in How We Assess Chemical Safety to Close the Gap Between Regulatory Testing and Modern Safety Science*. Alternatives to Laboratory Animals, 2021. **49**(4): p. 122-132.

48. Doke, S.K. and S.C. Dhawale, *Alternatives to animal testing: A review*. Saudi Pharm J, 2015. **23**(3): p. 223-9.
49. Bal-Price, A., et al., *Strategies to improve the regulatory assessment of developmental neurotoxicity (DNT) using in vitro methods*. Toxicol Appl Pharmacol, 2018. **354**: p. 7-18.
50. Tamaki, C., et al., *Potentials and limitations of nonclinical safety assessment for predicting clinical adverse drug reactions: correlation analysis of 142 approved drugs in Japan*. J Toxicol Sci, 2013. **38**(4): p. 581-98.
51. COUNCIL, T.C.T.T.E.P.A.T., *2019 report on the statistics on the use of animals for scientific purposes in the Member States of the European Union in 2015-2017*. 2020.
52. Russell, W.M.S. and R.L. Burch, *The principles of humane experimental technique*. 1959: Methuen.
53. Pallocca, G., C. Rovida, and M. Leist, *On the usefulness of animals as a model system (part I): Overview of criteria and focus on robustness*. ALTEX, 2022. **39**(2): p. 347-353.
54. Paparella, M., et al., *Uncertainty of testing methods--what do we (want to) know?* ALTEX, 2013. **30**(2): p. 131-44.
55. *Ban on animal testing*, E. Comission, Editor.
56. Schechtman, L.M. and W.S. Stokes, *ECVAM-ICCVAM: Prospects for Future Collaboration*. Alternatives to Laboratory Animals, 2002. **30**(2_suppl): p. 227-236.
57. ECVAM, E. *Alternatives to animal testing and safety assessment of chemicals*. Available from: https://joint-research-centre.ec.europa.eu/scientific-activities-z/alternatives-animal-testing-and-safety-assessment-chemicals_en.
58. Bressers, S., et al., *Policy driven changes in animal research practices: mapping researchers' attitudes towards animal-free innovations using the Netherlands as an example*. Res Integr Peer Rev, 2019. **4**: p. 8.
59. Council, N.R., *Toxicity Testing in the 21st Century: A Vision and a Strategy*. 2007, Washington, DC: The National Academies Press. 216.
60. Krewski, D., et al., *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Journal of Toxicology and Environmental Health, Part B, 2010. **13**(2-4): p. 51-138.
61. Rajagopal, R., et al., *Beyond AOPs: A Mechanistic Evaluation of NAMs in DART Testing*. Front Toxicol, 2022. **4**: p. 838466.
62. Ankley, G.T., et al., *Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment*. Environ Toxicol Chem, 2010. **29**(3): p. 730-41.
63. Vinken, M., *The adverse outcome pathway concept: A pragmatic tool in toxicology*. Toxicology, 2013. **312**: p. 158-165.
64. Ball, N., et al., *A framework for chemical safety assessment incorporating new approach methodologies within REACH*. Arch Toxicol, 2022. **96**(3): p. 743-766.
65. Williams, T.D., L. Mirbahai, and J.K. Chipman, *The toxicological application of transcriptomics and epigenomics in zebrafish and other teleosts*. Briefings in Functional Genomics, 2014. **13**(2): p. 157-171.
66. OECD, *Users' Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways*. 2018.
67. Wittwehr, C., et al., *How Adverse Outcome Pathways Can Aid the Development and Use of Computational Prediction Models for Regulatory Toxicology*. Toxicological Sciences, 2017. **155**(2): p. 326-336.
68. AOPs, S.f.A.o. *AOP-Wiki*. 2023 29/04/2023 [cited 2023 may]; release 2.6:[Available from: <http://aopwiki.org>].
69. Pico, A.R., et al., *WikiPathways: pathway editing for the people*. PLoS Biol, 2008. **6**(7): p. e184.
70. OECD. *Adverse Outcome Pathways, Molecular Screening and Toxicogenomics*. 2023 [cited 2023 06-2023]; Available from: <https://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm>.

71. Tonk, E.C., J.L. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis*. *Reprod Toxicol*, 2015. **55**: p. 104-13.
72. Brannen, K.C., et al., *Alternative Models of Developmental and Reproductive Toxicity in Pharmaceutical Risk Assessment and the 3Rs*. *ILAR Journal*, 2016. **57**(2): p. 144-156.
73. Lee, H.Y., et al., *Alternative models in developmental toxicology*. *Syst Biol Reprod Med*, 2012. **58**(1): p. 10-22.
74. Chapman, K.L., et al., *Pharmaceutical toxicology: designing studies to reduce animal use, while maximizing human translation*. *Regul Toxicol Pharmacol*, 2013. **66**(1): p. 88-103.
75. Chapin, R., et al., *State of the art in developmental toxicity screening methods and a way forward: a meeting report addressing embryonic stem cells, whole embryo culture, and zebrafish*. *Birth Defects Res B Dev Reprod Toxicol*, 2008. **83**(4): p. 446-56.
76. Genschow, E., et al., *Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests*. *Altern Lab Anim*, 2004. **32**(3): p. 209-44.
77. Bauer, B., A. Mally, and D. Liedtke, *Zebrafish Embryos and Larvae as Alternative Animal Models for Toxicity Testing*. *Int J Mol Sci*, 2021. **22**(24).
78. Hermesen, S., *Implementation of transcriptomics for developmental toxicity assessment in the zebrafish embryo model*. 2013.
79. Grunwald, D.J. and J.S. Eisen, *Headwaters of the zebrafish — emergence of a new model vertebrate*. *Nature Reviews Genetics*, 2002. **3**(9): p. 717-724.
80. Streisinger, G., et al., *Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*)*. *Nature*, 1981. **291**(5813): p. 293-296.
81. Varga, M., *The Doctor of Delayed Publications: The Remarkable Life of George Streisinger (1927–1984)*. *Zebrafish*, 2018. **15**(3): p. 314-319.
82. Haffter, P., et al., *The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio**. *Development*, 1996. **123**: p. 1-36.
83. Felsenfeld, A.L., et al., *Mutations affecting skeletal muscle myofibril structure in the zebrafish*. *Development*, 1990. **108**(3): p. 443-59.
84. Grunwald, D.J., et al., *A neural degeneration mutation that spares primary neurons in the zebrafish*. *Dev Biol*, 1988. **126**(1): p. 115-28.
85. Hatta, K., et al., *The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system*. *Nature*, 1991. **350**(6316): p. 339-41.
86. Song, Y.-S., et al., *Validation, Optimization, and Application of the Zebrafish Developmental Toxicity Assay for Pharmaceuticals Under the ICH S5(R3) Guideline*. *Frontiers in cell and developmental biology*, 2021. **9**: p. 721130-721130.
87. Ali, S., et al., *Zebrafish embryos and larvae: A new generation of disease models and drug screens*. *Birth Defects Research Part C: Embryo Today: Reviews*, 2011. **93**(2): p. 115-133.
88. Ali, S., H.G. van Mil, and M.K. Richardson, *Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing*. *PLoS One*, 2011. **6**(6): p. e21076.
89. OECD, *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*. 2013.
90. Wlodkowic, D. and O. Campana, *Toward High-Throughput Fish Embryo Toxicity Tests in Aquatic Toxicology*. *Environmental Science & Technology*, 2021. **55**(6): p. 3505-3513.
91. Wixon, J., *Featured organism: *Danio rerio*, the zebrafish*. *Yeast*, 2000. **17**(3): p. 225-31.
92. Teraoka, H., W. Dong, and T. Hiraga, *Zebrafish as a novel experimental model for developmental toxicology*. *Congenit Anom (Kyoto)*, 2003. **43**(2): p. 123-32.
93. Weigt, S., et al., *Zebrafish (*Danio rerio*) embryos as a model for testing proteratogens*. *Toxicology*, 2011. **281**(1-3): p. 25-36.
94. *ERGO -EndoCRine Guiline Opimisation*. [cited 2023 06-2023]; Available from: <https://ergo-project.eu/>.

95. Kraft, M., et al., *Developmental exposure to triclosan and benzophenone-2 causes morphological alterations in zebrafish (Danio rerio) thyroid follicles and eyes*. Environ Sci Pollut Res Int, 2023. **30**(12): p. 33711-33724.
96. PARC: Partnership for the Assessment of Risks from Chemicals. Available from: <https://www.eu-parc.eu/>.
97. Staal, Y.C.M., et al., *Head skeleton malformations in zebrafish (Danio rerio) to assess adverse effects of mixtures of compounds*. Archives of Toxicology, 2018. **92**(12): p. 3549-3564.
98. Ali, S., et al., *Zebrafish embryos and larvae: a new generation of disease models and drug screens*. Birth Defects Res C Embryo Today, 2011. **93**(2): p. 115-33.
99. Collazo, A., *Developmental variation, homology, and the pharyngula stage*. Syst Biol, 2000. **49**(1): p. 3-18.
100. Irie, N. and S. Kuratani, *Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis*. Nat Commun, 2011. **2**: p. 248.
101. Howe, K., et al., *The zebrafish reference genome sequence and its relationship to the human genome*. Nature, 2013. **496**(7446): p. 498-503.
102. de Souza Anselmo, C., et al., *Zebrafish (Danio rerio): A valuable tool for predicting the metabolism of xenobiotics in humans?* Comp Biochem Physiol C Toxicol Pharmacol, 2018. **212**: p. 34-46.
103. Teixeira, E., et al., *Assessment of developmental delay in the zebrafish embryo teratogenicity assay*. Toxicol In Vitro, 2013. **27**(1): p. 469-78.
104. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance. 2010; Available from: <http://data.europa.eu/eli/dir/2010/63/oj>.
105. Nagel, R., *DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology*. ALTEX, 2002. **19 Suppl 1**: p. 38-48.
106. Brannen, K.C., et al., *Development of a zebrafish embryo teratogenicity assay and quantitative prediction model*. Birth Defects Res B Dev Reprod Toxicol, 2010. **89**(1): p. 66-77.
107. Kimmel, C.B., et al., *Stages of embryonic development of the zebrafish*. Dev Dyn, 1995. **203**(3): p. 253-310.
108. Teixeira, E., et al., *Comparative Assessment of the Sensitivity of Fish Early-Life Stage, Daphnia, and Algae Tests to the Chronic Ecotoxicity of Xenobiotics: Perspectives for Alternatives to Animal Testing*. Environ Toxicol Chem, 2020. **39**(1): p. 30-41.
109. Hermesen, S.A., et al., *Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies*. Toxicol In Vitro, 2011. **25**(3): p. 745-53.
110. Herrmann, K., *Teratogenic effects of retinoic acid and related substances on the early development of the zebrafish (Brachydanio rerio) as assessed by a novel scoring system*. Toxicol In Vitro, 1995. **9**(3): p. 267-83.
111. Panzica-Kelly, J.M., et al., *Morphological score assignment guidelines for the dechlorinated zebrafish teratogenicity assay*. Birth Defects Res B Dev Reprod Toxicol, 2010. **89**(5): p. 382-95.
112. Song, Y.S., et al., *Validation, Optimization, and Application of the Zebrafish Developmental Toxicity Assay for Pharmaceuticals Under the ICH S5(R3) Guideline*. Front Cell Dev Biol, 2021. **9**: p. 721130.
113. Jarque, S., et al., *Morphometric analysis of developing zebrafish embryos allows predicting teratogenicity modes of action in higher vertebrates*. Reprod Toxicol, 2020. **96**: p. 337-348.
114. Yamashita, A., et al., *Improvement of the evaluation method for teratogenicity using zebrafish embryos*. J Toxicol Sci, 2014. **39**(3): p. 453-64.

115. Lee, S.H., et al., *Teratogenic potential of antiepileptic drugs in the zebrafish model*. Biomed Res Int, 2013. **2013**: p. 726478.
116. Van den Bulck, K., et al., *Zebrafish developmental toxicity assay: A fishy solution to reproductive toxicity screening, or just a red herring?* Reprod Toxicol, 2011. **32**(2): p. 213-9.
117. Sipes, N.S., S. Padilla, and T.B. Knudsen, *Zebrafish: as an integrative model for twenty-first century toxicity testing*. Birth Defects Res C Embryo Today, 2011. **93**(3): p. 256-67.
118. Daston, G.P., *Gene expression, dose-response, and phenotypic anchoring: applications for toxicogenomics in risk assessment*. Toxicol Sci, 2008. **105**(2): p. 233-4.
119. Gradin, R., et al., *The GARDpotency Assay for Potency-Associated Subclassification of Chemical Skin Sensitizers-Rationale, Method Development, and Ring Trial Results of Predictive Performance and Reproducibility*. Toxicol Sci, 2020. **176**(2): p. 423-432.
120. Hermsen, S.A., et al., *Transcriptomic analysis in the developing zebrafish embryo after compound exposure: individual gene expression and pathway regulation*. Toxicol Appl Pharmacol, 2013. **272**(1): p. 161-71.
121. Shankar, P., et al., *Gene co-expression network analysis in zebrafish reveals chemical class specific modules*. BMC Genomics, 2021. **22**(1): p. 658.
122. Li, R., et al., *Systems Toxicology Approach for Testing Chemical Cardiotoxicity in Larval Zebrafish*. Chem Res Toxicol, 2020. **33**(10): p. 2550-2564.
123. Reinwald, H., et al., *Toxicogenomic profiling after sublethal exposure to nerve- and muscle-targeting insecticides reveals cardiac and neuronal developmental effects in zebrafish embryos*. Chemosphere, 2022. **291**(Pt 1): p. 132746.
124. Min, E.K., et al., *Advantages of omics technology for evaluating cadmium toxicity in zebrafish*. Toxicological Research, 2021. **37**(4): p. 395-403.
125. Lawson, N.D., et al., *An improved zebrafish transcriptome annotation for sensitive and comprehensive detection of cell type-specific genes*. Elife, 2020. **9**.
126. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nat Protoc, 2009. **4**(1): p. 44-57.
127. Huang, D.W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nature Protocols, 2009. **4**(1): p. 44-57.
128. Eden, E., et al., *GORilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists*. BMC Bioinformatics, 2009. **10**(1): p. 48.
129. Fabregat, A., et al., *Reactome pathway analysis: a high-performance in-memory approach*. BMC Bioinformatics, 2017. **18**(1): p. 142.
130. Kanehisa, M., et al., *KEGG for taxonomy-based analysis of pathways and genomes*. Nucleic Acids Res, 2023. **51**(D1): p. D587-D592.
131. Szklarczyk, D., et al., *STITCH 5: augmenting protein-chemical interaction networks with tissue and affinity data*. Nucleic Acids Res, 2016. **44**(D1): p. D380-4.
132. Kramer, A., et al., *Causal analysis approaches in Ingenuity Pathway Analysis*. Bioinformatics, 2014. **30**(4): p. 523-30.
133. Brockmeier, E.K., et al., *The Role of Omics in the Application of Adverse Outcome Pathways for Chemical Risk Assessment*. Toxicol Sci, 2017. **158**(2): p. 252-262.
134. Balmer, J.E. and R. Blomhoff, *A robust characterization of retinoic acid response elements based on a comparison of sites in three species*. J Steroid Biochem Mol Biol, 2005. **96**(5): p. 347-54.
135. Elgar, G. and T. Vavouri, *Tuning in to the signals: noncoding sequence conservation in vertebrate genomes*. Trends Genet, 2008. **24**(7): p. 344-52.
136. Postlethwait, J.H., et al., *Vertebrate genome evolution and the zebrafish gene map*. Nat Genet, 1998. **18**(4): p. 345-9.

137. Chan, E.T., et al., *Conservation of core gene expression in vertebrate tissues*. J Biol, 2009. **8**(3): p. 33.
138. Muhr, J., T.C. Arbor, and K.M. Ackerman, *Embryology, Gastrulation*, in StatPearls. 2023: Treasure Island (FL).
139. Pamies, D., et al., *Chapter 11 - Mechanism-based models in reproductive and developmental toxicology*, in *Reproductive and Developmental Toxicology*, R.C. Gupta, Editor. 2011, Academic Press: San Diego. p. 135-146.
140. Sonnen, K.F. and C.Y. Janda, *Signalling dynamics in embryonic development*. Biochemical Journal, 2021. **478**(23): p. 4045-4070.
141. Takebayashi-Suzuki, K. and A. Suzuki, *Intracellular Communication among Morphogen Signaling Pathways during Vertebrate Body Plan Formation*. Genes (Basel), 2020. **11**(3).
142. Christian, J.L., *Morphogen gradients in development: from form to function*. Wiley Interdiscip Rev Dev Biol, 2012. **1**(1): p. 3-15.
143. Balmer, J.E. and R. Blomhoff, *Gene expression regulation by retinoic acid*. J Lipid Res, 2002. **43**(11): p. 1773-808.
144. Bernheim, S. and S.M. Meilhac, *Mesoderm patterning by a dynamic gradient of retinoic acid signalling*. Philos Trans R Soc Lond B Biol Sci, 2020. **375**(1809): p. 20190556.
145. Blomhoff, R. and H.K. Blomhoff, *Overview of retinoid metabolism and function*. J Neurobiol, 2006. **66**(7): p. 606-30.
146. Campo-Paysaa, F., et al., *Retinoic acid signaling in development: tissue-specific functions and evolutionary origins*. Genesis, 2008. **46**(11): p. 640-56.
147. Casci, T., *Retinoic acid passes the morphogen test*. Nature Reviews Genetics, 2008. **9**(1): p. 7-7.
148. Duester, G., *Retinoic acid synthesis and signaling during early organogenesis*. Cell, 2008. **134**(6): p. 921-31.
149. Bollig, F., et al., *A highly conserved retinoic acid responsive element controls wt1a expression in the zebrafish pronephros*. Development, 2009. **136**(17): p. 2883-92.
150. Cunningham, T.J., et al., *Retinoic Acid Activity in Undifferentiated Neural Progenitors Is Sufficient to Fulfill Its Role in Restricting Fgf8 Expression for Somitogenesis*. PLoS One, 2015. **10**(9): p. e0137894.
151. Diez del Corral, R. and K.G. Storey, *Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis*. Bioessays, 2004. **26**(8): p. 857-69.
152. Kudoh, T., S.W. Wilson, and I.B. Dawid, *Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm*. Development, 2002. **129**(18): p. 4335-46.
153. Nolte, C., B. De Kumar, and R. Krumlauf, *Hox genes: Downstream "effectors" of retinoic acid signaling in vertebrate embryogenesis*. Genesis, 2019. **57**(7-8): p. e23306.
154. Yamada, K., et al., *An atlas of seven zebrafish hox cluster mutants provides insights into sub/neofunctionalization of vertebrate Hox clusters*. Development, 2021. **148**(11).
155. Afzal, Z. and R. Krumlauf, *Transcriptional Regulation and Implications for Controlling Hox Gene Expression*. J Dev Biol, 2022. **10**(1).
156. Thompson, B., et al., *Genetics and functions of the retinoic acid pathway, with special emphasis on the eye*. Hum Genomics, 2019. **13**(1): p. 61.
157. Semba, R.D., *On the 'discovery' of vitamin A*. Ann Nutr Metab, 2012. **61**(3): p. 192-8.
158. Maden, M., *Vitamin A and the developing embryo*. Postgrad Med J, 2001. **77**(910): p. 489-91.
159. Cunningham, T.J. and G. Duester, *Mechanisms of retinoic acid signalling and its roles in organ and limb development*. Nature Reviews Molecular Cell Biology, 2015. **16**(2): p. 110-123.
160. Ghyselinck, N.B. and G. Duester, *Retinoic acid signaling pathways*. Development, 2019. **146**(13).

161. Navarro-Martin, L., et al., *Dysregulatory effects of retinoic acid isomers in late zebrafish embryos*. Environ Sci Pollut Res Int, 2018. **25**(4): p. 3849-3859.
162. Deak, K.L., et al., *Analysis of ALDH1A2, CYP26A1, CYP26B1, CRABP1, and CRABP2 in human neural tube defects suggests a possible association with alleles in ALDH1A2*. Birth Defects Res A Clin Mol Teratol, 2005. **73**(11): p. 868-75.
163. Goldstone, J.V., et al., *Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish*. BMC Genomics, 2010. **11**: p. 643.
164. Pennimpede, T., et al., *The role of CYP26 enzymes in defining appropriate retinoic acid exposure during embryogenesis*. Birth Defects Res A Clin Mol Teratol, 2010. **88**(10): p. 883-94.
165. Thatcher, J.E. and N. Isoherranen, *The role of CYP26 enzymes in retinoic acid clearance*. Expert Opin Drug Metab Toxicol, 2009. **5**(8): p. 875-86.
166. Billings, S.E., et al., *The retinaldehyde reductase DHRS3 is essential for preventing the formation of excess retinoic acid during embryonic development*. FASEB J, 2013. **27**(12): p. 4877-89.
167. Chenevix-Trench, G., et al., *Cleft lip with or without cleft palate: associations with transforming growth factor alpha and retinoic acid receptor loci*. Am J Hum Genet, 1992. **51**(6): p. 1377-85.
168. Okano, J., S. Suzuki, and K. Shiota, *Involvement of apoptotic cell death and cell cycle perturbation in retinoic acid-induced cleft palate in mice*. Toxicol Appl Pharmacol, 2007. **221**(1): p. 42-56.
169. Hendrickx, A.G. and H. Hummler, *Teratogenicity of all-trans retinoic acid during early embryonic development in the cynomolgus monkey (Macaca fascicularis)*. Teratology, 1992. **45**(1): p. 65-74.
170. Bouman, H.G., et al., *Diminished growth of atrioventricular cushion tissue in stage 24 retinoic acid-treated chicken embryos*. Dev Dyn, 1998. **213**(1): p. 50-8.
171. Griffith, C.M. and M.J. Wiley, *Effects of retinoic acid on chick tail bud development*. Teratology, 1991. **43**(3): p. 217-24.
172. Robens, J.F., *Teratogenic effects of hypervitaminosis A in the hamster and the guinea pig*. Toxicol Appl Pharmacol, 1970. **16**(1): p. 88-99.
173. Abbott, B.D. and R.M. Pratt, *Human embryonic palatal epithelial differentiation is altered by retinoic acid and epidermal growth factor in organ culture*. J Craniofac Genet Dev Biol, 1987. **7**(3): p. 241-65.
174. Laue, K., et al., *Craniosynostosis and multiple skeletal anomalies in humans and zebrafish result from a defect in the localized degradation of retinoic acid*. Am J Hum Genet, 2011. **89**(5): p. 595-606.
175. Cohan, S.Q., *Excessive intake of vitamin A during pregnancy as a cause of congenital anomalies in the rat*. AMA Am J Dis Child, 1953. **86**(3): p. 348-9.
176. Abbott, B.D. and R.M. Pratt, *Retinoids and epidermal growth factor alter embryonic mouse palatal epithelial and mesenchymal cell differentiation in organ culture*. J Craniofac Genet Dev Biol, 1987. **7**(3): p. 219-40.
177. Sandell, L.L., et al., *RDH10 oxidation of Vitamin A is a critical control step in synthesis of retinoic acid during mouse embryogenesis*. PLoS One, 2012. **7**(2): p. e30698.
178. Piersma, A.H., E.V. Hessel, and Y.C. Staal, *Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker*. Reprod Toxicol, 2017. **72**: p. 53-61.
179. Samarut, E., et al., *ZebRA: An overview of retinoic acid signaling during zebrafish development*. Biochim Biophys Acta, 2015. **1849**(2): p. 73-83.
180. Schilling, T.F., *Anterior-posterior patterning and segmentation of the vertebrate head*. Integr Comp Biol, 2008. **48**(5): p. 658-67.
181. Deschamps, J. and J. van Nes, *Developmental regulation of the Hox genes during axial morphogenesis in the mouse*. Development, 2005. **132**(13): p. 2931-42.

182. Dorey, K. and E. Amaya, *FGF signalling: diverse roles during early vertebrate embryogenesis*. Development, 2010. **137**(22): p. 3731-42.
183. Zimmer, B., et al., *Coordinated waves of gene expression during neuronal differentiation of embryonic stem cells as basis for novel approaches to developmental neurotoxicity testing*. Cell Death Differ, 2011. **18**(3): p. 383-95.
184. Fan, C.Y., et al., *Gene expression changes in developing zebrafish as potential markers for rapid developmental neurotoxicity screening*. Neurotoxicol Teratol, 2010. **32**(1): p. 91-8.
185. Chen, H., M.A. Chidboy, and J.F. Robinson, *Retinoids and developmental neurotoxicity: Utilizing toxicogenomics to enhance adverse outcome pathways and testing strategies*. Reprod Toxicol, 2020. **96**: p. 102-113.
186. Barison, M.J., et al., *Reorganization of Metabolism during Cardiomyogenesis Implies Time-Specific Signaling Pathway Regulation*. Int J Mol Sci, 2021. **22**(3).
187. Fan, Z., et al., *Molecular mechanism of ethylparaben on zebrafish embryo cardiotoxicity based on transcriptome analyses*. Sci Total Environ, 2022. **842**: p. 156785.
188. Han, X., et al., *Precise Dose of Folic Acid Supplementation Is Essential for Embryonic Heart Development in Zebrafish*. Biology (Basel), 2021. **11**(1).
189. Keegan, B.R., et al., *Retinoic acid signaling restricts the cardiac progenitor pool*. Science, 2005. **307**(5707): p. 247-9.
190. Zile, M.H., et al., *Retinoid signaling is required to complete the vertebrate cardiac left/right asymmetry pathway*. Dev Biol, 2000. **223**(2): p. 323-38.
191. Stefanovic, S. and S. Zaffran, *Mechanisms of retinoic acid signaling during cardiogenesis*. Mech Dev, 2017. **143**: p. 9-19.
192. Lammer, E.J., et al., *Retinoic acid embryopathy*. N Engl J Med, 1985. **313**(14): p. 837-41.
193. Mark, M., N.B. Ghyselinck, and P. Chambon, *Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis*. Annu Rev Pharmacol Toxicol, 2006. **46**: p. 451-80.
194. Fisher, S. and M.E. Halpern, *Patterning the zebrafish axial skeleton requires early chordin function*. Nat Genet, 1999. **23**(4): p. 442-6.
195. Gillis, J.A., M.S. Modrell, and C.V. Baker, *Developmental evidence for serial homology of the vertebrate jaw and gill arch skeleton*. Nat Commun, 2013. **4**: p. 1436.
196. Levi, G., et al., *Msx1 and Dlx5 act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation*. Mech Dev, 2006. **123**(1): p. 3-16.
197. Lopez-Delgado, A.C., et al., *Axial skeleton anterior-posterior patterning is regulated through feedback regulation between Meis transcription factors and retinoic acid*. Development, 2021. **148**(1).
198. Pierro, J.D., et al., *Computational model for fetal skeletal defects potentially linked to disruption of retinoic acid signaling*. Front Pharmacol, 2022. **13**: p. 971296.
199. See, A.W., et al., *A nutritional model of late embryonic vitamin A deficiency produces defects in organogenesis at a high penetrance and reveals new roles for the vitamin in skeletal development*. Dev Biol, 2008. **316**(2): p. 171-90.
200. Shifley, E.T. and S.E. Cole, *The vertebrate segmentation clock and its role in skeletal birth defects*. Birth Defects Res C Embryo Today, 2007. **81**(2): p. 121-33.
201. Roberts, C., *Regulating Retinoic Acid Availability during Development and Regeneration: The Role of the CYP26 Enzymes*. J Dev Biol, 2020. **8**(1).
202. Spoorendonk, K.M., et al., *Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton*. Development, 2008. **135**(22): p. 3765-74.
203. Menegola, E., et al., *An adverse outcome pathway on the disruption of retinoic acid metabolism leading to developmental craniofacial defects*. Toxicology, 2021. **458**: p. 152843.

Chapter 2

Dynamic regulation of gene expression and morphogenesis in the zebrafish embryo test after exposure to all-trans retinoic acid

Laura M.M. Samrani^{1,2,4*}, Jeroen L.A. Pennings¹,
Nina Hallmark⁵, Rémi Bars³, Helen Tinwell³,
Marc Pallardy², Aldert H. Piersma^{1,4}

Reproductive Toxicology, 2023 Jan, 115; 8-16

DOI: 10.1016/j.reprotox.2022.11.001

1. Centre for Health Protection National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
2. Université Paris-Saclay, Inflammation, Microbiome and Immunosurveillance, INSERM, Faculté Pharmacie, Châtenay-Malabry 92296, France
3. Bayer SAS, Lyon, France
4. Institute for Risk Assessment Sciences (IRAS), Utrecht University, the Netherlands
5. Bayer AG Crop Science Division, Monheim, Germany

Abstract

The zebrafish embryotoxicity test (ZET) is widely used in developmental toxicology. The analysis of gene expression regulation in ZET after chemical exposure provides mechanistic information about the effects of chemicals on morphogenesis in the test. The gene expression response magnitude has been shown to change with exposure duration. The objective of this work is to study the effect of the exposure duration on the magnitude of gene expression changes in the all-trans retinoic acid (ATRA) signaling pathway in the ZET. Retinoic acid regulation is a key driver of morphogenesis and is therefore employed here as an indicator for the regulation of developmental genes. A teratogenic concentration of 7.5nM of ATRA was given at 3 hours post fertilization (hpf) for a range of exposure durations until 120 hrs of development. The expression of a selection of genes related to ATRA signaling and downstream developmental genes was determined. The highest magnitudes of gene expression regulation were observed after 2-24 hours exposure with an optimal response after 4hrs. Longer exposures showed a decrease in the gene expression response, although continued exposure to 120hpf caused malformations and lethality. This study shows that assessment of gene expression regulation at early time points after the onset of exposure in the ZET may be optimal for the prediction of developmental toxicity. We believe these results could help optimize sensitivity in future studies with ZET.

Keywords: All-trans-retinoic acid, Zebrafish embryo test, Transcriptomic, Retinoic acid pathway, Developmental toxicity, Exposure duration

Introduction

In the context of reproductive and developmental regulatory toxicology, the animal model is often still perceived as the “gold standard.” The safety assessment of chemicals is extrapolated from observations of adverse outcomes such as malformations in the offspring of exposed animals. Given the ethical and scientific concerns raised by this approach [1, 2], a mechanistic approach is preferable. The zebrafish embryo offers an alternative lower vertebrate model to study the mechanism of chemical toxicology [3-5]. Although development is obviously different from that in human [6], early embryogenesis in all vertebrates is highly conserved through the common pharyngula stage [7, 8]. Early morphogenesis effects of chemicals in the zebrafish embryo can therefore be considered relevant for human risk assessment. Conservation between vertebrate species is especially relevant at the level of gene expression, since approximately 70% of human genes have at least one obvious zebrafish orthologue [6-9]. Gene expression regulation offers important information about mechanisms of action, which may aid interspecies extrapolation. It has therefore been introduced as additional readout of the zebrafish embryo [10-13], in addition to classical morphological scoring [9, 14-23]. Gene expression regulation after chemical exposures changes with duration and timing of exposure and differs dependent on the time point of gene expression assessment in development [24]. In order to optimize the measurement of gene expression changes in zebrafish embryo, we have determined, for known dosing of a developmentally toxic compound, the duration of exposure in zebrafish embryo development at which gene expression change magnitudes were maximal. This study can help inform a standardized optimal study design for gene expression regulation analysis in zebrafish embryo.

We focused our gene expression regulation study on the all-trans retinoic acid (ATRA) signaling pathway. ATRA is a highly conserved key regulator of early vertebrate embryogenesis [25], and therefore, genes regulating ATRA homeostasis and downstream responsive genes may provide crucial biomarkers of development and maldevelopment. ATRA functions through concentration gradients in the embryo that are modified according to developmental progression [25-31]. This gradient regulates other major developmental pathways such as *wnt*, *bmp*, *fgf8*, *shh* [32-37]. Together they act in the regulation of complex morphogenetic processes such as axial patterning [28, 31, 38], craniofacial morphogenesis, limb development

[39, 40] and neural tube closure [25, 41, 42]. The concentration of ATRA is strictly maintained locally by a balance between synthesizing enzymes such as the *raldh* family and metabolizing enzymes of the *cyp26* family [43]. An imbalance in this gradient caused by an excess or deficit of ATRA has been shown to lead to teratogenicity in multiple species, including humans [44-46]. Alteration of ATRA regulation has been studied since the 1950s [47], and has been shown to lead to cranial malformations [48-55] (cleft palate, external ear defects, mandibular hypoplasia), neural tube closure defects [56] (spina bifida, anencephaly), limb defects, caudal regression [57], and cardiac defects [58] in human, mouse, rat, cynomolgus monkey, chicken, guinea pig, and zebrafish [22, 44, 46-51, 54, 56, 58-61].

In the zebrafish embryo test (ZET), zebrafish embryos are exposed to a chemical for up to 120 hours post-fertilization (hpf), followed by an examination of morphologic parameters. Gene expression responses occur already within hours after the onset of chemical exposure. In order to refine effect assessment, we have studied chemical-induced exposure duration dependent gene expression in a selected ATRA related gene set, in order to determine the time, point in which the magnitude of gene expression changes were highest. Fertilized zebrafish eggs were exposed to a fixed concentration of ATRA for a range of durations (2-117hrs). Our findings provide useful information for optimizing the protocol for embryo gene expression analysis in the ZET.

Materials and Methods

Zebrafish (*Danio rerio*) husbandry, embryo generation

Zebrafish were bred in house (RIVM laboratory) under permit NVWA-32600 and handled according to Dutch regulations. They originated from an AB strain obtained from Karlsruhe Institute of Technology (KIT, European Zebrafish Resource Center, Institute of toxicology and Genetics, Eggenstein-Leopoldshafen, Germany). Temperature, pH, conductivity conditions were monitored and maintained between $27.5 \pm 0.5^{\circ}\text{C}$, 7.5 ± 0.5 , and $500 \pm 100\mu\text{S}$, with a light/dark cycle of 14/10hrs, respectively. Fish were maintained in 8L Tecniplast ZebTech tanks (Buguggiate, Italy) in a flow-through system. They were fed 3 times a day with dry flakes (special Diet Service Granules, Tecnilab-BMI, Someren, the Netherlands). The breeding took place in breeding tanks (Tecniplast), in which 2 males and 2

females were placed after 4pm. Spawning is triggered by daylight, the next morning eggs were collected in petri dishes with Dutch Standard Water (DSW; demineralized water supplemented with 100mg/l NaHCO_3 , 20mg/l KHCO_3 , 200mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 180mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and then aerated for 24 h at 27°C), 30 minutes after spawning, the fertilized good quality eggs were selected by stereo microscopical observation.

Zebrafish exposure

After selection, the eggs were placed individually into wells of a 24 wells plate containing 2ml of test medium. The test medium contained DSW plus 0,1% dimethyl sulfoxide (DMSO, Cas# 67-68-5; Sigma-Aldrich Zwijndrecht, Netherlands), defined as Vehicle water, for the control and vehicle water plus different concentrations of All-trans retinoic acid (ATRA, Cas# 302-79-4; Sigma-Aldrich Zwijndrecht, Netherlands) for the treated fish. The plates were kept in an incubator at 27.5°C, with a 14/10hrs light/dark cycle.

Dose-response modelling

Control embryos were observed for 72hrs to confirm the quality of the batch during later steps of embryogenesis. They fulfilled the acceptance criteria of more than 90% showing normal development. To quantify the effect of ATRA, 40 embryos per concentration (divided into 4 independent experiments) were used. The effective dose range was determined by testing 0/1/3/10/20/30/80/100/300nM ATRA, and scoring using an integrative semi-quantitative scoring system (General Morphology Scoring: GMS,) after 24-, 48- and 72- hours post-fertilization (hpf) [46]). This semi-quantitative assessment of specific developmental endpoints supports standardization of the evaluation. Embryonic development was evaluated with 12 endpoints: detachment of the tail, somite formation, development and pigmentation of the eye, movements, heartbeat, blood circulation, head and body pigmentation, tail pigmentation, presence of pectoral fin, presence of a protruding mouth, and hatching. The teratological assessment was recorded based on the observation of pericardial edema, yolk sac edema, eye edema, malformation of the head, absence or malformation of the sacculi/otoliths, malformation of the tail, malformation of the heart, modified chorda structure, scoliosis, rickets, yolk deformation. The scored GMS data of individual fish were collected and used for concentration-response modelling.

The CED20 (critical effect dose resulting in a 20% reduction in GMS) was established using PROAST (version 65.5, [62]) package in RStudio (version 4.0.0). The CED20 was calculated based on the concentration that change the mean GMS by 20% and the CEDL-CEDU (critical effect dose lower bound – critical effect dose upper bound) represents the 90% confidence interval around the CED.

Exposure duration experiment

Starting at 3hpf, the fish were exposed to 7.5nM ATRA for different durations (2, 4, 6, 24, 48, 72, 117hrs) and then used for morphologic or transcriptomic analysis (Figure 1). Three independent experiments were performed. For the transcriptomic assessment, embryos were removed from the exposure medium and put in liquid nitrogen for the next step of RNA isolation. Three independent experiments were performed, with four samples per experiment and at least 12 fish per sample. For the morphologic assessment, embryos were removed from the exposure medium after the different exposure durations and put into the vehicle medium until scoring at 120hpf; 30 fish per exposure duration (divided into three independent experiments) were used. Control embryos fulfilled the acceptance criteria of more than 90% morphologically normal embryos at 120hrs.

Morphological analysis

Each experimental group contained 10 fish. Embryos were placed individually in wells of a 24 wells plate with 2ml of the test medium. At the end of each exposure duration, embryos were transferred to a fresh plate containing 2ml of vehicle water and left in the incubator until 120hpf. In all cases, morphological development was scored using the GMS system [63] at 120hpf.

Transcriptomic analysis

Eggs were separated into 14 groups per independent experiment (7 exposure groups treated with ATRA for different durations and 7 control groups -Figure 1: green dots-). 4 samples per condition were used, containing 35 eggs per sample for the shortest durations (2/4/6hrs) and 12 eggs per sample for the longer durations (from 24hrs to 117hrs) for each of the 3 independent experiments. The samples were placed in wells of 6 well plates with 5ml of test medium. After exposure, the embryos were carefully dried using tissue paper as much as possible and collected in 2ml low-binding DNA tubes. The tubes were then placed in liquid nitrogen and stored at -80°C before RNA isolation.

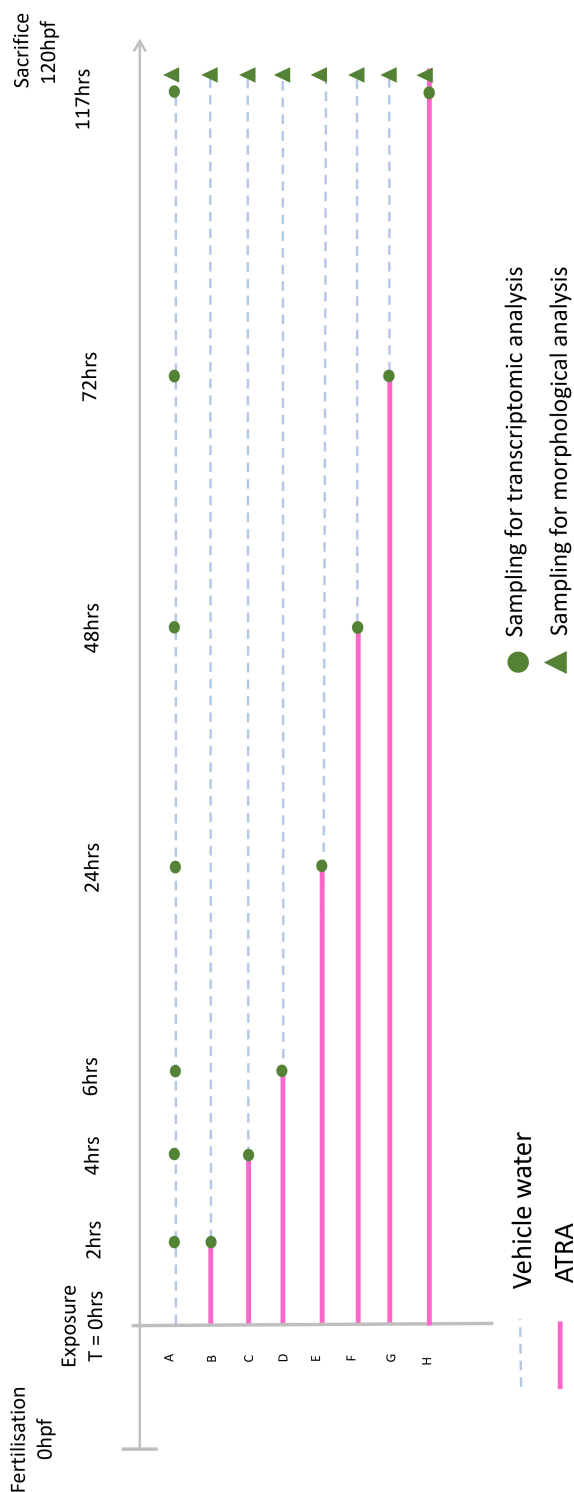


Figure 1 | ATRA exposure duration protocol of zebrafish embryos. 3 hours after fertilization, the zebrafish were exposed to 7.5nM ATRA during the indicated exposure. Morphological assessment was realized using 30 fish per time of exposure (divided into 3 independent experiments) scored at 120hpf. The transcriptomic assessment was performed using at least 12 fish per replicate, and 4 replicates per independent experiment, in total 3 independent experiments were carried out.

RNA isolation

RNA isolation was done using the RNeasy Mini kit (Qiagen, Cat. # 74104) following the manufacturer's protocol. The samples were homogenized with a prospectively autoclave blender in 700 μ l Quiazol Lysis Reagent (Qiagen, Cas# 79306), then 300 μ l of Quiazol was added. A DNase step was done using an RNase-Free DNase set (Qiagen, Cat # 79254). RNA quality and concentration of all samples were assessed using the Nanodrop (Nanodrop Technologies Inc., Wilmington, Delaware) and 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). In all cases, the 260nm/280nm absorbance ratios were between 1.9 and 2.2 and the RIN (RNA Integrity Number) scores were > 7 .

Gene expression analysis

RNA was converted to cDNA using the Fluidigm® Reverse Transcription Master Mix following the manufacturer's recommendations. A preamplification of 18 cycles was done, using the Fluidigm® PreAmp Master Mix and TaqMan® Assays (Thermo Fisher Scientific,). Gene expression was assessed with a 192.24 IFC of Fluidigm [64] in a BioMark (Fluidigm®, CA, USA). The housekeeping genes *actb1* and *hprt1* were used to normalize the average expression level of the genes listed in Supplementary Data Table 1. The median value of each condition was used as reference. The relative gene expression levels were assessed by measuring the difference of expression level between the mean of the treated for a specific duration against the mean of the control for the same duration. Gene expression data were further analyzed using R statistical software (version 4.0.0), to create a heatmap.

Gene interaction mapping

A gene interaction network of the genes studied in this investigation was obtained from the STITCH ('search tool for interactions of chemicals') web tool [65]. The inputs used were the gene symbols tested in the RT-qPCR test using the *Danio rerio* as an organism. This resulted in a network containing the input genes and additional genes that could be associated with the input set, using default settings for information sources and a moderate confidence level (0.4), representing the probability that a predicted link is present between two genes or proteins in the database. These data were used to analyze the degree of relationship between the genes and ATRA.

Statistical analysis

Dose response modelling

The Critical Effect Dose 20% (CED20), representing the concentration that reduces the mean GMS by 20%, and the CEDL-CEDU which represents the 90% confidence interval around the CED, was established using PROAST ([62]; version 65.5) in RStudio (version 4.0.0).

Morphological analysis

The statistical analysis of the morphologic data was realized with GraphPad prism 9 and R studio software. First, to verify the overall batch effect and duration variable as quality control, we used an ANOVA 2-way test (GMS: batch effect p-value=0.615, exposure duration p-value<2e-16, malformation score: batch effect p-value=0.00118, exposure duration p-value<2e-16). As the normality test did not pass, we applied a Kruskal-Wallis test for median (p-value<2.2e-16). For pairwise comparison, we applied the Wilcox test, the p-value is represented, in Figure 3, as ***p-value<0.001. An unpaired t-test was performed using the percentage of mortality per independent experimentations (3 were performed n=10 for each) as the independent variable. The samples were compared to the non-exposed control, the p-value is represented as *p-value<0.05.

Results

ATRA dose-response on morphological development

Zebrafish embryos were exposed at 3hpf to increasing concentrations of ATRA for 72hrs. We observed no mortality at 10nM and 89% mortality at 20nM (data not shown). At the non-lethal concentration of 3nM malformations and developmental delays were first observed, which became more severe with increased ATRA concentration (Figure 2B). The embryos exposed to 3nM exhibited scoliosis, a decrease in hatching rate and decreased blood circulation. At 10nM, heart malformations, pericardial edema as well as head and tail malformations were observed after exposure to ATRA. The embryos also displayed an absence of pectoral fin and protrusion of the mouth at this concentration. The embryos also displayed an absence of pectoral fin and protrusion of the mouth at this concentration.

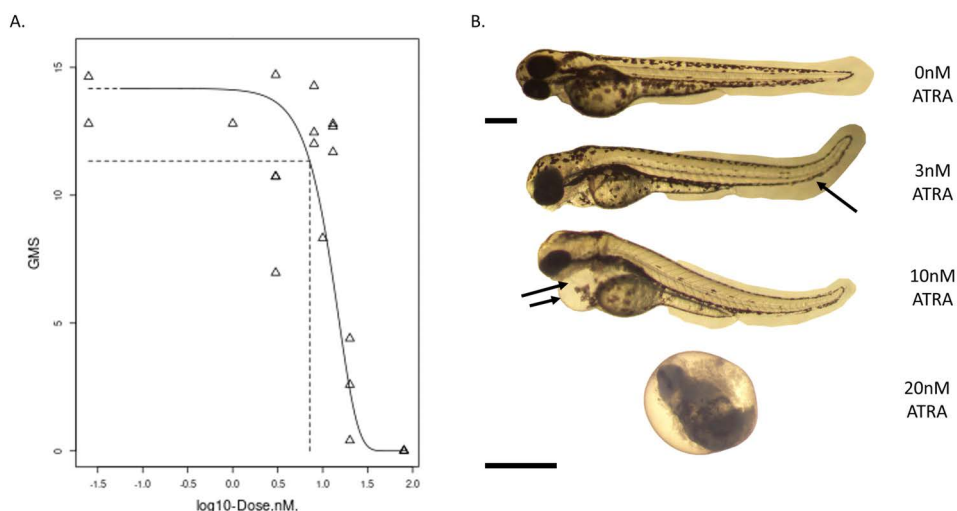


Figure 2 | Concentration-response of ATRA on zebrafish morphology. A. Dose-response curve of general morphology score scored at 72hpf after 3 days exposure to ATRA. Results were obtained from four independent experiments. Symbols represent average general morphology score per experiment per dose. The dotted line represents the CED20 point 7.16 (4.39-12.70) nM (CEDL-CEDU). B. Representative zebrafish embryos exposed to 0, 3, 10, and 20nM of ATRA. Malformations (highlighted by arrows) include scoliosis observed at 3nM and heart malformation and pericardial edema at 10nM. At 20nM hatching did not occur. Scale bar = 0.5mm

Using the GMS scoring system of Hermesen *et al.* [63] we determined a CED20 of 7.16 (4.39-12.70)nM after 72hrs of exposure (Figure 2A). Based on this observed CED20, we selected a non-lethal concentration of 7.5nM for further experiments.

Influence of ATRA exposure duration on morphogenesis and gene expression

Effect of ATRA exposure duration on morphological development

The zebrafish embryos were exposed to 7.5nM ATRA for different durations (from 2 to 117hrs) and the morphology of each embryo was evaluated at 120hpf. The embryos exposed for 2, 4, or 6hrs had a GMS comparable to the untreated controls (Figure 3A, Supplementary data Figure 1). Overall, the embryos did not display any morphological impact at 120hpf when exposed to ATRA for a duration of 6hrs or less.

After 24hpf of exposure the GMS average was 12 ($p<0.001$) and the teratogenicity score reached an average of 3 malformations per embryo ($p<0.001$) (Figure 3A). Longer exposure durations showed more severe effects. Affected embryos displayed similar developmental delays and malformations as observed previously when zebrafish embryos were exposed to ATRA from 3nM for 72hpf (Figure 2 and Figure 4B). A steadily increasing trend in mortality was observed from 24hpf exposure duration reaching statistical significance at 120hpf when 25% of the embryos were not viable (Figure 3B).

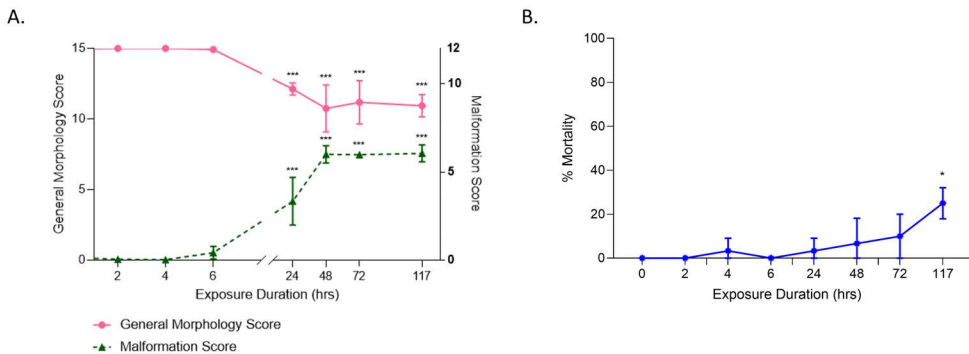


Figure 3 | Effect of ATRA exposure duration on general morphology score (GMS), malformations, and mortality. A. GMS (solid pink line) and malformation score (dashed green line) of embryos at 120hpf exposed at 3hpf for different durations to 7.5nM ATRA. Results from 3 independent experiments ($n=10$ embryos per experiment), analyzed using a pairwise Wilcoxon test, *** p -value <0.001 compared to control. B. Percentage mortality. Results were analyzed using a paired t -test, * p -value <0.05 compared to control.

As stated above, a significant increase in malformations and developmental delay was visible from 24 - 117hrs exposure duration as depicted in Figure

4A. Embryos showed a pattern of developmental delays and malformations consistent with a perturbation of the RA pathway (Figure 4). These include malformations of the head (95% of surviving embryos) and heart (86%), yolk sac and pericardium edemas (83% and 87% respectively), scoliosis (90%), yolk sac deformation (78%), absence of a protruding mouth (84%), absence of blood circulation (84%) and defect of the development of the eye (72%). 97% of embryos presented at least one of these malformations (Figure 4A, in green) and 71% presented all of them. In 82% of embryos at least one endpoint for developmental delay was affected (Figure 4A, in pink).

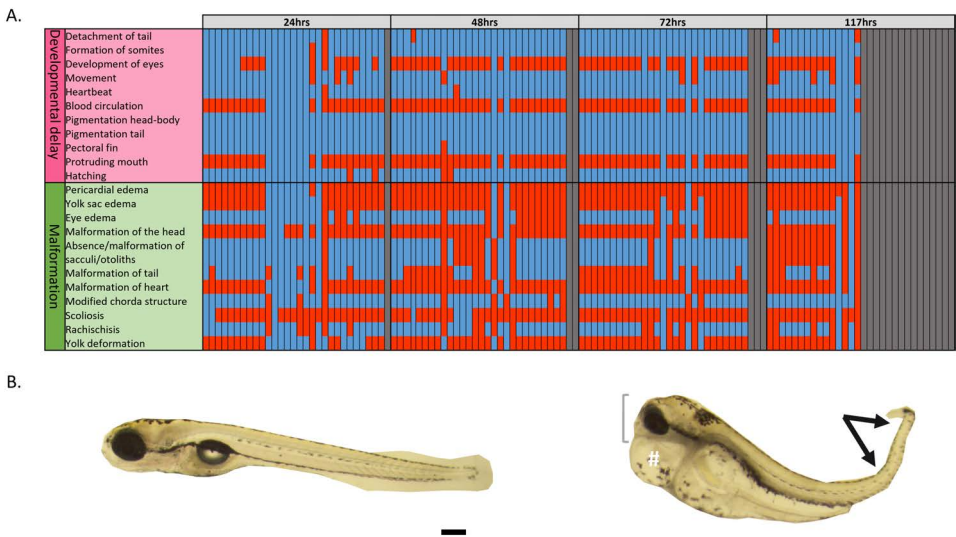


Figure 4 | Heatmap of morphological endpoints (A.) showing the impact of ATRA at different exposure durations in ZET at 120hpf. Each vertical line represents one embryo, each horizontal line represents one endpoint in the GMS. Blue cells indicate the absence of an effect, red cells indicate embryotoxicity, grey cells indicate dead embryos. B. Representative picture of zebrafish embryo control (left) and exposed to ATRA (7.5nM) until 120hpf (right). Grey bracket arrow shows a head malformation, black arrow shows scoliosis, # shows malformation of the heart and pericardial edema. Scale bar = 0.5mm

ATRA exposure duration response on transcriptomic changes

The genes (supplementary table 1) were chosen to cover actors in well-known developmental pathways known to be interacting with ATRA (such as *wnt*, *bmp*, *fgf*, *shh*). Additionally, some genes were involved in the regulation of ATRA concentrations (such as the *cyp26* family, *rdh10*, *aldh1a2*), and downstream developmental genes implicated in neural tube closure (such as the *hox* gene family, *zic1*, *gli1*). The 44 genes studied for the

transcriptomic readout were mapped in a network to show their relations by STITCH analysis (Figure 5). They were highly related within this network, visualized by the edges showing connections between the different nodes. We observed an overall dissociation between the genes responsible for the retinoic acid metabolism (colored in blue) and the other genes studied (in green and grey), however some important connections remain such as between *cyp26a* and *aldh1a2* on one side and *fgf8a* on the other side. In addition, *aldh1a2* relates to 3 developmental genes (*sox2*, *fgf8a*, *shha*) and *sox9b* relates to 5 ATRA metabolic genes (*cyp26a1*, *rarga*, *raraa*, *cyp26c1*, *cyp26b1*), suggesting a strong interplay between ATRA homeostasis and embryogenesis.

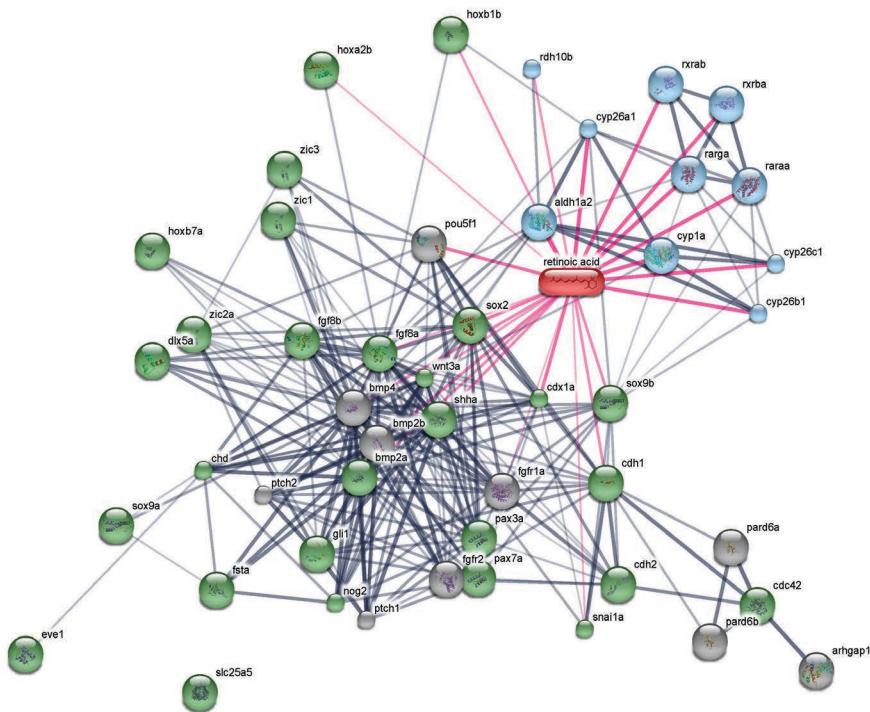


Figure 5 | Network showing the relations between ATRA-regulated and ATRA-metabolism genes (blue) and developmental genes (green) including additional genes proposed by STITCH (grey). First neighbor genes to ATRA are linked with a pink line. Node size highlights whether the 3D structure of the protein is known (big node) or not (small node). Line thickness represents the edge confidence from 0.150 (low) to 0.900 (highest).

The influence of exposure duration on the relative gene expression was studied. The most abundant gene expression changes were observed following a short exposure duration of up to 24hrs (Figure 6). The highest expression changes appeared after 4hrs of exposure (Figure 6) irrespective of whether genes were direct neighbors of ATRA (Figure 5) or had a more indirect relation. In particular, the most responsive genes included direct neighbors such as *hoxb1b*, *hoxa2b* and *cdx1a* with log2FC of 5.72, 2.50, -3.95, and indirect neighbors like *chrd*, *dlx5*, *eve1* with a log2FC of 3.53, 2.27, -2.43 respectively after 4hrs exposure. These genes all play a key role in neural tube closure, more precisely in the anterior/posterior pattern specification, dorsal/ventral pattern formation and the neural keel formation. The metabolism of ATRA is also highly regulated after 4hrs of exposure as especially observed in the expression change of *cyp26a* with log2FC of 2.92.

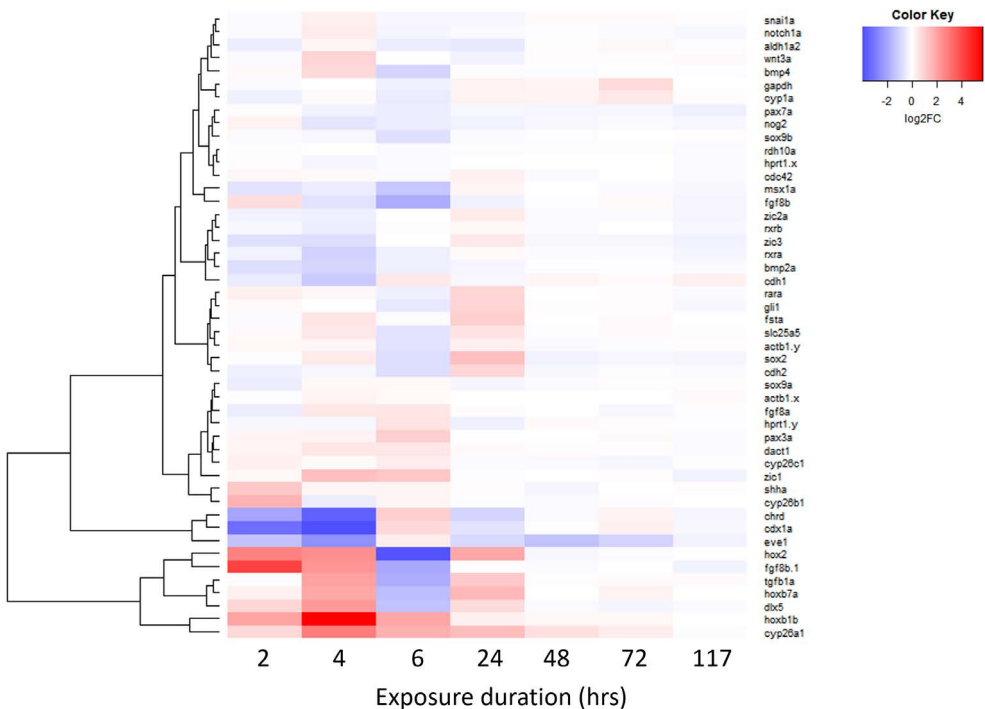


Figure 6 | Heatmap of relative gene expression in zebrafish embryos exposed to ATRA for different exposure durations. Levels are expressed as log2FC relative to the vehicle control group. Zebrafish were exposed starting 2.5/3 hours after fertilization to 0 or 7.5nM ATRA for 2, 4, 6, 24, 48, 72 or 117hrs and processed for analysis directly after exposure.

Discussion

In this study, we investigated the regulation of gene expression changes with time in ZET after exposure to the morphogenetic regulator ATRA. We observed exposure duration dependent changes in the magnitude of morphological and transcriptomic response parameters. The strongest signal in the regulation of gene expression appeared between 2-24hrs after the onset of exposure, while the morphological consequences were only visible after a longer exposure duration (24-117hrs) when gene expression changes were no longer apparent.

The exposure duration-dependent aspect in transcriptomic changes due to exposure to chemicals during zebrafish development is visible in several studies. For example, Kuhnert *et al.* show a difference in relative gene expression of biotransformation enzymes (e.g., *Cyp1a*) with an exposure duration from 2 to 26hpf [66]. Meyer-Alert [67] concludes on the relevance of the “period of exposure used for bioanalytical investigation” when studying the aryl-hydrocarbon-receptor pathway, but none of these studies confirms an optimal duration of exposure. To address this issue, we studied genes covering a large spectrum of main developmental pathways and secondary actors in the neural tube closure process (supplementary data table 1) allowing us to draw conclusions about an optimal exposure from this limited but representative gene selection. Thus, the strongest signal is between 2 and 24hrs. 4hrs exposure duration provided the optimal signals due to biological and practical reasons. The other duration timepoints lose some of their predictive power: at 24hrs some malformations are already visible (whilst the transcriptomic signal is not detected), at 6hrs even though the signal is strong, it shows different patterns than during the rest of the exposure complicating the interpretation, and at 2hrs synchronizing the zebrafish development is difficult leading to more variability in the signal. For these reasons the 4-8 hpf exposure window appears to be an effective exposure duration to explore the effect of ATRA in the ZET as regards the transcriptomic readout, compared to the morphological readout which required at least 24hrs. Whereas this illustrates the influence of the exposure duration on the transcriptomic readout, other exposure levels, compounds, and windows of exposure may result in different optima as to readout timing.

In this study, we first used the scoring system developed by Hermesen et al. [63] to determine the CED20 of ATRA, defined as inducing a 20% changes of GMS. The Hermesen morphology scoring method, well established in our laboratory, has been shown to be adequate to discriminate between chemicals within the same class [63]. An CED20 of 7.16nM for ATRA was established, which was comparable with the results of previous work in this laboratory [10]. This value is also comparable with published data from other laboratories [68-70].

Subsequently, the effects of exposure duration were investigated. The results indicated an exposure duration-dependent effect on the GMS score, the malformation score, and mortality. The signal strength increased with the increase in exposure duration (Figure 3). These results suggest that to discriminate the teratogenic potential of a chemical based on the morphological readout, exposure duration beyond 24hrs is more sensitive. To the best of our knowledge, the consequence of exposure duration on zebrafish development has not yet been investigated at this level of detail. However, the published literature does provide some insights into the role of exposure duration on the observed effects. Tu *et al.* [71] focused their study on behavior and calculated a malformation rate at 72, 96, and 120hpf, following exposure to cadmium, manganese, or lead. No statistical analysis comparing the different exposure durations was performed. Nevertheless, a duration-related increasing trend was observable for malformation rate and mortality. An increase in malformation rate and mortality between 72hrs exposure and 120hrs exposure was visible for each chemical. Exposure duration-dependent effects on mortality have also been seen in other studies. Chen *et al.* [72] highlighted an exposure duration-dependent effect on survival when zebrafish embryos were exposed to sodium benzoate at the same concentration. The survival rate was significantly and steadily decreased as the duration of exposure increased, from 93%, at 24hrs to 30% at 120hpf.

The transcriptomic analysis revealed a duration-dependent effect pattern, with gene expression signal strength being greater at the shorter exposure duration (Figure 6; 2hrs to 24hrs) irrespective of whether genes were direct neighbors of ATRA (Figure 5) or had a more indirect relationship.

Transcriptomic analysis represents a sensitive measure of molecular changes, as this signal has already been proven to be detectable at concentrations below those causing morphologically visible effects [10]. In this study, we

observed the same phenomenon concerning the duration of exposure to the tested molecule. We observed that the 6hrs timepoint showed a different pattern of effects as compared to adjacent time points with inversion of some gene expression (Figure 6). This may perhaps be explained by the fast-changing gene expression levels in early morphogenesis, with precise regional specification within the embryo. Consequently, ATRA can upregulate or downregulate selected genes depending on the stage of development, as measured in total fish samples.

In this study, a hypervitaminosis A is mimicked by the addition of ATRA in the water of the developing zebrafish embryos. This excess of ATRA leads to a perturbation of local concentration gradients, translating phenotypically to a pattern of developmental delays and malformations [61, 73]. These include scoliosis, heart defects, craniofacial defects (Figure 4), and have been related in literature to a dysregulation of patterning genes that are driven by the aforementioned concentration gradient [33-35, 37, 74-76]. Scoliosis malformations are known to be specifically sensitive to dysregulation of the anterior/ posterior pattern specification genes, such as, *hoxb7a* [77], *fgf8b* [37, 73] and *eve1* [78, 79]. Overexpression of even only one of these three genes leads to scoliosis in zebrafish and higher vertebrates. Mutation in *zic1* and *shh* is related to human syndromes (Dandy-Walker syndrome [80] and Klippel-Feil syndrome [81] respectively), which include scoliosis as associated anomalies. Craniofacial malformation in this study (more precisely absence of protrusion of the mouth) can be related to the observable dysregulation of the genes *dlx5* [82-86] and *chrd* [37, 74, 87-89], which is associated with skeletal malformation of the jaw in zebrafish [84, 86, 88], mice [82, 87] and human [83, 89]. A mutation of chordin is also believed to participate to the DiGeorge and VeloCardio-Facial syndromes, covering a spectrum of head and neck malformations and heart defects in humans [89]. The gene *eve1* contributes to the dorsal/ventral pattern formation. Its dysregulation in zebrafish leads to an anterior truncation and progressive loss of posterior patterning when overexpressed and suppresses the trunk and tail development when depleted. In some studies, this dysregulation translates into malformations such as pericardial edema, craniofacial malformation, and scoliosis [78, 79].

The zebrafish embryo is a promising model for testing embryotoxicity and providing insight into the toxicity mechanism of chemicals. One finding of our study is that four hours of exposure duration is optimal for observing

2

those changes related to ATRA signaling in the ZET, but additional knowledge is needed to precisely relate quantitative information about gene expression changes to morphological changes. Given the specific purpose of this study we did not take into consideration the study of a specific developmental endpoint that could necessitate a different exposure window, the study of different exposure starting points which could define the influence of the treatment on maternal versus zygotic mRNA, and the study of other signaling pathways. Moreover, a kinetic study to understand the distribution and half-life of ATRA in the embryo and chorion after the transfer into clean water will help define an ATRA-sensitive window for specific morphological endpoints. More teratogens and time points need to be tested to specify the time window for studying the gene expression changes in other signaling pathways.

In conclusion, this study offers further information to improve the mechanistic effect assessment in the zebrafish embryo model as a test system in developmental toxicology. These investigations show that a strong transcriptomic signal after 2-24hrs exposure to ATRA is observed in the absence of visible malformations or developmental delay at that stage of development. Beyond 24hrs the transcriptomic signal fades but when exposure is continued the morphological signal increases, seen as obvious malformations, developmental delays, and mortality. This research identified a relevant short duration timepoint (4hrs of exposure) to optimally measure early gene expression changes which could be used to predict the later appearance of developmental effects occurring after prolonged continued exposure. The metabolic and developmental pathways related to ATRA show clear gene expression changes that could be employed as early biomarkers for developmental toxicity, offering mechanistic information about chemical effects. Additional exposures with other chemicals should reveal whether these ATRA-related genes, or others known to be fundamental regulators in embryogenesis, can be used as a general biomarker to already detect teratogenicity [68] at time points before the onset of morphological malformations and provide insights into mechanistic information.

References

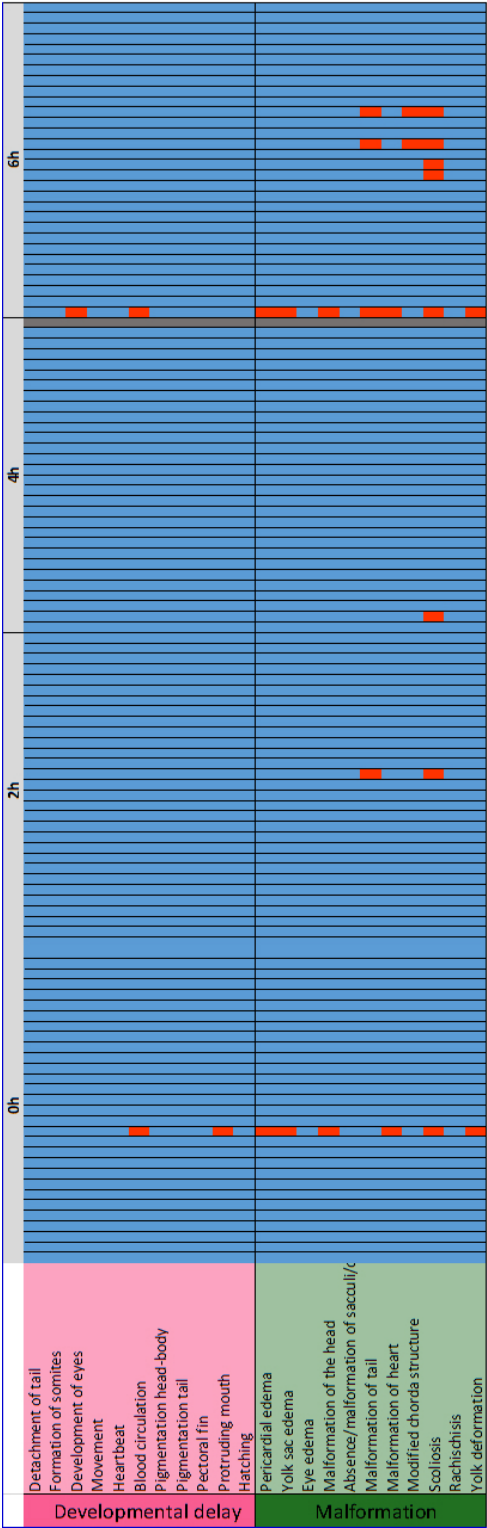
1. Hansen, J.M., *In vivo models of developmental toxicology*. Methods Mol Biol, 2012. **889**: p. 7-13.
2. Leenaars, C.H.C., et al., *Animal to human translation: a systematic scoping review of reported concordance rates*. J Transl Med, 2019. **17**(1): p. 223.
3. Wixon, J., *Featured organism: Danio rerio, the zebrafish*. Yeast, 2000. **17**(3): p. 225-31.
4. Teraoka, H., W. Dong, and T. Hiraga, *Zebrafish as a novel experimental model for developmental toxicology*. Congenit Anom (Kyoto), 2003. **43**(2): p. 123-32.
5. Weigt, S., et al., *Zebrafish (Danio rerio) embryos as a model for testing proteratogens*. Toxicology, 2011. **281**(1-3): p. 25-36.
6. Ali, S., et al., *Zebrafish embryos and larvae: a new generation of disease models and drug screens*. Birth Defects Res C Embryo Today, 2011. **93**(2): p. 115-33.
7. Collazo, A., *Developmental variation, homology, and the pharyngula stage*. Syst Biol, 2000. **49**(1): p. 3-18.
8. Irie, N. and S. Kuratani, *Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis*. Nat Commun, 2011. **2**: p. 248.
9. Ali, S., H.G. van Mil, and M.K. Richardson, *Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing*. PLoS One, 2011. **6**(6): p. e21076.
10. Hermesen, S.A., et al., *Transcriptomic analysis in the developing zebrafish embryo after compound exposure: individual gene expression and pathway regulation*. Toxicol Appl Pharmacol, 2013. **272**(1): p. 161-71.
11. Hermesen, S.A., et al., *Concentration-response analysis of differential gene expression in the zebrafish embryotoxicity test following flusilazole exposure*. Toxicol Sci, 2012. **127**(1): p. 303-12.
12. Schuttler, A., et al., *The Transcriptome of the Zebrafish Embryo After Chemical Exposure: A Meta-Analysis*. Toxicol Sci, 2017. **157**(2): p. 291-304.
13. Wu, C.C., et al., *The phenotypic and transcriptomic effects of developmental exposure to nanomolar levels of estrone and bisphenol A in zebrafish*. Sci Total Environ, 2021. **757**: p. 143736.
14. Nagel, R., *DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology*. ALTEX, 2002. **19 Suppl 1**: p. 38-48.
15. Busquet, F., et al., *Development of a new screening assay to identify proteratogenic substances using zebrafish danio rerio embryo combined with an exogenous mammalian metabolic activation system (mDarT)*. Toxicol Sci, 2008. **104**(1): p. 177-88.
16. Lessman, C.A., *The developing zebrafish (Danio rerio): a vertebrate model for high-throughput screening of chemical libraries*. Birth Defects Res C Embryo Today, 2011. **93**(3): p. 268-80.
17. Peterson, R.T., et al., *Small molecule developmental screens reveal the logic and timing of vertebrate development*. Proc Natl Acad Sci U S A, 2000. **97**(24): p. 12965-9.
18. Rennekamp, A.J. and R.T. Peterson, *15 years of zebrafish chemical screening*. Curr Opin Chem Biol, 2015. **24**: p. 58-70.
19. Sipes, N.S., S. Padilla, and T.B. Knudsen, *Zebrafish: as an integrative model for twenty-first century toxicity testing*. Birth Defects Res C Embryo Today, 2011. **93**(3): p. 256-67.
20. Nishimura, Y., et al., *Using zebrafish in systems toxicology for developmental toxicity testing*. Congenit Anom (Kyoto), 2016. **56**(1): p. 18-27.
21. Selderslaghs, I.W., et al., *Locomotor activity in zebrafish embryos: a new method to assess developmental neurotoxicity*. Neurotoxicol Teratol, 2010. **32**(4): p. 460-71.
22. Brannen, K.C., et al., *Development of a zebrafish embryo teratogenicity assay and quantitative prediction model*. Birth Defects Res B Dev Reprod Toxicol, 2010. **89**(1): p. 66-77.

23. Van den Bulck, K., et al., *Zebrafish developmental toxicity assay: A fishy solution to reproductive toxicity screening, or just a red herring?* *Reprod Toxicol*, 2011. **32**(2): p. 213-9.
24. Goldstone, J.V., et al., *Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish*. *BMC Genomics*, 2010. **11**: p. 643.
25. Piersma, A.H., E.V. Hessel, and Y.C. Staal, *Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker*. *Reprod Toxicol*, 2017. **72**: p. 53-61.
26. Bernheim, S. and S.M. Meilhac, *Mesoderm patterning by a dynamic gradient of retinoic acid signalling*. *Philos Trans R Soc Lond B Biol Sci*, 2020. **375**(1809): p. 20190556.
27. Shimoazono, S., et al., *Visualization of an endogenous retinoic acid gradient across embryonic development*. *Nature*, 2013. **496**(7445): p. 363-6.
28. Duester, G., *Retinoic acid synthesis and signaling during early organogenesis*. *Cell*, 2008. **134**(6): p. 921-31.
29. White, R.J., et al., *Complex regulation of cyp26a1 creates a robust retinoic acid gradient in the zebrafish embryo*. *PLoS Biol*, 2007. **5**(11): p. e304.
30. Schilling, T.F., Q. Nie, and A.D. Lander, *Dynamics and precision in retinoic acid morphogen gradients*. *Curr Opin Genet Dev*, 2012. **22**(6): p. 562-9.
31. Samarut, E., et al., *ZebRA: An overview of retinoic acid signaling during zebrafish development*. *Biochim Biophys Acta*, 2015. **1849**(2): p. 73-83.
32. Durston, A.J., *Time, space and the vertebrate body axis*. *Semin Cell Dev Biol*, 2015. **42**: p. 66-77.
33. Kudoh, T., S.W. Wilson, and I.B. Dawid, *Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm*. *Development*, 2002. **129**(18): p. 4335-46.
34. Onai, T., et al., *Retinoic acid and Wnt/beta-catenin have complementary roles in anterior/posterior patterning embryos of the basal chordate amphioxus*. *Dev Biol*, 2009. **332**(2): p. 223-33.
35. Reynolds, K., et al., *Wnt signaling in orofacial clefts: crosstalk, pathogenesis and models*. *Dis Model Mech*, 2019. **12**(2).
36. Schilling, T.F., J. Sosnik, and Q. Nie, *Visualizing retinoic acid morphogen gradients*. *Methods Cell Biol*, 2016. **133**: p. 139-63.
37. Furthauer, M., et al., *Fgf signalling controls the dorsoventral patterning of the zebrafish embryo*. *Development*, 2004. **131**(12): p. 2853-64.
38. Sive, H.L., et al., *Identification of a retinoic acid-sensitive period during primary axis formation in Xenopus laevis*. *Genes Dev*, 1990. **4**(6): p. 932-42.
39. Conlon, R.A., *Retinoic acid and pattern formation in vertebrates*. *Trends Genet*, 1995. **11**(8): p. 314-9.
40. Casci, T., *Retinoic acid passes the morphogen test*. *Nature Reviews Genetics*, 2008. **9**(1): p. 7-7.
41. Heusinkveld, H.J., et al., *An ontology for developmental processes and toxicities of neural tube closure*. *Reprod Toxicol*, 2021. **99**: p. 160-167.
42. Maden, M., *Vitamin A and the developing embryo*. *Postgrad Med J*, 2001. **77**(910): p. 489-91.
43. Reijntjes, S., et al., *The control of morphogen signalling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo*. *Dev Biol*, 2005. **285**(1): p. 224-37.
44. Sandell, L.L., et al., *RDH10 oxidation of Vitamin A is a critical control step in synthesis of retinoic acid during mouse embryogenesis*. *PLoS One*, 2012. **7**(2): p. e30698.
45. Rhinn, M. and P. Dolle, *Retinoic acid signalling during development*. *Development*, 2012. **139**(5): p. 843-58.
46. Laue, K., et al., *Craniosynostosis and multiple skeletal anomalies in humans and zebrafish result from a defect in the localized degradation of retinoic acid*. *Am J Hum Genet*, 2011. **89**(5): p. 595-606.
47. Cohlan, S.Q., *Excessive intake of vitamin A during pregnancy as a cause of congenital anomalies in the rat*. *AMA Am J Dis Child*, 1953. **86**(3): p. 348-9.

48. Hendrickx, A.G. and H. Hummler, *Teratogenicity of all-trans retinoic acid during early embryonic development in the cynomolgus monkey (Macaca fascicularis)*. *Teratology*, 1992. **45**(1): p. 65-74.
49. Abbott, B.D. and R.M. Pratt, *Human embryonic palatal epithelial differentiation is altered by retinoic acid and epidermal growth factor in organ culture*. *J Craniofac Genet Dev Biol*, 1987. **7**(3): p. 241-65.
50. Abbott, B.D. and R.M. Pratt, *Retinoids and epidermal growth factor alter embryonic mouse palatal epithelial and mesenchymal cell differentiation in organ culture*. *J Craniofac Genet Dev Biol*, 1987. **7**(3): p. 219-40.
51. Chenevix-Trench, G., et al., *Cleft lip with or without cleft palate: associations with transforming growth factor alpha and retinoic acid receptor loci*. *Am J Hum Genet*, 1992. **51**(6): p. 1377-85.
52. Geelen, J.A., *Hypervitaminosis A induced teratogenesis*. *CRC Crit Rev Toxicol*, 1979. **6**(4): p. 351-75.
53. Kochhar, D.M., J.D. Penner, and C.I. Tellone, *Comparative teratogenic activities of two retinoids: effects on palate and limb development*. *Teratog Carcinog Mutagen*, 1984. **4**(4): p. 377-87.
54. Okano, J., S. Suzuki, and K. Shiota, *Involvement of apoptotic cell death and cell cycle perturbation in retinoic acid-induced cleft palate in mice*. *Toxicol Appl Pharmacol*, 2007. **221**(1): p. 42-56.
55. Shaw, D., et al., *Further evidence of a relationship between the retinoic acid receptor alpha locus and nonsyndromic cleft lip with or without cleft palate (CL +/- P)*. *Am J Hum Genet*, 1993. **53**(5): p. 1156-7.
56. Robens, J.F., *Teratogenic effects of hypervitaminosis A in the hamster and the guinea pig*. *Toxicol Appl Pharmacol*, 1970. **16**(1): p. 88-99.
57. Jelinek, R. and A. Kistler, *Effect of retinoic acid upon the chick embryonic morphogenetic systems. I. The embryotoxicity dose range*. *Teratology*, 1981. **23**(2): p. 191-5.
58. Bouman, H.G., et al., *Diminished growth of atrioventricular cushion tissue in stage 24 retinoic acid-treated chicken embryos*. *Dev Dyn*, 1998. **213**(1): p. 50-8.
59. Billings, S.E., et al., *The retinaldehyde reductase DHRS3 is essential for preventing the formation of excess retinoic acid during embryonic development*. *FASEB J*, 2013. **27**(12): p. 4877-89.
60. Pennimpede, T., et al., *The role of CYP26 enzymes in defining appropriate retinoic acid exposure during embryogenesis*. *Birth Defects Res A Clin Mol Teratol*, 2010. **88**(10): p. 883-94.
61. Griffith, C.M. and M.J. Wiley, *Effects of retinoic acid on chick tail bud development*. *Teratology*, 1991. **43**(3): p. 217-24.
62. Slob, W., *Dose-response modeling of continuous endpoints*. *Toxicol Sci*, 2002. **66**(2): p. 298-312.
63. Hermesen, S.A., et al., *Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies*. *Toxicol In Vitro*, 2011. **25**(3): p. 745-53.
64. Pieprzyk, M. and H. High, *Fluidigm Dynamic Arrays provide a platform for single-cell gene expression analysis*. *Nature Methods*, 2009. **6**(7): p. iii-iv.
65. Szklarczyk, D., et al., *STITCH 5: augmenting protein-chemical interaction networks with tissue and affinity data*. *Nucleic Acids Res*, 2016. **44**(D1): p. D380-4.
66. Kuhnert, A., et al., *Biotransformation in the zebrafish embryo -temporal gene transcription changes of cytochrome P450 enzymes and internal exposure dynamics of the AhR binding xenobiotic benz[a]anthracene*. *Environ Pollut*, 2017. **230**: p. 1-11.
67. Meyer-Alert, H., et al., *A temporal high-resolution investigation of the Ah-receptor pathway during early development of zebrafish (Danio rerio)*. *Aquat Toxicol*, 2018. **204**: p. 117-129.

68. Wang, Y., et al., *Characterization of retinoic acid-induced neurobehavioral effects in developing zebrafish*. Environ Toxicol Chem, 2014. **33**(2): p. 431-7.
69. Jarque, S., et al., *Morphometric analysis of developing zebrafish embryos allows predicting teratogenicity modes of action in higher vertebrates*. Reprod Toxicol, 2020. **96**: p. 337-348.
70. Teixeira, E., et al., *Assessment of developmental delay in the zebrafish embryo teratogenicity assay*. Toxicol In Vitro, 2013. **27**(1): p. 469-78.
71. Tu, H., et al., *Effects of cadmium, manganese, and lead on locomotor activity and neurexin 2a expression in zebrafish*. Environ Toxicol Chem, 2017. **36**(8): p. 2147-2154.
72. Chen, Q., et al., *Sodium benzoate exposure downregulates the expression of tyrosine hydroxylase and dopamine transporter in dopaminergic neurons in developing zebrafish*. Birth Defects Res B Dev Reprod Toxicol, 2009. **86**(2): p. 85-91.
73. Duester, G., *Retinoic acid regulation of the somitogenesis clock*. Birth Defects Res C Embryo Today, 2007. **81**(2): p. 84-92.
74. Stottmann, R.W., R.M. Anderson, and J. Klingensmith, *The BMP antagonists Chordin and Noggin have essential but redundant roles in mouse mandibular outgrowth*. Dev Biol, 2001. **240**(2): p. 457-73.
75. Hashiguchi, M. and M.C. Mullins, *Anteroposterior and dorsoventral patterning are coordinated by an identical patterning clock*. Development, 2013. **140**(9): p. 1970-80.
76. Schilling, T.F., *Anterior-posterior patterning and segmentation of the vertebrate head*. Integr Comp Biol, 2008. **48**(5): p. 658-67.
77. Rochtus, A., et al., *DNA methylation analysis of Homeobox genes implicates HOXB7 hypomethylation as risk factor for neural tube defects*. Epigenetics, 2015. **10**(1): p. 92-101.
78. Cruz, C., et al., *Induction and patterning of trunk and tail neural ectoderm by the homeobox gene eve1 in zebrafish embryos*. Proc Natl Acad Sci U S A, 2010. **107**(8): p. 3564-9.
79. Seebald, J.L. and D.P. Szeto, *Zebrafish eve1 regulates the lateral and ventral fates of mesodermal progenitor cells at the onset of gastrulation*. Dev Biol, 2011. **349**(1): p. 78-89.
80. Vandervore, L.V., et al., *Mutated zinc finger protein of the cerebellum 1 leads to microcephaly, cortical malformation, callosal agenesis, cerebellar dysplasia, tethered cord and scoliosis*. Eur J Med Genet, 2018. **61**(12): p. 783-789.
81. Umamaheshwar, K.L., et al., *Two case reports of an unusual association between Klippel-Feil syndrome and amyotrophic lateral sclerosis: Do they share same genetic defect?* Ann Indian Acad Neurol, 2013. **16**(4): p. 705-7.
82. Acampora, D., et al., *Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5*. Development, 1999. **126**(17): p. 3795-809.
83. Brown, K.K., et al., *Deletion of an enhancer near DLX5 and DLX6 in a family with hearing loss, craniofacial defects, and an inv(7)(q21.3q35)*. Hum Genet, 2010. **127**(1): p. 19-31.
84. Levi, G., et al., *Msx1 and Dlx5 act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation*. Mech Dev, 2006. **123**(1): p. 3-16.
85. Robledo, R.F., et al., *The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development*. Genes Dev, 2002. **16**(9): p. 1089-101.
86. Talbot, J.C., S.L. Johnson, and C.B. Kimmel, *hand2 and Dlx genes specify dorsal, intermediate and ventral domains within zebrafish pharyngeal arches*. Development, 2010. **137**(15): p. 2507-17.
87. Choi, M. and J. Klingensmith, *Chordin is a modifier of tbx1 for the craniofacial malformations of 22q11 deletion syndrome phenotypes in mouse*. PLoS Genet, 2009. **5**(2): p. e1000395.
88. Fisher, S. and M.E. Halpern, *Patterning the zebrafish axial skeleton requires early chordin function*. Nat Genet, 1999. **23**(4): p. 442-6.
89. Bachiller, D., et al., *The role of chordin/Bmp signals in mammalian pharyngeal development and DiGeorge syndrome*. Development, 2003. **130**(15): p. 3567-78.

Supplementary data



Supplementary data Figure 1 | Heat map of morphological endpoints showing impact of the duration exposure to ATRA (for 0 to 6hrs) in zebrafish embryo assay at 120 hours post fertilization. Blue cells indicate the absence of an effect, red cells indicate embryotoxicity, grey cells indicate dead embryos.

Supplementary data Table 1 | Gene selection list and Primers list used for gene expression analysis and quantification by qPCR.

	Abbreviation	Assay ID/primer sequence
Retinoic acid metabolism		
cytochrome P450, subfamily XXVIA, polypeptide 1	cyp26a1	Dr03086662_m1
cytochrome P450, family 26, subfamily b, polypeptide 1	cyp26b1	Dr03088547_m1
cytochrome P450, family 26, subfamily c, polypeptide 1	cyp26c2	Dr03072287_m1
aldehyde dehydrogenase 1 family, member A2	aldh1a2	Dr03131682_m1
retinol dehydrogenase 10a	rdh10a	Dr03428954_m1
retinoid x receptor, beta a	rxra	Dr03131425_m1
retinoic acid receptor gamma a	rarg	Dr03176411_s1
retinoic acid receptor, alpha a	rara	Dr03079987_m1
retinoid x receptor, beta b	rxrb	Dr03150236_m1
cytochrome P450, family 1, subfamily A	cyp1a	Dr03112444_m1
homeobox B1b	Hoxb1b	Dr03432669_g1
Major developmental pathways		
wingless-type MMTV integration site family, member 3A	wnt3a	Dr03134760_m1
bone morphogenetic protein 2a	bmp2a	Dr03105736_m1
sonic hedgehog a	shha	Dr03432632_m1
fibroblast growth factor 8a	fgf8a	Dr03105657_m1
fibroblast growth factor 8 b	fgf8b	Dr03433601_m1
notch homolog 1a	notch1a	Dr03112151_m1
SRY-box containing gene 9a	sox9a	Dr03112282_m1
SRY-box containing gene 9b	sox9b	Dr03080049_m1
transforming growth factor, beta 1a	tgfb1a	Dr03087345_m1
Neural tube closure		
Snail Family Transcriptional Repressor 1	snai1a	Dr03112012_m1
paired box gene 3a	pax3a	Dr03144248_m1
paired box gene 7a	pax7a	Dr03125022_m1
zic family member 1	zic1	Dr03073932_m1
distal-less homeobox gene 5a	dlx5a	Dr03150313_m1
homeobox A2b	hoxa2b	Dr03112035_m1
noggin 2	nog2	Dr03200727_s1
B-catenin	dact1	Dr03152516_m1
chordin	chrd	Dr03124897_m1
zic family member 3 heterotaxy 1	zic 3	Dr03425716_m1
zic family member 2 a	zic2a	Dr03086973_g1
GLI family zinc finger 1	Gli1	Dr03093666_m1

	Abbreviation	Assay ID/primer sequence
SRY (sex determining region Y)-box 2	sox2	Dr03203853_s1
cell division cycle 42	cdc42	Dr03139234_m1
N-cadherin	cdh2	Dr03150153_m1
E-cadherin	cdh1	Dr03433209_m1
follistatin	fsta	Dr03138160_m1
caudal type homeobox 1a	cdx2	Dr03203649_s1
muscle segment homeobox 1a	msx1a	Dr03986683_uH
solute carrier family 25-member 5	slc25a5	Dr03433439_Mh
even-skipped-like1	eve1	Dr03112037_m1
homeobox B7a	hoxb7a	Dr03436736_m1
Housekeeping gene		
hypoxanthine phosphoribosyl transferase 1	hprt1	Dr03095135_m1
actin, beta 1	actb1	Dr03432610_m1
glyceraldehyde-3-phosphate dehydrogenase	gapdh	Dr03436842_m1

Chapter 3

Nervous system development related gene expression regulation in the zebrafish embryo after exposure to valproic acid and retinoic acid: a genome wide approach

Laura M.M. Samrani^{1,2,3}, Florent Dumont⁴,
Nina Hallmark⁵, Rémi Bars⁶, Helen Tinwell⁶,
Marc Pallardy², Aldert H. Piersma^{1,3}

Toxicology Letters, 2023 Aug, 384; 96-104

DOI: 10.1016/j.toxlet.2023.07.005

1. Centre for Health Protection National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
2. Université Paris-Saclay, Inflammation, Microbiome and Immunosurveillance, INSERM, Faculté Pharmacie, 91104 Orsay, France
3. Institute for Risk Assessment Sciences (IRAS), Utrecht University, the Netherlands
4. Université Paris-Saclay, UMS IPSIT, 91104 Orsay, France
5. Bayer AG Crop Science Division, Monheim, Germany
6. Bayer SAS, Lyon, France

Abstract

The evaluation of chemical and pharmaceutical safety for humans is moving from animal studies to New Approach Methodologies (NAM), reducing animal use and focusing on mechanism of action, whilst enhancing human relevance. In developmental toxicology, the mechanistic approach is facilitated by the assessment of predictive biomarkers, which allow mechanistic pathways perturbation monitoring at the basis of human hazard assessment. In our search for biomarkers of maldevelopment, we focused on chemically-induced perturbation of the retinoic acid signaling pathway (RA-SP), a major pathway implicated in a plethora of developmental processes. A genome-wide expression screening was performed on zebrafish embryos treated with two teratogens, all-trans retinoic acid (ATRA) and valproic acid (VPA), and a non-teratogen reference compound, folic acid (FA). Each compound was found to have a specific mRNA expression profile with 248 genes commonly dysregulated by both teratogenic compounds but not by FA. These genes were implicated in several developmental processes (e.g., the circulatory and nervous system). Given the prominent response of neurodevelopmental gene sets, and the crucial need to better understand developmental neurotoxicity, our study then focused on nervous system development. We found 62 genes that are potential early neurodevelopmental toxicity biomarker candidates. These results advance NAM-based safety assessment evaluation by highlighting the usefulness of the RA-SP in providing early toxicity biomarker candidates.

Keywords: Toxicogenomics, neurodevelopmental toxicity, retinoic acid signaling pathway, valproic acid, teratogen, maldevelopment, zebrafish embryo, all-trans retinoic acid, folic acid

Introduction

Developmental toxicity assessment of chemicals and pharmaceuticals for regulatory risk assessment and market approval is traditionally performed in mammalian species. This meets with scientific challenges, as the extrapolation of *in vivo* findings in animal models to the situation in humans is not straightforward [1, 2]. In addition, the extensive use of animals meets with ethical concerns [3]. Considering these limitations, alternative strategies are being developed, focusing on mechanistic understanding of toxicity, often described in adverse outcome pathways (AOPs) [2]. Key events in AOPs may be monitored through biomarkers, using *in vitro* assays [2, 4, 5]. This approach is ethically preferable and may be scientifically more valuable as it would facilitate extrapolation to the human situation while limiting animal use [2].

The current study focuses on identifying predictive embryotoxicity biomarker candidates, using the zebrafish embryo (ZE) as a non-mammalian vertebrate model. As it is a complete vertebrate organism, the ZE offers a broader range of mechanisms to study, compared to reductionist *in vitro* cell and tissue culture models. The ZE model is relevant for early human embryo development because early mechanisms of embryogenesis are highly conserved in all vertebrates [6-9]. It has been used to study a wide range of basic mechanisms of developmental toxicity relevant to human risk assessment, such as perturbation of body axis patterning, organ development (e.g., heart), and developmental neurotoxicity [10-13]. When compared to mammalian models, in addition to some general practical advantages (e.g., large sample sizes, external development, lower cost) [14, 15], the ZE also has significant biological relevance as a model for developmental studies, which includes a fully sequenced genome and a high genetic homology and conserved gene function with humans. Since before 120 hours post-fertilization (hpf) the ZE is not subjected to the EU Directive 2010/63/EU for animal protection [16], and given the rapid developmental time frame of ZE, they can be efficiently used to study a large spectrum of developmental processes. Thus, given these features, and considering the limitations of mammalian models, the ZE is a useful model to study human developmental toxicity.

An extensive number of studies in a variety of vertebrate models (e.g., zebrafish, chicken, mice, monkeys) have highlighted the prevalent role of the retinoic acid signaling pathway (RA-SP) in development [17-21]. To understand how this pathway influences development, knockout animals

and dietary restrictions have been used leading to perturbation of the main RA-SP actor, all-trans retinoic acid (ATRA). Physiologically, the concentration of ATRA is regulated locally by a specific balance between enzymes of the *Raldh* family that catabolizes Vitamin A (retinol) to ATRA, and the *cyp26* family that metabolizes ATRA into an inactive form [22]. This local concentration gradient controls the correct expression of a host of patterning genes [23-25], which are then responsible for developmental processes such as neural tube closure and craniofacial development [24, 26-28]. Its perturbation leads to maldevelopments, such as neural tube closure, heart and craniofacial defects [29]. As this pathway is central to development, it has been suggested that it could be a good source of embryotoxicity biomarkers [27].

The aim of this study, was to explore the perturbation of the RA-SP, so as to identify responding genes that can potentially be used as embryotoxicity biomarkers in safety assessment. ATRA was used as a positive control for RA-SP perturbation. Valproic acid (VPA) is a well-studied antiepileptic drug known as a teratogen in vertebrate models and in humans [30-36]. In humans, this drug increases the incidence rate of congenital anomalies (e.g., heart defects, cleft palate, neural tube closure defects) and neurodevelopmental anomalies (e.g., autistic spectrum disorder, including communication problems) [30-35, 37]. Although believed to be due to VPA-induced HDAC (histone deacetylase) inhibition, the mechanism of action leading to these anomalies remains unclear [38]. Some of them, such as neural tube defects, axial patterning defects, and heart malformations, are believed to be related to an RA-SP perturbation by the compound [39-41]. This has been suggested since VPA regulates ATRA central metabolic enzymes (e.g., *aldh1a2* and *cyp26a,c*) [39-42] and gene expression of developmental regulators known to be transcriptional targets of the RA-SP (e.g., *cdx1* and *hoxa1*) [39]. Consequently, studying the VPA-induced perturbation of this signaling pathway could help uncover RA-SP related embryotoxicity biomarker genes. In this study, the folic acid (FA) is used as a non-teratogen reference compound. It has been shown to protect embryos from malformations caused by ATRA excess [43], as well as cardiotoxicity and neurotoxicity caused by VPA [44, 45]. It is given as a supplement before and during pregnancy to help reduce the chance of neural tube closure defects. This protective action is believed to be due to the role of FA as source of one carbon groups used to methylate DNA during early development [46]. Additionally FA is a cofactor and suggested regulator of MTHFD1, a rate-determining enzyme in the one-carbon cycle, recently observed to regulate the retinoic acid receptor (RAR) [47, 48].

This study aims to select biomarker genes in the RA-SP by using a whole genome expression analysis approach (RNAseq). Shortly after fertilization, we exposed groups of zebrafish embryos to FA (FA=75 μ M) or a teratogen (ATRA=10nM or VPA=1000 μ M) for 4 hours. This exposure duration was selected since it has been found previously to be the optimal duration for observing significant transcriptomic changes in the RA-SP [49]. During the current study data analysis, we clearly observed the perturbation of genes related to several developmental processes, among which we focused on nervous system development. This decision was driven by the prominent neurodevelopmental gene expression response in this study, and the currently growing concern on the impact of chemicals on the neurodevelopment of the growing fetus.

Methods

Zebrafish maintenance and embryo collection

The zebrafish (*Danio rerio* AB strain) were bred in-house (RIVM) and originally acquired from Karlsruhe Institute of Technology (KIT, European Zebrafish Resource Center, Institute of toxicology and Genetics, Eggenstein-Leopoldshafen, Germany). They were handled according to the Dutch regulation under permit NVWA-32600. They were fed three times a day with dry flake (Special Diet Service Granules, Technilab-BMI, Someren, the Netherlands). They were maintained in 8L Tecniplast Zebtech tanks (Buguggiate, Italy) with a photoperiod of 14hrs light: 10hrs dark, a temperature of 27.5 \pm 0.5 $^{\circ}$ C, a pH of 7.5 \pm 0.5, and conductivity of 500 \pm 100 μ S. The breeding took place under the same conditions; at the end of the afternoon two females and two males were placed in 8L tanks with a grid at the bottom. The eggs were collected and selected in Petri dishes with Dutch standard water (DSW; demineralized water supplemented with 100 mg/l NaHCO₃, 20 mg/l KHCO₃, 200 mg/l CaCl₂ 2H₂O, and 180 mg/l MgSO₄·7H₂O and then aerated for 24hrs at 27 $^{\circ}$ C). The pH of the medium was checked and stayed stable at around 8 for all treatments. If the fertilization rate of the batch was at least 90%, good-quality eggs were selected and used for exposure.

Compounds

All compounds were purchased from Sigma-Aldrich, Zwijndrecht, Netherlands, including dimethyl sulfoxide (DMSO, CAS# 67-68-5). The test compounds, all-trans retinoic acid (ATRA, CAS# 302-79-4) and valproic acid (VPA, CAS# 1069-66-5), were selected for their abilities to induce neural tube closure defects in animal testing and in humans. All teratogenic compounds were used at equipotent concentrations equal to their CED20 (critical effect dose 20%); this represents the concentration at which the general morphology score (GMS) decreases by 20%, as established with the scoring system published by Hermesen and al. 2011[50]. CED20 is established using PROAST (version 69). ATRA was tested at 10nM, and VPA was tested at 1000μM. Folic acid (FA, CAS# 59-30-3) was included as a non-teratogen reference compound at 75μM. FA was not embryotoxic in the assay up to at least the highest concentration tested of 1 mM.

Exposure

After egg selection, 15 good-quality eggs were placed in one well of a six-well plate with 5ml of test medium. The test medium contained DSW plus 0.1% DMSO for the control, and DSW plus the different chemicals with 0.1% DMSO for the treated fish. The plates were incubated at 27.5°C, in light condition for 4hrs. Thirty eggs (2 wells) were used for one sample, collected in 2 ml low-binding DNA tubes. Eight samples per condition were collected, two samples per condition in each of four independent experiments. The tubes were then placed in liquid nitrogen and stored at -80°C before RNA isolation. For each experiment, 5 to 10 eggs per condition were allowed to develop for 72 hours post fertilization (hpf) to confirm the effect of the compound on development.

RNA isolation

The samples were processed according to the manufacturer's protocol using the RNeasy Mini kit (Qiagen, Cat# 74104). The samples were homogenized with a prospective autoclave blender in 700 μl Quiazol Lysis Reagent (Qiagen, Cat# 79306), and then 300 μl of Quiazol was added. A DNase step was done using an RNase-Free DNase set (Qiagen, Cat # 79254). The sample RNA quantity and quality were assessed using the Qubit3 (Invitrogen, Carlsbad, CA, USA) and 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). All samples scored greater than 7.5 on the RIN (RNA Integrity Number) scale.

RNA sequencing

RNA sequencing

GenomeScan (Leiden, The Netherlands) processed and analyzed the RNA samples, following the same protocol as Mennen et al. 2022 [51]. The gene expression transcript was mapped and annotated using the Ensembl zebrafish genome assembly GRCz11 + corresponding GTF file.

Data analysis

Genes with a read count mean for all samples lower than 10 were discarded for downstream analysis. Statistical analysis was done using a read data count matrix for 32 samples and 15710 Ensembl genes. Data were first linearized using the voom function from the Limma R package [52], then a quantile normalize and log2 transformation were applied.

Then, differentially expressed genes were found by applying a two-way analysis of variance of MUTATION and BATCH factors and by performing a pairwise Tukey post hoc test between groups. Differentially Expressed Genes (DEGs) were obtained by filtering results for $p < 0.05$, fold-change > 1.1 for upregulation, and fold-change < -1.1 for downregulation. Enrichment analysis was performed using the MSigDB v7.5 [52] as a gene-set database, and a Fisher exact test were applied with false discovery rate (FDR) correction (5%) [53]. Interaction networks were generated using STITCH interaction networks of chemicals and proteins (STITCH, <http://stitch.embl.de>), QIAGEN IPA (QIAGEN Inc., <https://digitalinsights.qiagen.com/IPA>) and Cytoscape software were used for visualization of the interaction network.

All processed data have been submitted to GEO NCBI database with the accession number **GSE226367**.

Results

Gene expression changes after compound exposures

The effects of three compounds on whole ZE gene expression were measured after 4 hrs of exposure. In the principal component analysis (PCA) plot (Figure 1), FA clustered with DSW control samples as expected. The two teratogenic compounds (VPA and ATRA) showed different responses, with

the largest distance to control of VPA on PC1 and ATRA on PC3. Overall, 6858 out of 15710 genes were differentially expressed by at least one of the treatments. Among the three different treatments (FA, VPA, ATRA), a similar number of up-and down-regulated genes per treatment was found. Also noted was a difference between the total number of differentially expressed genes (DEGs) per compound (Figure 2A).

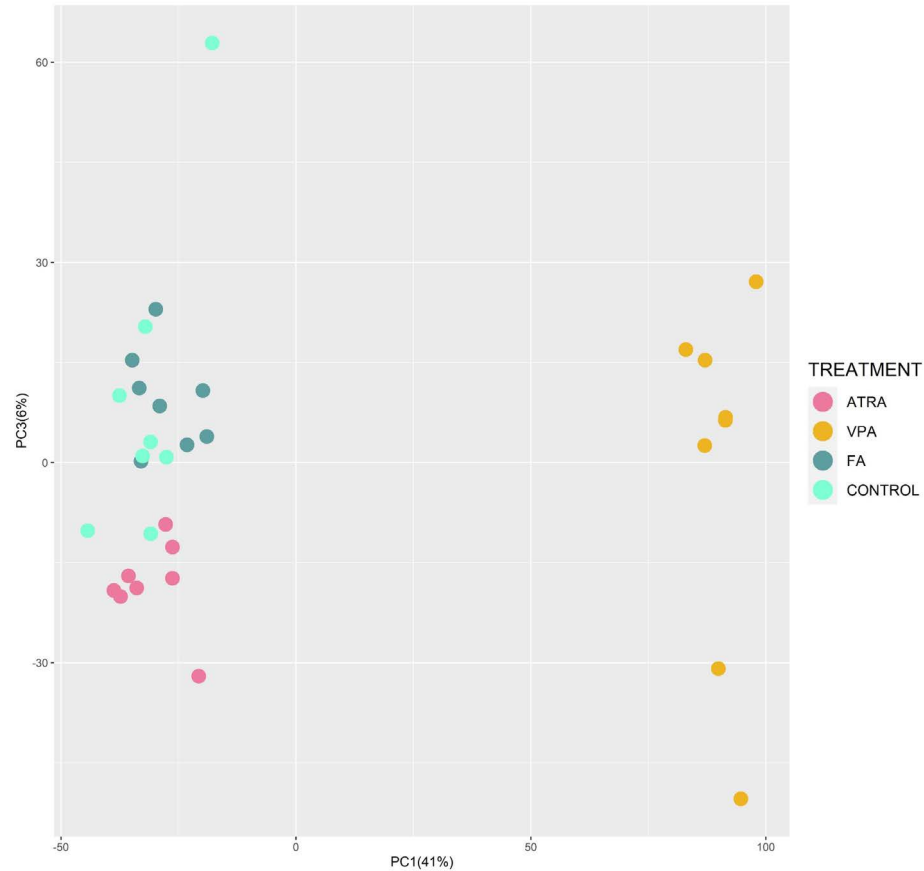


Figure 1 | Principal component analysis (PCA) of the 32 samples color-coded by treatment: all-trans retinoic acid (ATRA), Valproic acid (VPA), Folic acid (FA) and control, organized on axis PC1 (41%) and PC3 (6%).

Using a Venn diagram, we could illustrate the comparison of the DEGs for each treatment (Figure 2A). This comparison revealed that eight genes were dysregulated by all three treatments, that the negative control FA shared 53 DEGs with VPA and 9 with ATRA, and that the 2 teratogenic chemicals had 248 DEGs in common not regulated by FA. To investigate this gene selection,

we displayed these 248 DEGs in a heatmap (Figure 2B). The heatmap shows specific signature profile responses for the two teratogenic chemicals. Four clusters could be discriminated, two responding with a similar profile of expression to both compounds, one with 50 DEGs down regulated, and one with 73 DEGs upregulated, and two clusters with opposite responses, one with 35 DEGs down regulated for ATRA and up regulated for VPA, and one with 90 DEGs down regulated for VPA and up regulated for ATRA.

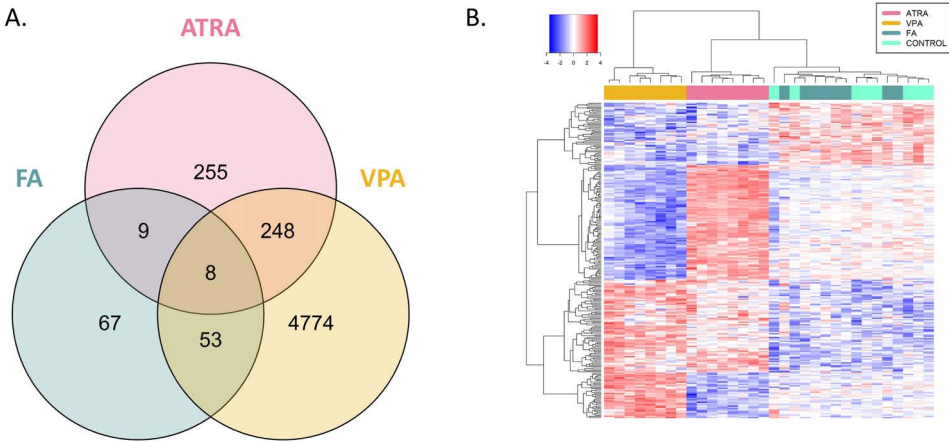


Figure 2 | A. Venn diagram of the DEGs regulated by at least one treatment ($p < 0.05$, fold-change < -1.1 and fold-change > 1.1) per treatment. B. Heatmap of the 248 DEGs common between the two teratogenic compounds hierarchically clustered by genes and treatment.

Analysis of gene interaction with RA-SP

To investigate this responsive gene set, we used the STITCH database to create an ATRA-related gene network of the 248 DEGs (Figure 3). We observed connected genes involved in retinoic acid metabolism (e.g., *ugt5g1*, *cyp26a*, *cyp26c* - Figure 3 left below of the retinoic acid node-), well-known patterning gene targets of ATRA regulation (e.g., *hoxb1b*, *meis1*, *2a*, and *3* - Figure 3 right below of the retinoic acid node-), and genes involved in other major developmental pathways known to have cross-regulation with the RA-SP (e.g., *wnt8b*, *fgf8a*, *fgfr4*, and *fzd8a*).

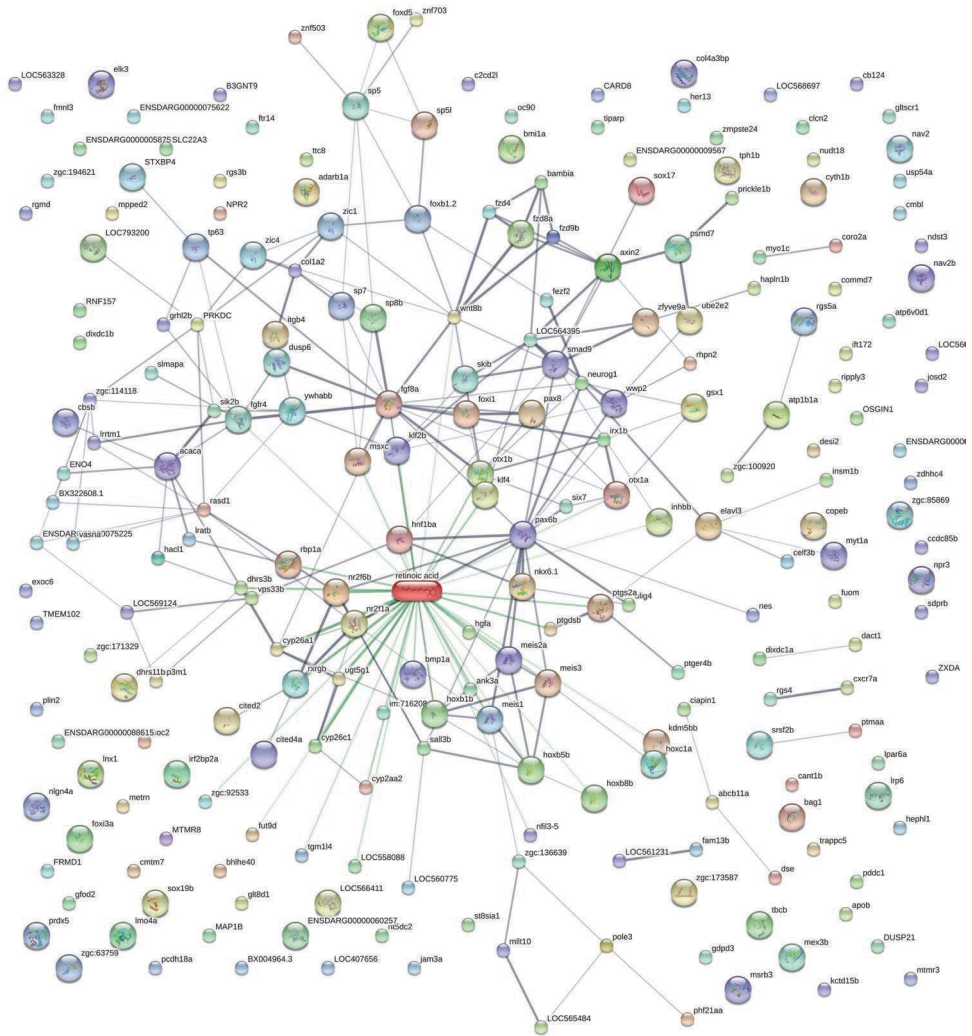


Figure 3 | STITCH network of the relation with ATRA of the 248 DEGs. First neighbor genes to ATRA are linked with a green line. Node size highlights whether the 3D structure of the protein is known (big node) or not (small node). Line thickness represents the edge confidence from 0.150 (low) to 0.900 (highest).

Analysis of effects on GO-terms

Given the observed chemically induced gene expression signature, we investigated which biological processes were perturbed and found that a wide range of 533 gene ontology terms (GO-terms) were regulated (Figure 4 and Supplementary Data Table 1). These GO-terms include very general processes such as regulation of transcription-DNA template, general developmental processes such as system development, and specific developmental processes such as central nervous system development. Two specific developmental processes appear in the top 10 GO-terms: nervous system development, and central nervous system development (Figure 4). We decided to focus on nervous system development as it is a prominent response area in our data and considering that xenobiotic-related neurotoxicity leading to neurodevelopmental disorders is a growing societal concern.

	GO term	Regulated/ total genes	P-value	Enrichment score
1	Anatomical structure development	121/4527	1,40E-23	2,3
2	Developmental process	122/4705	1,20E-22	2,2
3	Multicellular organism dev.	111/4159	7,90E-21	2,3
4	System dev.	99/3689	4.50e-18	2,3
5	Central nervous system dev.	39/686	1.70e-16	4,7
6	Multicellular organismal process	115/5018	2.40e-16	1,9
7	regulation of DNA-templated transcription	71/2202	3,00E-16	2,7
8	regulation of nucleic acid-templated transcription	71/2203	3,00E-16	2,7
9	regulation of RNA biosynthetic process	71/2205	3,20E-16	2,7
10	Nervous system dev.	62/1728	3,30E-16	3

Figure 4 | Table of top 10 GO-terms most statistically enriched in the genes studied organized by P-value.

Analysis of effects on nervous system development related gene expression

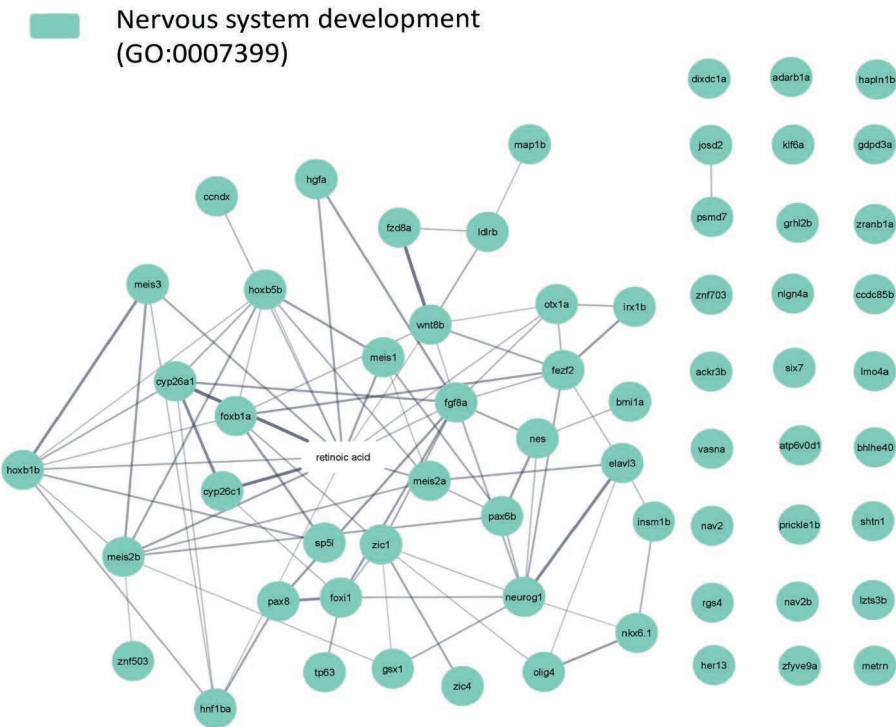
The STITCH database was used to create an ATRA-related gene network (Figure 5A). The DEGs related to the Nervous system development GO-term participate to the regulation of its child terms (or direct descendant; figure 5B). Multiple DEGs were found to be implicated in multiple child terms, such as *fgf8a*, *foxb1a*, *cyp26a*, some were implicated in only one nervous system development process such as: *ccdc85b*, *map1b* in neural tube development; *dixdc1a*, *gdpc3a*, and *lmo4a* in brain development; or *pax8* in peripheral nervous system development (Supplementary Data Table 3). The observation of clear links between ATRA, the DEGs and neurodevelopmental GO-terms was considered to be a good basis to search for potential neurodevelopmental toxicity biomarkers in the RA-SP.

In this study, 62 out of the 1120 genes specific to the nervous system development GO-term were regulated (Figure 4). As in the larger 248 gene set analysis (Figure 2B), we observed in the heatmap (Figure 6A), a division of 4 clusters: two clusters show an inverse response pattern with 34 DEGs up-regulated by ATRA but down-regulated by VPA and 4 DEGs down-regulated by ATRA but up-regulated by VPA. Two clusters responded similarly in both treatments, with 11 DEGs commonly down-regulated and 13 DEGs commonly up-regulated. Two distinct gene expression signature profiles can be observed with the teratogenic treatments (Figure 6B). For example, VPA down-regulates the enzyme family *cyp26*, while ATRA upregulates the family.

Discussion

In this study, we mapped the gene expression response in the ZE using two model teratogenic compounds expected to perturb the RA-SP. Using a genome-wide study (RNAseq), a type of data-driven approach, we investigated gene expression changes after exposure to known teratogens ATRA and VPA, as compared to a negative control FA. We observed changes in the expression of genes regulated directly by the RA-SP as well as genes associated with developmental processes downstream of the RA-SP and thereby determined possible early neurodevelopmental toxicity biomarkers

A.



B.

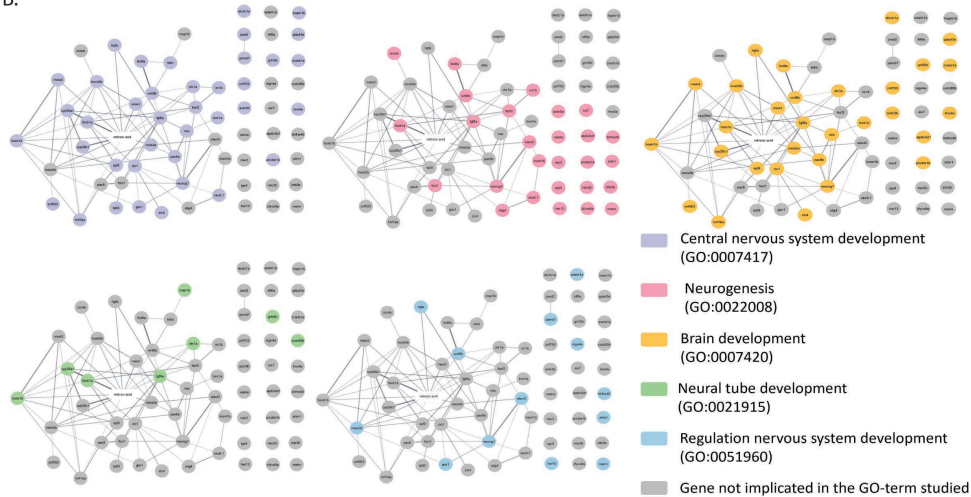


Figure 5 | A. ATRA-related STITCH network of the 62 DEGs implicated in the GO-term nervous system development. B. Same STITCH network response for related neurodevelopmental GO-terms, regulated genes highlighted with colors.

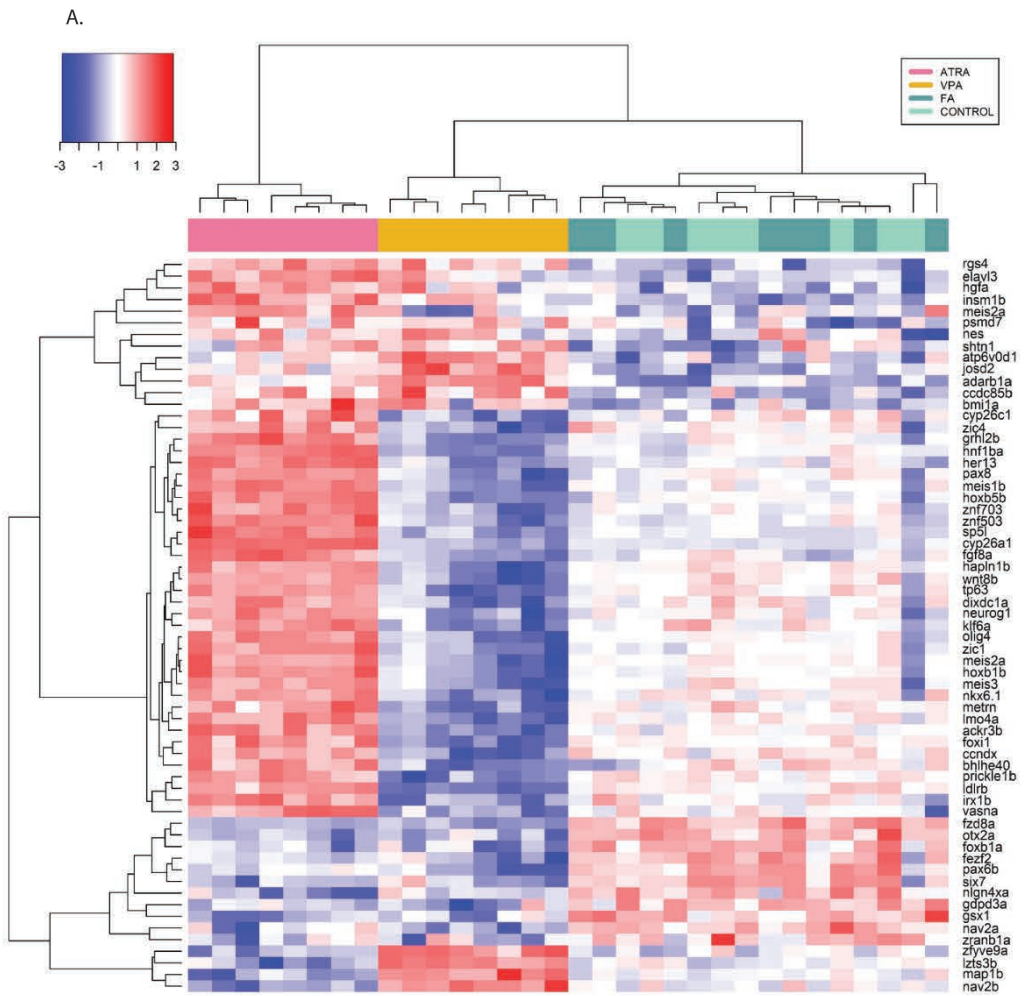
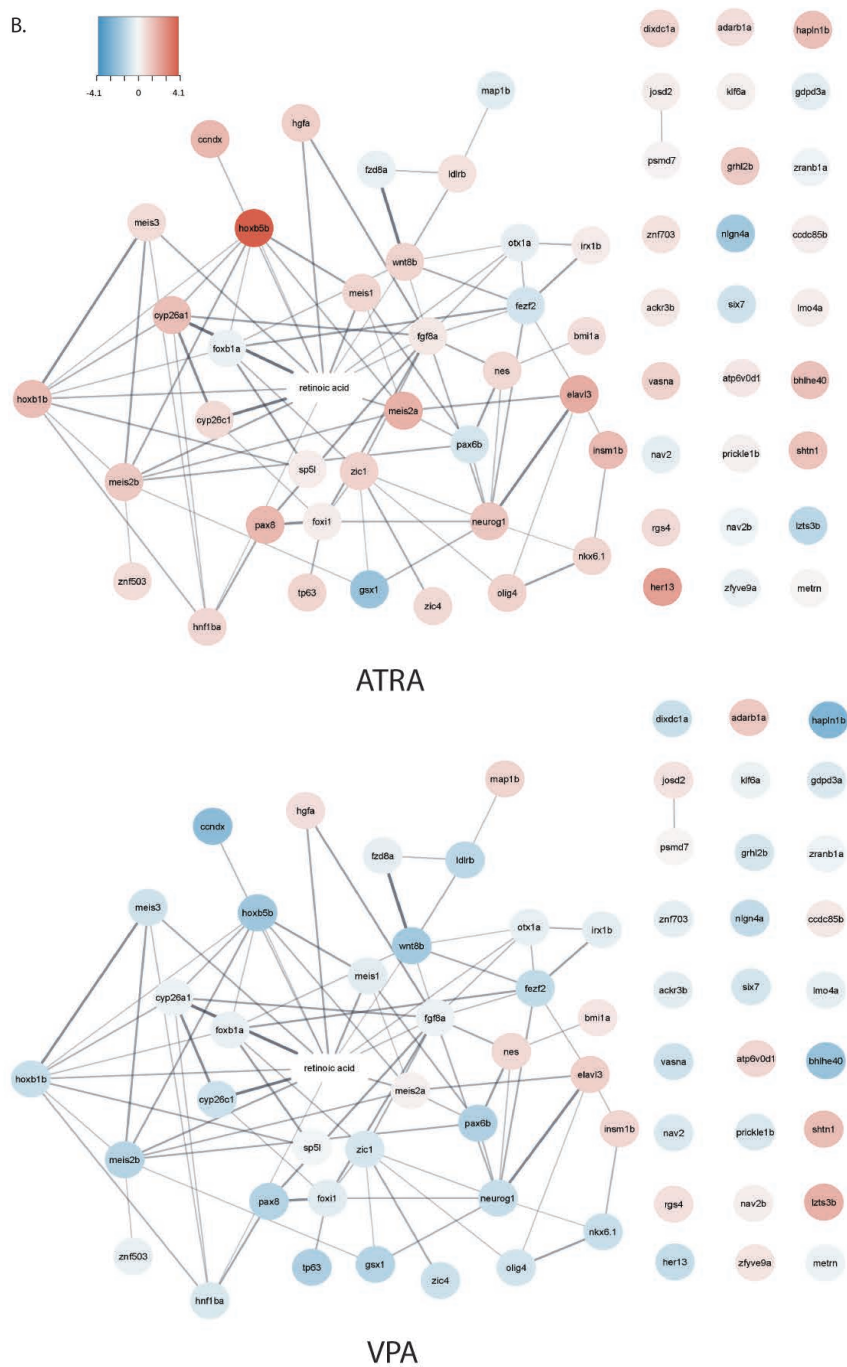


Figure 6 | A. Heatmap of the 62 genes regulated in the nervous system development GO-term. B. Network of nervous system development Go-term DEGs of the two teratogenic compounds (log2 fold change).

related to the RA-SP for both teratogenic compounds. VPA and ATRA were selected due to their similar toxicity and adverse outcomes [54, 55] (e.g., neural tube closure defect) and their well-studied MOA, which includes expected perturbation of RA-SP genes.

Our results show that using RNAseq, we can observe that RA-SP is perturbed by both compounds as anticipated. Further, following only 4h exposure to the ZE, it can be observed that different compounds induce compound-specific changes to the RA-SP related gene are regulated differently depending on the compound, in two ways. First, a discrepancy in number



of DEGs between compounds was found in this study, with VPA significantly regulating 5083 genes and ATRA 520 genes. This discrepancy has also been observed in other studies. For example, microarray experimentation identified 6 times more DEGs after VPA treatment than after ATRA treatment using equipotent compound concentrations based on embryotoxicity [56, 57]. This difference could be explained by the different mechanisms of action of these two molecules on gene transcription. VPA acts directly on the machinery of gene transcription by inhibiting histone deacetylation and making transcription sites more accessible. We believe that this effect translates into a broader impact on gene expression in comparison to ATRA, a well-known activator of transcription factors. Secondly, although not in the scope of this work, differences in the mode of action were observed for the two compounds. For example, the *cyp26* gene family, which is essential for the regulation of ATRA gradients [58], is up-regulated by ATRA and down-regulated by VPA treatment (Figure 6). The same pattern of regulation is observed for *fgf8a* and the gene families of *meis*, *hox*, and *zic* which are regulators of ATRA concentration through feedback loops [59], and direct effectors of RA-SP respectively. These RNAseq findings are consistent with other studies using zebrafish and other models (e.g., cardiac embryonic stem cell test, rat post implantation whole embryo culture) [24, 42, 49, 60], and suggest that valproic acid and ATRA have opposite effects on ATRA-related developmental gene expression, whilst still both affecting brain development, stressing the importance of homeostatic balance for proper development. Additional mechanistic studies including the characterization of the toxicity profile of each compound in terms of concentration and time response will be necessary to fully understand the mode of action of each compound. To our knowledge, this is the first study using whole-genome-scale approach performed on ZE early development (7 hpf) after a short exposure duration of 4hrs, as most recent research has focused on the 24 – 120 hpf time points [61, 62]. When compared to existing transcriptomic data on ZE, the current RNAseq based study offers a more comprehensive approach than a curated, preselected gene list [63, 64], enabling us to select the studied gene list unbiasedly. Nevertheless, analyzing all the data at once can be challenging, due to the high amount of data available. Additionally, defining the downstream gene expression regulation of the RA-SP can also be challenging since the RA-SP includes direct target genes but also indirect targets that are regulated through transcriptional intermediaries, for example, through interconnection with other morphogen gradients

(e.g., *wnt*, *fgf8*, *shh*, *bmp* - Figure 3) [60, 61]. Taking into consideration these challenges, we designed the analysis by defining the RA-SP as all genes regulated by ATRA treatment, and consequently focused on the common DEGs between the two teratogen compounds and not the non teratogen reference compound (Figure 2A) to study the toxicity MOA of the RA-SP. In the future, an interesting next step would be to analyze these data with other design strategies.

The DEGs commonly regulated by ATRA and VPA but not FA were organized in GO-terms to understand the teratogenic implication in the different biological processes (Figure 4). Perturbed GO-terms included specific developmental processes such as the circulatory system, head, skeletal, urogenital, and nervous system development. These results correlate with previously established adverse outcomes [54, 65-67] for the teratogens studied in this work. We also found that the GO-term for nervous system development (GO:0007399) was represented with the highest number of regulated genes and central nervous system was the most enriched, with 4.8 times more regulated genes than when gene expression changes are random (Figure 4). This fact and a societal need for mechanistic developmental neurotoxicity tests [68] shaped our focus for the rest of this study.

To find potential neurodevelopmental toxicity biomarkers related to the RA-SP, we investigated the DEGs related to nervous system development GO-terms (Figure 5). This resulted in 62 DEGs that are candidate for potential early biomarkers. These genes are involved in several of the fundamental neurodevelopmental processes such as neural precursor proliferation, neuronal differentiation, and glycogenesis, often linked to neurodevelopmental adverse outcomes such as reduced learning ability, shortened attention span, and autism spectrum disorders in humans [54]. Other studies using different neurodevelopmental toxicants have also observed dysregulation of these candidate genes. Indeed, genes such as *nes*, *elavl3*, *map1* and *neurog1*, were dysregulated following exposure to neurodevelopmental toxicants (e.g., ethanol, lead chloride, and carbamazepine) in different models (e.g., ZE, primary culture of rat CGCs, human embryonic stem cells) [64, 68-71]. This increases the credibility of these candidates as early neurodevelopmental toxicity biomarkers, however further evaluation of their responses after different exposures is needed. Existing lists of example neurodevelopmental toxicants with different modes of action [54, 72] could be used to that purpose.

3

The present selection of potential biomarkers is limited to the developmental neurotoxicity aspect of the data. Researching other developmental processes that appear to be affected, such as the circulatory system, skeletal system, and urogenital system development, would be an interesting next step. Additionally, we focused on the RA-SP as a source of biomarkers, whereas other publications, for example, Sashana et al. [73], propose the study of other signaling pathways to mine for developmental neurotoxicity biomarkers such as the MAPK/ERK, the PI3-K, and the JNK pathways. In addition, the zebrafish model has its limitations as to the prediction of human hazards. For example, some software (e.g., IPA, ConsensusPathDB - mpg.de -) was created or optimized for human or rodent data, not zebrafish. Furthermore, several zebrafish genes can have the same human orthologue or may not yet be linked with human orthologues. Once additional expertise and tools become available for this model, the present data could be revisited.

In conclusion, our investigation advances the mechanistic knowledge about chemical MOA by showing that, while the two teratogen xenobiotics ATRA and VPA perturbed the RA-SP differently, they did have some common DEGs during early development. When looking further into the link between MOA and adverse outcomes, we also observed that these DEGs perturbed several major developmental processes, including circulatory, urogenital, and nervous system development. This points to the central role of the RA-SP in a plethora of developmental processes, which renders gene expression biomarkers in this area especially attractive as tools to detect the possible developmental impact of compounds. Studying these candidate gene biomarkers with different models and chemical exposures should reveal whether they can be more universally used as biomarkers and could contribute to predictive tools in animal-free chemical hazard and risk assessment.

Funding

This work was supported by the French National Association for Research and Technology (ANRT) through a CIFRE PhD grant no. 2017/1154.

Acknowledgements

We gratefully thank Harm Heusinkveld, Edwin Zwart, Eric Gremmer for the technical support and their guidance on experimental design and the Academic Writing Center, Université Paris-Saclay for proofreading this article.

References

1. Shanks, N., R. Greek, and J. Greek, *Are animal models predictive for humans?* Philos Ethics Humanit Med, 2009. **4**: p. 2.
2. Ball, N., et al., *A framework for chemical safety assessment incorporating new approach methodologies within REACH*. Arch Toxicol, 2022. **96**(3): p. 743-766.
3. Schechtman, L.M., *The safety assessment process--setting the scene: an FDA perspective*. ILAR J, 2002. **43 Suppl**: p. S5-10.
4. Doke, S.K. and S.C. Dhawale, *Alternatives to animal testing: A review*. Saudi Pharm J, 2015. **23**(3): p. 223-9.
5. Bal-Price, A., et al., *Strategies to improve the regulatory assessment of developmental neurotoxicity (DNT) using in vitro methods*. Toxicol Appl Pharmacol, 2018. **354**: p. 7-18.
6. Ali, S., et al., *Zebrafish embryos and larvae: a new generation of disease models and drug screens*. Birth Defects Res C Embryo Today, 2011. **93**(2): p. 115-33.
7. Ali, S., H.G. van Mil, and M.K. Richardson, *Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing*. PLoS One, 2011. **6**(6): p. e21076.
8. Collazo, A., *Developmental variation, homology, and the pharyngula stage*. Syst Biol, 2000. **49**(1): p. 3-18.
9. Irie, N. and S. Kuratani, *Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis*. Nat Commun, 2011. **2**: p. 248.
10. Nishimura, Y., et al., *Using zebrafish in systems toxicology for developmental toxicity testing*. Congenit Anom (Kyoto), 2016. **56**(1): p. 18-27.
11. Teixeira, E., et al., *Assessment of developmental delay in the zebrafish embryo teratogenicity assay*. Toxicol In Vitro, 2013. **27**(1): p. 469-78.
12. Nagel, R., *DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology*. ALTEX, 2002. **19 Suppl 1**: p. 38-48.
13. Selderslaghs, I.W., et al., *Locomotor activity in zebrafish embryos: a new method to assess developmental neurotoxicity*. Neurotoxicol Teratol, 2010. **32**(4): p. 460-71.
14. Lee, H.Y., et al., *Alternative models in developmental toxicology*. Syst Biol Reprod Med, 2012. **58**(1): p. 10-22.
15. Hill, A.J., et al., *Zebrafish as a model vertebrate for investigating chemical toxicity*. Toxicol Sci, 2005. **86**(1): p. 6-19.
16. *Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance*. 2010; Available from: <http://data.europa.eu/eli/dir/2010/63/oj>.
17. Hendrickx, A.G. and H. Hummler, *Teratogenicity of all-trans retinoic acid during early embryonic development in the cynomolgus monkey (Macaca fascicularis)*. Teratology, 1992. **45**(1): p. 65-74.
18. Bouman, H.G., et al., *Diminished growth of atrioventricular cushion tissue in stage 24 retinoic acid-treated chicken embryos*. Dev Dyn, 1998. **213**(1): p. 50-8.
19. Robens, J.F., *Teratogenic effects of hypervitaminosis A in the hamster and the guinea pig*. Toxicol Appl Pharmacol, 1970. **16**(1): p. 88-99.
20. Laue, K., et al., *Craniosynostosis and multiple skeletal anomalies in humans and zebrafish result from a defect in the localized degradation of retinoic acid*. Am J Hum Genet, 2011. **89**(5): p. 595-606.
21. Pennimpede, T., et al., *The role of CYP26 enzymes in defining appropriate retinoic acid exposure during embryogenesis*. Birth Defects Res A Clin Mol Teratol, 2010. **88**(10): p. 883-94.
22. Reijntjes, S., et al., *The control of morphogen signalling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo*. Dev Biol, 2005. **285**(1): p. 224-37.
23. Shimozone, S., et al., *Visualization of an endogenous retinoic acid gradient across embryonic development*. Nature, 2013. **496**(7445): p. 363-6.

24. Duester, G., *Retinoic acid synthesis and signaling during early organogenesis*. Cell, 2008. **134**(6): p. 921-31.
25. Schilling, T.F., Q. Nie, and A.D. Lander, *Dynamics and precision in retinoic acid morphogen gradients*. Curr Opin Genet Dev, 2012. **22**(6): p. 562-9.
26. Samarut, E., et al., *ZebRA: An overview of retinoic acid signaling during zebrafish development*. Biochim Biophys Acta, 2015. **1849**(2): p. 73-83.
27. Piersma, A.H., E.V. Hessel, and Y.C. Staal, *Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker*. Reprod Toxicol, 2017. **72**: p. 53-61.
28. Heusinkveld, H.J., et al., *An ontology for developmental processes and toxicities of neural tube closure*. Reprod Toxicol, 2021. **99**: p. 160-167.
29. Tonk, E.C., J.L. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis*. Reprod Toxicol, 2015. **55**: p. 104-13.
30. Clayton-Smith, J., et al., *Diagnosis and management of individuals with Fetal Valproate Spectrum Disorder; a consensus statement from the European Reference Network for Congenital Malformations and Intellectual Disability*. Orphanet J Rare Dis, 2019. **14**(1): p. 180.
31. Parodi, C., et al., *Chromatin Imbalance as the Vertex Between Fetal Valproate Syndrome and Chromatinopathies*. Front Cell Dev Biol, 2021. **9**: p. 654467.
32. Koren, G., et al., *Major malformations with valproic acid*. Can Fam Physician, 2006. **52**: p. 441-2, 444, 447.
33. Jentink, J., et al., *Valproic acid monotherapy in pregnancy and major congenital malformations*. N Engl J Med, 2010. **362**(23): p. 2185-93.
34. Ornoy, A., *Valproic acid in pregnancy: how much are we endangering the embryo and fetus?* Reprod Toxicol, 2009. **28**(1): p. 1-10.
35. Tomson, T., et al., *Dose-dependent risk of malformations with antiepileptic drugs: an analysis of data from the EURAP epilepsy and pregnancy registry*. Lancet Neurol, 2011. **10**(7): p. 609-17.
36. Oberemm, A. and F. Kirschbaum, *Valproic acid induced abnormal development of the central nervous system of three species of amphibians: implications for neural tube defects and alternative experimental systems*. Teratog Carcinog Mutagen, 1992. **12**(6): p. 251-62.
37. Robert, E. and P. Guibaud, *Maternal valproic acid and congenital neural tube defects*. Lancet, 1982. **2**(8304): p. 937.
38. Kowalski, T.W., et al., *Transcriptome meta-analysis of valproic acid exposure in human embryonic stem cells*. Eur Neuropsychopharmacol, 2022. **60**: p. 76-88.
39. Beker van Woudenberg, A., et al., *Zebrafish embryotoxicity test for developmental (neuro) toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs*. Reprod Toxicol, 2014. **49**: p. 101-16.
40. Li, A.S. and Y. Marikawa, *Adverse effect of valproic acid on an in vitro gastrulation model entails activation of retinoic acid signaling*. Reprod Toxicol, 2016. **66**: p. 68-83.
41. Jergil, M., et al., *Valproic acid-induced deregulation in vitro of genes associated in vivo with neural tube defects*. Toxicol Sci, 2009. **108**(1): p. 132-48.
42. Mennen, R.H.G., J. Pennings, and A.H.A. Piersma, *Neural crest related gene transcript regulation by valproic acid analogues in the cardiac embryonic stem cell test*. Reprod Toxicol, 2019. **90**: p. 44-52.
43. Wang, H. and W. Chen, *Dose-Dependent Antiteratogenic Effects of Folic Acid on All-Trans Retinoic Acid-Induced Cleft Palate in Fetal Mice*. Cleft Palate Craniofac J, 2016. **53**(6): p. 720-726.
44. Muhsen, M., et al., *Folic acid supplementation rescues valproic acid-induced developmental neurotoxicity and behavioral alterations in zebrafish embryos*. Epilepsia, 2021. **62**(7): p. 1689-1700.

45. Padmanabhan, R. and M.M. Shafiullah, *Amelioration of sodium valproate-induced neural tube defects in mouse fetuses by maternal folic acid supplementation during gestation*. Congenit Anom (Kyoto), 2003. **43**(1): p. 29-40.
46. Crider, K.S., et al., *Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role*. Adv Nutr, 2012. **3**(1): p. 21-38.
47. Xiaolu, X., et al., *MTHFD1 is critical for the negative regulation of retinoic acid receptor signalling in anencephaly*. Brain, 2023.
48. Protiva, P., et al., *Altered folate availability modifies the molecular environment of the human colorectum: implications for colorectal carcinogenesis*. Cancer Prev Res (Phila), 2011. **4**(4): p. 530-43.
49. Samrani, L.M.M., et al., *Dynamic regulation of gene expression and morphogenesis in the zebrafish embryo test after exposure to all-trans retinoic acid*. Reprod Toxicol, 2022.
50. Hermesen, S.A., et al., *Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies*. Toxicol In Vitro, 2011. **25**(3): p. 745-53.
51. Mennen, R.H., et al., *Genome-wide expression screening in the cardiac embryonic stem cell test shows additional differentiation routes that are regulated by morpholines and piperidines*. Curr Res Toxicol, 2022. **3**: p. 100086.
52. Ritchie, M.E., et al., *limma powers differential expression analyses for RNA-sequencing and microarray studies*. Nucleic Acids Res, 2015. **43**(7): p. e47.
53. Liberzon, A., et al., *Molecular signatures database (MSigDB) 3.0*. Bioinformatics, 2011. **27**(12): p. 1739-40.
54. Aschner, M., et al., *Reference compounds for alternative test methods to indicate developmental neurotoxicity (DNT) potential of chemicals: example lists and criteria for their selection and use*. ALTEX, 2017. **34**(1): p. 49-74.
55. Christen, V., et al., *Developmental neurotoxicity of different pesticides in PC-12 cells in vitro*. Toxicol Appl Pharmacol, 2017. **325**: p. 25-36.
56. Hermesen, S.A., et al., *Transcriptomic analysis in the developing zebrafish embryo after compound exposure: individual gene expression and pathway regulation*. Toxicol Appl Pharmacol, 2013. **272**(1): p. 161-71.
57. Krug, A.K., et al., *Human embryonic stem cell-derived test systems for developmental neurotoxicity: a transcriptomics approach*. Arch Toxicol, 2013. **87**(1): p. 123-43.
58. Thatcher, J.E. and N. Isoherranen, *The role of CYP26 enzymes in retinoic acid clearance*. Expert Opin Drug Metab Toxicol, 2009. **5**(8): p. 875-86.
59. Dorey, K. and E. Amaya, *FGF signalling: diverse roles during early vertebrate embryogenesis*. Development, 2010. **137**(22): p. 3731-42.
60. Jergil, M., et al., *Short-time gene expression response to valproic acid and valproic acid analogs in mouse embryonic stem cells*. Toxicol Sci, 2011. **121**(2): p. 328-42.
61. Ducharme, N.A., et al., *Meta-analysis of toxicity and teratogenicity of 133 chemicals from zebrafish developmental toxicity studies*. Reprod Toxicol, 2013. **41**: p. 98-108.
62. Schuttler, A., et al., *The Transcriptome of the Zebrafish Embryo After Chemical Exposure: A Meta-Analysis*. Toxicol Sci, 2017. **157**(2): p. 291-304.
63. Liu, M.Y., W.C. Hsieh, and B.C. Yang, *In vitro aberrant gene expression as the indicator of lead-induced neurotoxicity in U-373MG cells*. Toxicology, 2000. **147**(1): p. 59-64.
64. Fan, C.Y., et al., *Gene expression changes in developing zebrafish as potential markers for rapid developmental neurotoxicity screening*. Neurotoxicol Teratol, 2010. **32**(1): p. 91-8.
65. Phiel, C.J., et al., *Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen*. J Biol Chem, 2001. **276**(39): p. 36734-41.
66. Rihtman, T., S. Parush, and A. Ornoy, *Developmental outcomes at preschool age after fetal exposure to valproic acid and lamotrigine: cognitive, motor, sensory and behavioral function*. Reprod Toxicol, 2013. **41**: p. 115-25.

67. Roullet, F.I., J.K. Lai, and J.A. Foster, *In utero exposure to valproic acid and autism--a current review of clinical and animal studies*. Neurotoxicol Teratol, 2013. **36**: p. 47-56.
68. Smirnova, L., et al., *Developmental neurotoxicity - challenges in the 21st century and in vitro opportunities*. ALTEX, 2014. **31**(2): p. 129-56.
69. Robinson, J.F., M.J. Gormley, and S.J. Fisher, *A genomics-based framework for identifying biomarkers of human neurodevelopmental toxicity*. Reprod Toxicol, 2016. **60**: p. 1-10.
70. Hogberg, H.T., et al., *mRNA expression is a relevant tool to identify developmental neurotoxicants using an in vitro approach*. Toxicol Sci, 2010. **113**(1): p. 95-115.
71. Laurenza, I., et al., *A human pluripotent carcinoma stem cell-based model for in vitro developmental neurotoxicity testing: effects of methylmercury, lead and aluminum evaluated by gene expression studies*. Int J Dev Neurosci, 2013. **31**(7): p. 679-91.
72. Mundy, W.R., et al., *Expanding the test set: Chemicals with potential to disrupt mammalian brain development*. Neurotoxicol Teratol, 2015. **52**(Pt A): p. 25-35.
73. Sachana, M., J. Flaskos, and A.J. Hargreaves, *Chapter 15 - In Vitro Biomarkers of Developmental Neurotoxicity*, in *Reproductive and Developmental Toxicology (Second Edition)*, R.C. Gupta, Editor. 2017, Academic Press. p. 255-288.

Supplementary data

Supplementary Data Table 1 | List of all GO-terms perturbed by the gene list studied with a P-Value<0.05, order of display P value, the Significant column represents the number of genes regulated in the GO-term.

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0048856	anatomical structure development	4527	121	53.17	1.4E-23
GO:0032502	developmental process	4705	122	55.26	1.2E-22
GO:0007275	multicellular organism development	4159	111	48.84	7.9E-21
GO:0048731	system development	3689	99	43.32	4.5E-18
GO:0007417	central nervous system development	686	39	8.06	1.7E-16
GO:0032501	multicellular organismal process	5018	115	58.93	2.4E-16
GO:0006355	regulation of transcription, DNA-templated	2202	71	25.86	3E-16
GO:1903506	regulation of nucleic acid-templated transcription	2203	71	25.87	3E-16
GO:2001141	regulation of RNA biosynthetic process	2205	71	25.9	3.2E-16
GO:0007399	nervous system development	1728	62	20.29	3.3E-16
GO:0060322	head development	499	33	5.86	6.4E-16
GO:0007420	brain development	480	32	5.64	1.5E-15
GO:0051252	regulation of RNA metabolic process	2388	73	28.04	1.6E-15
GO:0006351	transcription, DNA-templated	2312	71	27.15	3.8E-15
GO:0097659	nucleic acid-templated transcription	2313	71	27.16	3.9E-15
GO:0032774	RNA biosynthetic process	2318	71	27.22	4.4E-15
GO:0031326	regulation of cellular biosynthetic process	2454	73	28.82	7E-15
GO:0009889	regulation of biosynthetic process	2472	73	29.03	1E-14
GO:2000112	regulation of cellular macromolecule biosynthetic process	2415	72	28.36	1E-14
GO:0019219	regulation of nucleobase-containing compound metabolic process	2477	73	29.09	1.1E-14
GO:0010556	regulation of macromolecule biosynthetic process	2422	72	28.44	1.2E-14
GO:0030902	hindbrain development	132	18	1.55	1.8E-14
GO:0034654	nucleobase-containing compound biosynthetic process	2575	73	30.24	8.6E-14
GO:0019438	aromatic compound biosynthetic process	2636	74	30.96	8.8E-14
GO:0018130	heterocycle biosynthetic process	2641	74	31.02	9.7E-14
GO:1901362	organic cyclic compound biosynthetic process	2718	75	31.92	1.3E-13
GO:0010468	regulation of gene expression	2726	75	32.01	1.6E-13
GO:0048513	animal organ development	2667	74	31.32	1.6E-13
GO:0006357	regulation of transcription by RNA polymerase II	1725	57	20.26	2.6E-13
GO:0080090	regulation of primary metabolic process	3170	81	37.23	5.7E-13
GO:0031323	regulation of cellular metabolic process	3312	83	38.9	7.2E-13
GO:0009653	anatomical structure morphogenesis	2181	64	25.61	1.2E-12
GO:0006366	transcription by RNA polymerase II	1793	57	21.06	1.3E-12
GO:0051171	regulation of nitrogen compound metabolic process	3101	79	36.42	1.6E-12
GO:0003002	regionalization	407	26	4.78	2.2E-12
GO:0007389	pattern specification process	621	31	7.29	9.2E-12

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0019222	regulation of metabolic process	3577	84	42.01	1.7E-11
GO:0034645	cellular macromolecule biosynthetic process	3250	78	38.17	5E-11
GO:0009059	macromolecule biosynthetic process	3260	78	38.29	5.9E-11
GO:0060255	regulation of macromolecule metabolic process	3327	79	39.07	6E-11
GO:0009790	embryo development	1184	42	13.91	8.5E-11
GO:0016070	RNA metabolic process	3103	74	36.44	2.9E-10
GO:0044271	cellular nitrogen compound biosynthetic process	3133	74	36.79	4.5E-10
GO:0009888	tissue development	1489	46	17.49	8.7E-10
GO:0050794	regulation of cellular process	7745	134	90.96	1.2E-09
GO:0007423	sensory organ development	716	30	8.41	1.4E-09
GO:0050789	regulation of biological process	8130	138	95.48	1.8E-09
GO:0065007	biological regulation	8766	145	102.95	2.2E-09
GO:0044249	cellular biosynthetic process	3926	84	46.11	2.3E-09
GO:0009058	biosynthetic process	4043	85	47.48	4.1E-09
GO:1901576	organic substance biosynthetic process	3999	84	46.96	5.7E-09
GO:0048598	embryonic morphogenesis	734	29	8.62	1E-08
GO:0090304	nucleic acid metabolic process	3497	76	41.07	1.1E-08
GO:0060429	epithelium development	904	32	10.62	2.2E-08
GO:1901360	organic cyclic compound metabolic process	4115	84	48.33	2.3E-08
GO:0010467	gene expression	3705	78	43.51	2.7E-08
GO:0071696	ectodermal placode development	43	8	0.5	3.2E-08
GO:0006139	nucleobase-containing compound metabolic process	3880	80	45.57	4E-08
GO:0060788	ectodermal placode formation	30	7	0.35	4.5E-08
GO:0030154	cell differentiation	2427	58	28.5	5.2E-08
GO:0046483	heterocycle metabolic process	3979	81	46.73	5.6E-08
GO:0048869	cellular developmental process	2448	58	28.75	7.1E-08
GO:0006725	cellular aromatic compound metabolic process	4000	81	46.98	7.1E-08
GO:0071697	ectodermal placode morphogenesis	35	7	0.41	1.4E-07
GO:0048880	sensory system development	647	25	7.6	1.8E-07
GO:0031076	embryonic camera-type eye development	53	8	0.62	1.8E-07
GO:0043049	otic placode formation	23	6	0.27	2.1E-07
GO:0045892	negative regulation of transcription, DNA-templated	324	17	3.81	0.0000003
GO:1902679	negative regulation of RNA biosynthetic process	325	17	3.82	3.1E-07
GO:1903507	negative regulation of nucleic acid-templated transcription	325	17	3.82	3.1E-07
GO:0009887	animal organ morphogenesis	830	28	9.75	4.9E-07
GO:0030916	otic vesicle formation	27	6	0.32	5.9E-07
GO:0048568	embryonic organ development	609	23	7.15	8.4E-07
GO:0051253	negative regulation of RNA metabolic process	351	17	4.12	0.0000009
GO:0048839	inner ear development	173	12	2.03	9.7E-07
GO:0016055	Wnt signaling pathway	278	15	3.26	0.0000011
GO:0198738	cell-cell signaling by wnt	278	15	3.26	0.0000011
GO:0043583	ear development	175	12	2.06	0.0000011
GO:0072175	epithelial tube formation	67	8	0.79	0.0000011

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0009952	anterior/posterior pattern specification	244	14	2.87	0.0000012
GO:1905114	cell surface receptor signaling pathway involved in cell-cell signaling	288	15	3.38	0.0000017
GO:0060070	canonical Wnt signaling pathway	184	12	2.16	0.0000019
GO:0034641	cellular nitrogen compound metabolic process	4464	83	52.43	0.000002
GO:0071599	otic vesicle development	51	7	0.6	0.0000021
GO:0035148	tube formation	73	8	0.86	0.0000022
GO:0071600	otic vesicle morphogenesis	34	6	0.4	0.0000025
GO:0044260	cellular macromolecule metabolic process	5959	102	69.98	0.0000029
GO:2000113	negative regulation of cellular macromolecule biosynthetic process	428	18	5.03	0.0000032
GO:0010558	negative regulation of macromolecule biosynthetic process	429	18	5.04	0.0000033
GO:0021546	rhombomere development	21	5	0.25	0.0000037
GO:0045934	negative regulation of nucleobase-containing compound metabolic process	392	17	4.6	0.000004
GO:0048729	tissue morphogenesis	575	21	6.75	0.0000044
GO:0031327	negative regulation of cellular biosynthetic process	441	18	5.18	0.0000049
GO:0009890	negative regulation of biosynthetic process	444	18	5.21	0.0000053
GO:0001654	eye development	539	20	6.33	0.0000059
GO:0150063	visual system development	539	20	6.33	0.0000059
GO:0030900	forebrain development	140	10	1.64	0.0000061
GO:0048646	anatomical structure formation involved in morphogenesis	856	26	10.05	0.0000087
GO:0009880	embryonic pattern specification	63	7	0.74	0.0000088
GO:0043010	camera-type eye development	464	18	5.45	0.0000097
GO:0035295	tube development	810	25	9.51	0.00001
GO:0048596	embryonic camera-type eye morphogenesis	44	6	0.52	0.000012
GO:0048562	embryonic organ morphogenesis	427	17	5.01	0.000012
GO:0090596	sensory organ morphogenesis	301	14	3.53	0.000013
GO:0021570	rhombomere 4 development	5	3	0.06	0.000016
GO:0021661	rhombomere 4 morphogenesis	5	3	0.06	0.000016
GO:0031016	pancreas development	156	10	1.83	0.000016
GO:0002009	morphogenesis of an epithelium	484	18	5.68	0.000017
GO:0044238	primary metabolic process	8103	125	95.16	0.00002
GO:0044237	cellular metabolic process	8210	126	96.42	0.000024
GO:0010817	regulation of hormone levels	102	8	1.2	0.000027
GO:0034672	anterior/posterior pattern specification involved in pronephros development	6	3	0.07	0.000031
GO:0055011	atrial cardiac muscle cell differentiation	6	3	0.07	0.000031
GO:0072098	anterior/posterior pattern specification involved in kidney development	6	3	0.07	0.000031
GO:0048048	embryonic eye morphogenesis	54	6	0.63	0.000039
GO:0021915	neural tube development	108	8	1.27	0.000041
GO:0060562	epithelial tube morphogenesis	252	12	2.96	0.000045

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0009987	cellular process	15001	195	176.17	0.000046
GO:0021593	rhombomere morphogenesis	19	4	0.22	0.000062
GO:0048699	generation of neurons	1020	27	11.98	0.000065
GO:0022008	neurogenesis	1136	29	13.34	0.000065
GO:0021575	hindbrain morphogenesis	37	5	0.43	0.000068
GO:0021592	fourth ventricle development	8	3	0.09	0.000086
GO:0039017	pattern specification involved in pronephros development	8	3	0.09	0.000086
GO:0061004	pattern specification involved in kidney development	8	3	0.09	0.000086
GO:0072048	renal system pattern specification	8	3	0.09	0.000086
GO:0009953	dorsal/ventral pattern formation	154	9	1.81	0.000087
GO:0042472	inner ear morphogenesis	90	7	1.06	0.000091
GO:0042471	ear morphogenesis	91	7	1.07	0.000098
GO:0021879	forebrain neuron differentiation	24	4	0.28	0.00016
GO:0048738	cardiac muscle tissue development	101	7	1.19	0.00019
GO:0001764	neuron migration	46	5	0.54	0.0002
GO:0010453	regulation of cell fate commitment	26	4	0.31	0.00023
GO:0071704	organic substance metabolic process	8553	126	100.45	0.00023
GO:0003310	pancreatic A cell differentiation	11	3	0.13	0.00025
GO:0048593	camera-type eye morphogenesis	178	9	2.09	0.00026
GO:0021872	forebrain generation of neurons	27	4	0.32	0.00026
GO:0030073	insulin secretion	28	4	0.33	0.0003
GO:0045165	cell fate commitment	185	9	2.17	0.00035
GO:0007267	cell-cell signaling	781	21	9.17	0.00036
GO:0008283	cell population proliferation	412	14	4.84	0.00038
GO:0008152	metabolic process	8994	130	105.63	0.00041
GO:0003406	retinal pigment epithelium development	13	3	0.15	0.00042
GO:0031058	positive regulation of histone modification	13	3	0.15	0.00042
GO:0002790	peptide secretion	32	4	0.38	0.00051
GO:0003309	type B pancreatic cell differentiation	32	4	0.38	0.00051
GO:0030072	peptide hormone secretion	32	4	0.38	0.00051
GO:0003007	heart morphogenesis	238	10	2.8	0.00052
GO:0010454	negative regulation of cell fate commitment	14	3	0.16	0.00053
GO:0022037	metencephalon development	58	5	0.68	0.00059
GO:0042886	amide transport	59	5	0.69	0.00064
GO:0043170	macromolecule metabolic process	7056	106	82.87	0.0007
GO:0002067	glandular epithelial cell differentiation	35	4	0.41	0.00073
GO:0035883	enteroendocrine cell differentiation	35	4	0.41	0.00073
GO:0007179	transforming growth factor beta receptor signaling pathway	62	5	0.73	0.0008
GO:0009957	epidermal cell fate specification	4	2	0.05	0.00081
GO:0021571	rhombomere 5 development	4	2	0.05	0.00081
GO:0021572	rhombomere 6 development	4	2	0.05	0.00081
GO:0021767	mamillary body development	4	2	0.05	0.00081

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0055014	atrial cardiac muscle cell development	4	2	0.05	0.00081
GO:0060898	eye field cell fate commitment involved in camera-type eye formation	4	2	0.05	0.00081
GO:0007166	cell surface receptor signaling pathway	1449	31	17.02	0.00081
GO:0006807	nitrogen compound metabolic process	7584	112	89.07	0.00083
GO:0031324	negative regulation of cellular metabolic process	718	19	8.43	0.00083
GO:0007507	heart development	553	16	6.49	0.00085
GO:0071559	response to transforming growth factor beta	63	5	0.74	0.00086
GO:0071560	cellular response to transforming growth factor beta stimulus	63	5	0.74	0.00086
GO:0061351	neural precursor cell proliferation	37	4	0.43	0.0009
GO:0009892	negative regulation of metabolic process	846	21	9.94	0.00101
GO:0051172	negative regulation of nitrogen compound metabolic process	673	18	7.9	0.00101
GO:1904888	cranial skeletal system development	217	9	2.55	0.00109
GO:0001523	retinoid metabolic process	39	4	0.46	0.0011
GO:0050793	regulation of developmental process	795	20	9.34	0.00113
GO:0050796	regulation of insulin secretion	18	3	0.21	0.00114
GO:0045595	regulation of cell differentiation	411	13	4.83	0.00118
GO:0007369	gastrulation	265	10	3.11	0.00119
GO:0016101	diterpenoid metabolic process	40	4	0.47	0.00121
GO:0046879	hormone secretion	40	4	0.47	0.00121
GO:0120254	olefinic compound metabolic process	40	4	0.47	0.00121
GO:0048592	eye morphogenesis	221	9	2.6	0.00123
GO:0010605	negative regulation of macromolecule metabolic process	804	20	9.44	0.00129
GO:0030917	midbrain-hindbrain boundary development	41	4	0.48	0.00133
GO:0003151	outflow tract morphogenesis	5	2	0.06	0.00134
GO:0035773	insulin secretion involved in cellular response to glucose stimulus	5	2	0.06	0.00134
GO:0061178	regulation of insulin secretion involved in cellular response to glucose stimulus	5	2	0.06	0.00134
GO:0035051	cardiocyte differentiation	70	5	0.82	0.00139
GO:0009914	hormone transport	42	4	0.49	0.00146
GO:0021549	cerebellum development	42	4	0.49	0.00146
GO:0072359	circulatory system development	994	23	11.67	0.00148
GO:0035239	tube morphogenesis	639	17	7.5	0.00148
GO:0031018	endocrine pancreas development	71	5	0.83	0.00148
GO:0048519	negative regulation of biological process	1911	37	22.44	0.00155
GO:0002791	regulation of peptide secretion	20	3	0.23	0.00157
GO:0090087	regulation of peptide transport	20	3	0.23	0.00157
GO:0090276	regulation of peptide hormone secretion	20	3	0.23	0.00157
GO:0009798	axis specification	72	5	0.85	0.00157
GO:0002065	columnar/cuboidal epithelial cell differentiation	43	4	0.5	0.00159
GO:0035265	organ growth	43	4	0.5	0.00159

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0006721	terpenoid metabolic process	44	4	0.52	0.00174
GO:0015833	peptide transport	44	4	0.52	0.00174
GO:0034754	cellular hormone metabolic process	44	4	0.52	0.00174
GO:0035567	non-canonical Wnt signaling pathway	45	4	0.53	0.00189
GO:0048523	negative regulation of cellular process	1734	34	20.36	0.00199
GO:0021534	cell proliferation in hindbrain	6	2	0.07	0.002
GO:0035066	positive regulation of histone acetylation	6	2	0.07	0.002
GO:0097150	neuronal stem cell population maintenance	6	2	0.07	0.002
GO:0001822	kidney development	193	8	2.27	0.00205
GO:0072001	renal system development	193	8	2.27	0.00205
GO:0016477	cell migration	718	18	8.43	0.00207
GO:0060419	heart growth	22	3	0.26	0.00209
GO:0001704	formation of primary germ layer	77	5	0.9	0.00212
GO:0021903	rostrocaudal neural tube patterning	47	4	0.55	0.00222
GO:0000122	negative regulation of transcription by RNA polymerase II	196	8	2.3	0.00225
GO:0001655	urogenital system development	197	8	2.31	0.00233
GO:0042659	regulation of cell fate specification	23	3	0.27	0.00238
GO:0001708	cell fate specification	117	6	1.37	0.00259
GO:0042592	homeostatic process	734	18	8.62	0.00263
GO:0048863	stem cell differentiation	247	9	2.9	0.00264
GO:0021953	central nervous system neuron differentiation	118	6	1.39	0.0027
GO:0035065	regulation of histone acetylation	7	2	0.08	0.00277
GO:0046619	lens placode formation involved in camera-type eye formation	7	2	0.08	0.00277
GO:1901985	positive regulation of protein acetylation	7	2	0.08	0.00277
GO:2000758	positive regulation of peptidyl-lysine acetylation	7	2	0.08	0.00277
GO:0035270	endocrine system development	119	6	1.4	0.00282
GO:0071363	cellular response to growth factor stimulus	298	10	3.5	0.00282
GO:0070848	response to growth factor	299	10	3.51	0.00289
GO:0055007	cardiac muscle cell differentiation	52	4	0.61	0.00322
GO:0021536	diencephalon development	85	5	1	0.00327
GO:0045893	positive regulation of transcription, DNA-templated	463	13	5.44	0.00334
GO:1902680	positive regulation of RNA biosynthetic process	463	13	5.44	0.00334
GO:1903508	positive regulation of nucleic acid-templated transcription	463	13	5.44	0.00334
GO:0009950	dorsal/ventral axis specification	26	3	0.31	0.0034
GO:0048857	neural nucleus development	26	3	0.31	0.0034
GO:0048468	cell development	1458	29	17.12	0.00356
GO:0051173	positive regulation of nitrogen compound metabolic process	879	20	10.32	0.00363
GO:0045944	positive regulation of transcription by RNA polymerase II	309	10	3.63	0.00365
GO:0001743	lens placode formation	8	2	0.09	0.00367
GO:1901983	regulation of protein acetylation	8	2	0.09	0.00367

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:2000756	regulation of peptidyl-lysine acetylation	8	2	0.09	0.00367
GO:0017015	regulation of transforming growth factor beta receptor signaling pathway	27	3	0.32	0.00379
GO:0021854	hypothalamus development	27	3	0.32	0.00379
GO:0050708	regulation of protein secretion	27	3	0.32	0.00379
GO:1903844	regulation of cellular response to transforming growth factor beta stimulus	27	3	0.32	0.00379
GO:0030855	epithelial cell differentiation	261	9	3.07	0.0038
GO:0051960	regulation of nervous system development	169	7	1.98	0.00387
GO:0048925	lateral line system development	129	6	1.51	0.0042
GO:0003206	cardiac chamber morphogenesis	28	3	0.33	0.00421
GO:0010565	regulation of cellular ketone metabolic process	28	3	0.33	0.00421
GO:0021591	ventricular system development	28	3	0.33	0.00421
GO:0021761	limbic system development	28	3	0.33	0.00421
GO:0021532	neural tube patterning	56	4	0.66	0.00421
GO:0021545	cranial nerve development	56	4	0.66	0.00421
GO:0014706	striated muscle tissue development	218	8	2.56	0.00431
GO:0060059	embryonic retina morphogenesis in camera-type eye	29	3	0.34	0.00466
GO:0071322	cellular response to carbohydrate stimulus	9	2	0.11	0.00468
GO:0071326	cellular response to monosaccharide stimulus	9	2	0.11	0.00468
GO:0071331	cellular response to hexose stimulus	9	2	0.11	0.00468
GO:0071333	cellular response to glucose stimulus	9	2	0.11	0.00468
GO:0048793	pronephros development	132	6	1.55	0.0047
GO:0048870	cell motility	777	18	9.13	0.00476
GO:0051674	localization of cell	777	18	9.13	0.00476
GO:0070887	cellular response to chemical stimulus	1030	22	12.1	0.0049
GO:0060537	muscle tissue development	226	8	2.65	0.00534
GO:0006720	isoprenoid metabolic process	60	4	0.7	0.00539
GO:0060041	retina development in camera-type eye	276	9	3.24	0.00545
GO:0007167	enzyme linked receptor protein signaling pathway	549	14	6.45	0.00551
GO:0031056	regulation of histone modification	31	3	0.36	0.00563
GO:0042445	hormone metabolic process	61	4	0.72	0.00572
GO:0072576	liver morphogenesis	10	2	0.12	0.00581
GO:0040011	locomotion	982	21	11.53	0.00587
GO:0009719	response to endogenous stimulus	556	14	6.53	0.00614
GO:0046856	phosphatidylinositol dephosphorylation	32	3	0.38	0.00616
GO:0046883	regulation of hormone secretion	32	3	0.38	0.00616
GO:0048384	retinoic acid receptor signaling pathway	32	3	0.38	0.00616
GO:0060795	cell fate commitment involved in formation of primary germ layer	33	3	0.39	0.00672
GO:0009996	negative regulation of cell fate specification	11	2	0.13	0.00704
GO:0010002	cardioblast differentiation	11	2	0.13	0.00704
GO:0032088	negative regulation of NF-kappaB transcription factor activity	11	2	0.13	0.00704
GO:0010557	positive regulation of macromolecule biosynthetic process	509	13	5.98	0.00729

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0060900	embryonic camera-type eye formation	34	3	0.4	0.00731
GO:0071495	cellular response to endogenous stimulus	512	13	6.01	0.00764
GO:0008544	epidermis development	104	5	1.22	0.00766
GO:0030901	midbrain development	35	3	0.41	0.00793
GO:0051090	regulation of DNA-binding transcription factor activity	67	4	0.79	0.00796
GO:0007422	peripheral nervous system development	68	4	0.8	0.00838
GO:0021555	midbrain-hindbrain boundary morphogenesis	12	2	0.14	0.00838
GO:0035701	hematopoietic stem cell migration	12	2	0.14	0.00838
GO:0042572	retinol metabolic process	12	2	0.14	0.00838
GO:0060581	cell fate commitment involved in pattern specification	12	2	0.14	0.00838
GO:0071679	commissural neuron axon guidance	12	2	0.14	0.00838
GO:0030182	neuron differentiation	950	20	11.16	0.0084
GO:0006928	movement of cell or subcellular component	1148	23	13.48	0.00856
GO:0009306	protein secretion	69	4	0.81	0.00882
GO:0035592	establishment of protein localization to extracellular region	69	4	0.81	0.00882
GO:0043009	chordate embryonic development	522	13	6.13	0.0089
GO:0010604	positive regulation of macromolecule metabolic process	958	20	11.25	0.00916
GO:0009792	embryo development ending in birth or egg hatching	524	13	6.15	0.00917
GO:0051254	positive regulation of RNA metabolic process	524	13	6.15	0.00917
GO:0033500	carbohydrate homeostasis	37	3	0.43	0.00925
GO:0042593	glucose homeostasis	37	3	0.43	0.00925
GO:0071692	protein localization to extracellular region	70	4	0.82	0.00927
GO:0031328	positive regulation of cellular biosynthetic process	525	13	6.17	0.00931
GO:0050767	regulation of neurogenesis	153	6	1.8	0.00947
GO:0008543	fibroblast growth factor receptor signaling pathway	71	4	0.83	0.00973
GO:0001678	cellular glucose homeostasis	13	2	0.15	0.00983
GO:0021551	central nervous system morphogenesis	13	2	0.15	0.00983
GO:0048898	anterior lateral line system development	13	2	0.15	0.00983
GO:0009891	positive regulation of biosynthetic process	529	13	6.21	0.00988
GO:0044344	cellular response to fibroblast growth factor stimulus	72	4	0.85	0.01021
GO:0071774	response to fibroblast growth factor	72	4	0.85	0.01021
GO:0048732	gland development	253	8	2.97	0.01023
GO:0031325	positive regulation of cellular metabolic process	972	20	11.42	0.01063
GO:0062012	regulation of small molecule metabolic process	73	4	0.86	0.0107
GO:0048878	chemical homeostasis	478	12	5.61	0.01109
GO:0048882	lateral line development	114	5	1.34	0.01114
GO:0021675	nerve development	74	4	0.87	0.01121
GO:0001501	skeletal system development	366	10	4.3	0.01148
GO:0071310	cellular response to organic substance	789	17	9.27	0.01189
GO:0007178	transmembrane receptor protein serine/threonine kinase signaling pathway	212	7	2.49	0.01278

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0014020	primary neural tube formation	15	2	0.18	0.01303
GO:0022612	gland morphogenesis	15	2	0.18	0.01303
GO:0009893	positive regulation of metabolic process	1064	21	12.5	0.01381
GO:0048915	posterior lateral line system development	79	4	0.93	0.014
GO:0044332	Wnt signaling pathway involved in dorsal/ventral axis specification	16	2	0.19	0.01478
GO:0045935	positive regulation of nucleobase-containing compound metabolic process	560	13	6.58	0.01526
GO:0001706	endoderm formation	45	3	0.53	0.01578
GO:0003205	cardiac chamber development	45	3	0.53	0.01578
GO:0048881	mechanosensory lateral line system development	82	4	0.96	0.01586
GO:1901615	organic hydroxy compound metabolic process	222	7	2.61	0.01609
GO:0021510	spinal cord development	83	4	0.97	0.01652
GO:0060828	regulation of canonical Wnt signaling pathway	126	5	1.48	0.01657
GO:0030512	negative regulation of transforming growth factor beta receptor signaling pathway	17	2	0.2	0.01662
GO:0035622	intrahepatic bile duct development	17	2	0.2	0.01662
GO:0036065	fucosylation	17	2	0.2	0.01662
GO:0042573	retinoic acid metabolic process	17	2	0.2	0.01662
GO:0042663	regulation of endodermal cell fate specification	17	2	0.2	0.01662
GO:0060038	cardiac muscle cell proliferation	17	2	0.2	0.01662
GO:0046903	secretion	277	8	3.25	0.01686
GO:0044255	cellular lipid metabolic process	635	14	7.46	0.01804
GO:0030111	regulation of Wnt signaling pathway	177	6	2.08	0.0183
GO:0003208	cardiac ventricle morphogenesis	18	2	0.21	0.01856
GO:0021548	pons development	18	2	0.21	0.01856
GO:0043433	negative regulation of DNA-binding transcription factor activity	18	2	0.21	0.01856
GO:0086001	cardiac muscle cell action potential	18	2	0.21	0.01856
GO:0033692	cellular polysaccharide biosynthetic process	48	3	0.56	0.01874
GO:0046839	phospholipid dephosphorylation	48	3	0.56	0.01874
GO:0008284	positive regulation of cell population proliferation	131	5	1.54	0.01927
GO:0030258	lipid modification	131	5	1.54	0.01927
GO:0032787	monocarboxylic acid metabolic process	285	8	3.35	0.01964
GO:0000271	polysaccharide biosynthetic process	49	3	0.58	0.0198
GO:0009749	response to glucose	19	2	0.22	0.02058
GO:0019827	stem cell population maintenance	19	2	0.22	0.02058
GO:0055017	cardiac muscle tissue growth	19	2	0.22	0.02058
GO:0060897	neural plate regionalization	19	2	0.22	0.02058
GO:1903224	regulation of endodermal cell differentiation	19	2	0.22	0.02058
GO:0051223	regulation of protein transport	50	3	0.59	0.02088
GO:0070201	regulation of establishment of protein localization	50	3	0.59	0.02088
GO:0051093	negative regulation of developmental process	184	6	2.16	0.02169
GO:0048278	vesicle docking	51	3	0.6	0.022
GO:0009743	response to carbohydrate	20	2	0.23	0.0227

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0009746	response to hexose	20	2	0.23	0.0227
GO:0014855	striated muscle cell proliferation	20	2	0.23	0.0227
GO:0034284	response to monosaccharide	20	2	0.23	0.0227
GO:0045814	negative regulation of gene expression, epigenetic	20	2	0.23	0.0227
GO:0060896	neural plate pattern specification	20	2	0.23	0.0227
GO:0061009	common bile duct development	20	2	0.23	0.0227
GO:0014031	mesenchymal cell development	137	5	1.61	0.02289
GO:0014032	neural crest cell development	137	5	1.61	0.02289
GO:0048864	stem cell development	137	5	1.61	0.02289
GO:0001944	vasculature development	530	12	6.22	0.02299
GO:0055113	epiboly involved in gastrulation with mouth forming second	52	3	0.61	0.02315
GO:0060284	regulation of cell development	187	6	2.2	0.02326
GO:0001745	compound eye morphogenesis	2	1	0.02	0.02335
GO:0002568	somatic diversification of T cell receptor genes	2	1	0.02	0.02335
GO:0002681	somatic recombination of T cell receptor gene segments	2	1	0.02	0.02335
GO:0003223	ventricular compact myocardium morphogenesis	2	1	0.02	0.02335
GO:0006535	cysteine biosynthetic process from serine	2	1	0.02	0.02335
GO:0008057	eye pigment granule organization	2	1	0.02	0.02335
GO:0009182	purine deoxyribonucleoside diphosphate metabolic process	2	1	0.02	0.02335
GO:0009191	ribonucleoside diphosphate catabolic process	2	1	0.02	0.02335
GO:0015722	canalicular bile acid transport	2	1	0.02	0.02335
GO:0021531	spinal cord radial glial cell differentiation	2	1	0.02	0.02335
GO:0022029	telencephalon cell migration	2	1	0.02	0.02335
GO:0030205	dermatan sulfate metabolic process	2	1	0.02	0.02335
GO:0031065	positive regulation of histone deacetylation	2	1	0.02	0.02335
GO:0033153	T cell receptor V(D)J recombination	2	1	0.02	0.02335
GO:0034333	adherens junction assembly	2	1	0.02	0.02335
GO:0046066	dGDP metabolic process	2	1	0.02	0.02335
GO:0048749	compound eye development	2	1	0.02	0.02335
GO:0048890	lateral line ganglion development	2	1	0.02	0.02335
GO:0060290	transdifferentiation	2	1	0.02	0.02335
GO:0060363	cranial suture morphogenesis	2	1	0.02	0.02335
GO:0061378	corpora quadrigemina development	2	1	0.02	0.02335
GO:0062236	ionocyte differentiation	2	1	0.02	0.02335
GO:0086012	membrane depolarization during cardiac muscle cell action potential	2	1	0.02	0.02335
GO:0090312	positive regulation of protein deacetylation	2	1	0.02	0.02335
GO:0098902	regulation of membrane depolarization during action potential	2	1	0.02	0.02335
GO:1900825	regulation of membrane depolarization during cardiac muscle cell action potential	2	1	0.02	0.02335
GO:1902033	regulation of hematopoietic stem cell proliferation	2	1	0.02	0.02335
GO:1905178	regulation of cardiac muscle tissue regeneration	2	1	0.02	0.02335

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:2001295	malonyl-CoA biosynthetic process	2	1	0.02	0.02335
GO:0010033	response to organic substance	990	19	11.63	0.02429
GO:0048840	otolith development	53	3	0.62	0.02433
GO:0098727	maintenance of cell number	21	2	0.25	0.02489
GO:0009799	specification of symmetry	299	8	3.51	0.02526
GO:0009855	determination of bilateral symmetry	299	8	3.51	0.02526
GO:0040007	growth	420	10	4.93	0.027
GO:0021538	epithalamus development	22	2	0.26	0.02718
GO:0030522	intracellular receptor signaling pathway	56	3	0.66	0.02807
GO:0034637	cellular carbohydrate biosynthetic process	56	3	0.66	0.02807
GO:0002011	morphogenesis of an epithelial sheet	98	4	1.15	0.02839
GO:0001714	endodermal cell fate specification	23	2	0.27	0.02954
GO:0021537	telencephalon development	23	2	0.27	0.02954
GO:0021587	cerebellum morphogenesis	23	2	0.27	0.02954
GO:0050821	protein stabilization	23	2	0.27	0.02954
GO:0001755	neural crest cell migration	100	4	1.17	0.03027
GO:0048332	mesoderm morphogenesis	58	3	0.68	0.03072
GO:0060215	primitive hemopoiesis	58	3	0.68	0.03072
GO:0006629	lipid metabolic process	817	16	9.59	0.03172
GO:0001711	endodermal cell fate commitment	24	2	0.28	0.03198
GO:0034308	primary alcohol metabolic process	24	2	0.28	0.03198
GO:0072329	monocarboxylic acid catabolic process	59	3	0.69	0.0321
GO:0061053	somite development	150	5	1.76	0.03214
GO:0001756	somitogenesis	102	4	1.2	0.03222
GO:0065008	regulation of biological quality	1734	29	20.36	0.03274
GO:0014033	neural crest cell differentiation	151	5	1.77	0.03294
GO:0048589	developmental growth	374	9	4.39	0.03297
GO:0022406	membrane docking	60	3	0.7	0.0335
GO:0140056	organelle localization by membrane tethering	60	3	0.7	0.0335
GO:0048705	skeletal system morphogenesis	205	6	2.41	0.0343
GO:0060972	left/right pattern formation	25	2	0.29	0.03449
GO:0001712	ectodermal cell fate commitment	3	1	0.04	0.03482
GO:0001715	ectodermal cell fate specification	3	1	0.04	0.03482
GO:0003254	regulation of membrane depolarization	3	1	0.04	0.03482
GO:0006521	regulation of cellular amino acid metabolic process	3	1	0.04	0.03482
GO:0006642	triglyceride mobilization	3	1	0.04	0.03482
GO:0009155	purine deoxyribonucleotide catabolic process	3	1	0.04	0.03482
GO:0010668	ectodermal cell differentiation	3	1	0.04	0.03482
GO:0019371	cyclooxygenase pathway	3	1	0.04	0.03482
GO:0021588	cerebellum formation	3	1	0.04	0.03482
GO:0021797	forebrain anterior/posterior pattern specification	3	1	0.04	0.03482
GO:0021885	forebrain cell migration	3	1	0.04	0.03482
GO:0030091	protein repair	3	1	0.04	0.03482
GO:0030326	embryonic limb morphogenesis	3	1	0.04	0.03482
GO:0033137	negative regulation of peptidyl-serine phosphorylation	3	1	0.04	0.03482

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0033238	regulation of cellular amine metabolic process	3	1	0.04	0.03482
GO:0034110	regulation of homotypic cell-cell adhesion	3	1	0.04	0.03482
GO:0035621	ER to Golgi ceramide transport	3	1	0.04	0.03482
GO:0035774	positive regulation of insulin secretion involved in cellular response to glucose stimulus	3	1	0.04	0.03482
GO:0042662	negative regulation of mesodermal cell fate specification	3	1	0.04	0.03482
GO:0042665	regulation of ectodermal cell fate specification	3	1	0.04	0.03482
GO:0042766	nucleosome mobilization	3	1	0.04	0.03482
GO:0043586	tongue development	3	1	0.04	0.03482
GO:0043587	tongue morphogenesis	3	1	0.04	0.03482
GO:0045719	negative regulation of glycogen biosynthetic process	3	1	0.04	0.03482
GO:0048785	hatching gland development	3	1	0.04	0.03482
GO:0050655	dermatan sulfate proteoglycan metabolic process	3	1	0.04	0.03482
GO:0061193	taste bud development	3	1	0.04	0.03482
GO:0061194	taste bud morphogenesis	3	1	0.04	0.03482
GO:0061195	taste bud formation	3	1	0.04	0.03482
GO:0061299	retina vasculature morphogenesis in camera-type eye	3	1	0.04	0.03482
GO:0070874	negative regulation of glycogen metabolic process	3	1	0.04	0.03482
GO:0071586	CAAX-box protein processing	3	1	0.04	0.03482
GO:0080120	CAAX-box protein maturation	3	1	0.04	0.03482
GO:0090104	pancreatic epsilon cell differentiation	3	1	0.04	0.03482
GO:0097094	craniofacial suture morphogenesis	3	1	0.04	0.03482
GO:0098529	neuromuscular junction development, skeletal muscle fiber	3	1	0.04	0.03482
GO:1901881	positive regulation of protein depolymerization	3	1	0.04	0.03482
GO:1905771	negative regulation of mesodermal cell differentiation	3	1	0.04	0.03482
GO:2000471	regulation of hematopoietic stem cell migration	3	1	0.04	0.03482
GO:2001293	malonyl-CoA metabolic process	3	1	0.04	0.03482
GO:0001702	gastrulation with mouth forming second	62	3	0.73	0.03641
GO:0044264	cellular polysaccharide metabolic process	62	3	0.73	0.03641
GO:0003231	cardiac ventricle development	26	2	0.31	0.03708
GO:0033002	muscle cell proliferation	26	2	0.31	0.03708
GO:2000027	regulation of animal organ morphogenesis	26	2	0.31	0.03708
GO:0007498	mesoderm development	107	4	1.26	0.03743
GO:0005976	polysaccharide metabolic process	63	3	0.74	0.03791
GO:0090090	negative regulation of canonical Wnt signaling pathway	63	3	0.74	0.03791
GO:0048522	positive regulation of cellular process	1834	30	21.54	0.03877
GO:0048920	posterior lateral line neuromast primordium migration	27	2	0.32	0.03975
GO:0060872	semicircular canal development	27	2	0.32	0.03975
GO:0031401	positive regulation of protein modification process	213	6	2.5	0.04014
GO:2000026	regulation of multicellular organismal development	389	9	4.57	0.0407

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:0016331	morphogenesis of embryonic epithelium	65	3	0.76	0.041
GO:0007368	determination of left/right symmetry	271	7	3.18	0.0413
GO:0090287	regulation of cellular response to growth factor stimulus	161	5	1.89	0.04157
GO:0048883	neuromast primordium migration	28	2	0.33	0.04248
GO:0048916	posterior lateral line development	66	3	0.78	0.04259
GO:0048701	embryonic cranial skeleton morphogenesis	164	5	1.93	0.0444
GO:0006904	vesicle docking involved in exocytosis	29	2	0.34	0.04528
GO:0008286	insulin receptor signaling pathway	29	2	0.34	0.04528
GO:0001705	ectoderm formation	4	1	0.05	0.04616
GO:0003139	secondary heart field specification	4	1	0.05	0.04616
GO:0003311	pancreatic D cell differentiation	4	1	0.05	0.04616
GO:0006272	leading strand elongation	4	1	0.05	0.04616
GO:0007412	axon target recognition	4	1	0.05	0.04616
GO:0009186	deoxyribonucleoside diphosphate metabolic process	4	1	0.05	0.04616
GO:0010889	regulation of sequestering of triglyceride	4	1	0.05	0.04616
GO:0010890	positive regulation of sequestering of triglyceride	4	1	0.05	0.04616
GO:0016103	diterpenoid catabolic process	4	1	0.05	0.04616
GO:0016115	terpenoid catabolic process	4	1	0.05	0.04616
GO:0019343	cysteine biosynthetic process via cystathionine	4	1	0.05	0.04616
GO:0019344	cysteine biosynthetic process	4	1	0.05	0.04616
GO:0021730	trigeminal sensory nucleus development	4	1	0.05	0.04616
GO:0021855	hypothalamus cell migration	4	1	0.05	0.04616
GO:0030730	sequestering of triglyceride	4	1	0.05	0.04616
GO:0031581	hemidesmosome assembly	4	1	0.05	0.04616
GO:0033152	immunoglobulin V(D)J recombination	4	1	0.05	0.04616
GO:0033688	regulation of osteoblast proliferation	4	1	0.05	0.04616
GO:0034653	retinoic acid catabolic process	4	1	0.05	0.04616
GO:0034720	histone H3-K4 demethylation	4	1	0.05	0.04616
GO:0046329	negative regulation of JNK cascade	4	1	0.05	0.04616
GO:0048730	epidermis morphogenesis	4	1	0.05	0.04616
GO:0048909	anterior lateral line nerve development	4	1	0.05	0.04616
GO:0060019	radial glial cell differentiation	4	1	0.05	0.04616
GO:0061303	cornea development in camera-type eye	4	1	0.05	0.04616
GO:0070650	actin filament bundle distribution	4	1	0.05	0.04616
GO:0070986	left/right axis specification	4	1	0.05	0.04616
GO:0072574	hepatocyte proliferation	4	1	0.05	0.04616
GO:0072575	epithelial cell proliferation involved in liver morphogenesis	4	1	0.05	0.04616
GO:0098773	skin epidermis development	4	1	0.05	0.04616
GO:1901166	neural crest cell migration involved in autonomic nervous system development	4	1	0.05	0.04616
GO:2000095	regulation of Wnt signaling pathway, planar cell polarity pathway	4	1	0.05	0.04616

GOID	GO-term	Annotated	Significant	Expected	pvalFisher
GO:2000179	positive regulation of neural precursor cell proliferation	4	1	0.05	0.04616
GO:0007492	endoderm development	69	3	0.81	0.04755
GO:0070121	Kupffer's vesicle development	69	3	0.81	0.04755
GO:0140352	export from cell	280	7	3.29	0.04775
GO:0040036	regulation of fibroblast growth factor receptor signaling pathway	30	2	0.35	0.04815
GO:0048854	brain morphogenesis	30	2	0.35	0.04815
GO:1902036	regulation of hematopoietic stem cell differentiation	30	2	0.35	0.04815
GO:0042221	response to chemical	1652	27	19.4	0.04923
GO:0042180	cellular ketone metabolic process	70	3	0.82	0.04927

Supplementary Data Table 2 | complete list of all nervous system related GO-terms in the studied gene list, the Significant column represents the number of genes regulated in the GO-term.

GOID	GO-term	Annotated	Significant	Expected	pvalFisher	Enrichment
GO:0007417	central nervous system development	686	39	8,06	0,0	4,8
GO:0007399	nervous system development	1728	62	20,29	0,0	3,1
GO:0007420	brain development	480	32	5,64	0,0	5,7
GO:0030902	hindbrain development	132	18	1,55	0,0	11,6
GO:0021546	rhombomere development	21	5	0,25	0,000	20,0
GO:0030900	forebrain development	140	10	1,64	0,000	6,1
GO:0021570	rhombomere 4 development	5	3	0,06	0,000	50,0
GO:0021661	rhombomere 4 morphogenesis	5	3	0,06	0,000	50,0
GO:0021915	neural tube development	108	8	1,27	0,000	6,3
GO:0021593	rhombomere morphogenesis	19	4	0,22	0,000	18,2
GO:0048699	generation of neurons	1020	27	11,98	0,000	2,3
GO:0022008	neurogenesis	1136	29	13,34	0,000	2,2
GO:0021575	hindbrain morphogenesis	37	5	0,43	0,000	11,6
GO:0021592	fourth ventricle development	8	3	0,09	0,000	33,3
GO:0021879	forebrain neuron differentiation	24	4	0,28	0,000	14,3
GO:0001764	neuron migration	46	5	0,54	0,000	9,3
GO:0021872	forebrain generation of neurons	27	4	0,32	0,000	12,5
GO:0022037	metencephalon development	58	5	0,68	0,001	7,4
GO:0021571	rhombomere 5 development	4	2	0,05	0,001	40,0
GO:0021572	rhombomere 6 development	4	2	0,05	0,001	40,0
GO:0021767	mammillary body development	4	2	0,05	0,001	40,0
GO:0061351	neural precursor cell proliferation	37	4	0,43	0,001	9,3
GO:0030917	midbrain-hindbrain boundary development	41	4	0,48	0,001	8,3
GO:0021549	cerebellum development	42	4	0,49	0,001	8,2
GO:0021534	cell proliferation in hindbrain	6	2	0,07	0,002	28,6
GO:0097150	neuronal stem cell population maintenance	6	2	0,07	0,002	28,6

GOLD	GO-term	Annotated	Significant	Expected	pvalFisher	Enrichment
GO:0021903	rostrocaudal neural tube patterning	47	4	0,55	0,002	7,3
GO:0021953	central nervous system neuron differentiation	118	6	1,39	0,003	4,3
GO:0021536	diencephalon development	85	5	1	0,003	5,0
GO:0048857	neural nucleus development	26	3	0,31	0,003	9,7
GO:0021854	hypothalamus development	27	3	0,32	0,004	9,4
GO:0051960	regulation of nervous system development	169	7	1,98	0,004	3,5
GO:0021591	ventricular system development	28	3	0,33	0,004	9,1
GO:0021761	limbic system development	28	3	0,33	0,004	9,1
GO:0021532	neural tube patterning	56	4	0,66	0,004	6,1
GO:0021545	cranial nerve development	56	4	0,66	0,004	6,1
GO:0030901	midbrain development	35	3	0,41	0,008	7,3
GO:0007422	peripheral nervous system development	68	4	0,8	0,008	5,0
GO:0021555	midbrain-hindbrain boundary morphogenesis	12	2	0,14	0,008	14,3
GO:0071679	commissural neuron axon guidance	12	2	0,14	0,008	14,3
GO:0030182	neuron differentiation	950	20	11,16	0,008	1,8
GO:0050767	regulation of neurogenesis	153	6	1,8	0,009	3,3
GO:0021551	central nervous system morphogenesis	13	2	0,15	0,010	13,3
GO:0021675	nerve development	74	4	0,87	0,011	4,6
GO:0014020	primary neural tube formation	15	2	0,18	0,013	11,1
GO:0021510	spinal cord development	83	4	0,97	0,017	4,1
GO:0021548	pons development	18	2	0,21	0,019	9,5
GO:0060897	neural plate regionalization	19	2	0,22	0,021	9,1
GO:0060896	neural plate pattern specification	20	2	0,23	0,023	8,7
GO:0021531	spinal cord radial glial cell differentiation	2	1	0,02	0,023	50,0
GO:0022029	telencephalon cell migration	2	1	0,02	0,023	50,0
GO:0048890	lateral line ganglion development	2	1	0,02	0,023	50,0
GO:0061378	corpora quadrigemina development	2	1	0,02	0,023	50,0
GO:0021537	telencephalon development	23	2	0,27	0,030	7,4
GO:0021587	cerebellum morphogenesis	23	2	0,27	0,030	7,4
GO:0021588	cerebellum formation	3	1	0,04	0,035	25,0
GO:0021797	forebrain anterior/posterior pattern specification	3	1	0,04	0,035	25,0
GO:0021885	forebrain cell migration	3	1	0,04	0,035	25,0
GO:0021730	trigeminal sensory nucleus development	4	1	0,05	0,046	20,0
GO:0021855	hypothalamus cell migration	4	1	0,05	0,046	20,0
GO:0048909	anterior lateral line nerve development	4	1	0,05	0,046	20,0
GO:0060019	radial glial cell differentiation	4	1	0,05	0,046	20,0
GO:1901166	neural crest cell migration involved in autonomic nervous system development	4	1	0,05	0,046	20,0
GO:2000179	positive regulation of neural precursor cell proliferation	4	1	0,05	0,046	20,0
GO:0048854	brain morphogenesis	30	2	0,35	0,048	5,7

Supplementary Data Table 3 | 62 DEGs implicated in the principal GO-term of nervous system development GO-term, the bold GO-term represent the ancestor term to the GO-term of the same color.

GO-term	Neural tube development Rostral neural tube patterning Primary neural tube formation	Neural crest cell migration involvement in autonomic nervous system development Neural plate regionalization	Neural precursor cell proliferation Positive regulation of neural precursor	Neurogenesis Neuron differentiation Neuron migration	Central nervous system development Central nervous system morphogenesis	Brain development Hindbrain development Forebrain development Midbrain development Trigeminal sensory nucleus development Brain morphogenesis Ventricular system dev	Nerve development Pheriperic nervous system development	Regulation nervous system development
zranb1a					X	X		
znf703					X	X X		
znf503					X	X X		
zic4			X		X	X X	X	
zic1			X		X	X X	X X	
zfyve9a				X				
wnt8b				X X X	X	X		X
vasna				X X X				
tp63					X			
sp5l					X	X X		
six7				X X X				
shtn1				X X x				X
rgs4				X X X				
psmd7					X X			
prickle1b				X	X	X X		
pax8							X	
pax6b					X	X X		
otx2a	X X				X	X X X		
olig4				X X X				
nlgn4xa								
nkx6.1				X X X	X			
neurog1				X X X	X	X X	X X X	
nes			X X		X	X	X	
nav2b				X				
nav2a				X				
metrn				X X X				X
meis3		X			X	X X		
meis2b								
meis2a					X	X		
meis1b					X	X		
map1b	X							
lzt3b				X X X				
lmo4a					X	X		

GO-term	Neural tube development Rostrocaudal neural tube patterning Primary neural tube formation	Neural crest cell migration involvement in autonomic nervous system development Neural plate regionalization	Neural precursor cell proliferation Positive regulation of neural precursor	Neurogenesis Neuron differentiation Neuron migration	Central nervous system development Central nervous system morphogenesis	Brain development Hindbrain development Forebrain development Midbrain development Trigeminal sensory nucleus development Brain morphogenesis Ventricular system dev	Nerve development Pheripheric nervous system development	Regulation nervous system development
Idlrb					X			
klf6a							X	
josed2					X X			
irx1b				X X X	X	X X		
insm1b				X X X				
hoxb5b					X	X		
hoxb1b	X X				X	X X	X	
hnf1ba					X	X X		
hgfa				X X X	X	X X		
her13				X				X
hapln1b					X			
gsx1				X X X	X			
grhl2b	X X				X	X		
gdpd3a					X	X		
fzd8a				X X X	X	X X		
foxi1				X X X			X X	
foxb1a	X			X X X	X	X X X		
fgf8a	X X			X X X	X	X X X	X	
fezf2				X X X	X	X X		
elavl3				X				X
dixdc1a					X	X		
cyp26c1					X	X X		
cyp26a1	X X				X	X X X	X	
ccndx			X	X				
ccdc85b	X X							
bmi1a					X	X X		
bhlhe40				X				X
atp6v0d1				X X X				
adarb1a								
ackr3b				X	X	X X		

Chapter 4

Retinoic acid signaling pathway perturbation impacts mesodermal-tissue development in the zebrafish embryo: biomarker candidate identification using transcriptomics

Laura M.M. Samrani^{1,2,3}, Florent Dumont⁴,
Nina Hallmark⁵, Rémi Bars⁶, Helen Tinwell⁶,
Marc Pallardy², Aldert H. Piersma^{1,3}

Reproductive Toxicology, 2023 Aug, 108404

DOI: 10.1016/j.reprotox.2023.108404

1. Centre for Health Protection, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
2. Université Paris-Saclay, Inflammation, Microbiome and Immunosurveillance, INSERM, Faculté Pharmacie, 91104 Orsay, France
3. Institute for Risk Assessment Sciences (IRAS), Utrecht University, the Netherlands
4. Université Paris-Saclay, UMS IPSIT, 91104 Orsay, France
5. Bayer AG Crop Science Division, Monheim, Germany
6. Bayer SAS, Lyon, France

Abstract

The zebrafish embryo (ZE) model provides a developmental model well conserved throughout vertebrate embryogenesis, with relevance for early human embryo development. It was employed to search for gene expression biomarkers of compound-induced disruption of mesodermal development. We were particularly interested in the expression of genes related to the retinoic acid signaling pathway (RA-SP), as a major morphogenetic regulating mechanism. We exposed ZE to teratogenic concentrations of valproic acid (VPA) and all-trans retinoic acid (ATRA), using folic acid (FA) as a non-teratogenic control compound shortly after fertilization for 4 hours, and performed gene expression analysis by RNA-sequencing. We identified 248 genes specifically regulated by both teratogens but not by FA. Further analysis of this gene set revealed 54 GO-terms related to the development of mesodermal tissues, distributed along the paraxial, intermediate, and lateral plate sections of the mesoderm. Gene expression regulation was specific to tissues and was observed for somites, striated muscle, bone, kidney, circulatory system, and blood. Stitch analysis revealed 47 regulated genes related to the RA-SP, which were differentially expressed in the various mesodermal tissues. These genes provide potential molecular biomarkers of mesodermal tissue and organ (mal)formation in the early vertebrate embryo.

Keywords: Toxicogenomics, mesoderm, retinoic acid signaling pathway, valproic acid, teratogen, maldevelopment, zebrafish embryo, toxicity mode of action, all-trans retinoic acid, folic acid

Introduction

Current regulatory strategies for human chemical safety assessment rely on the observation of adverse outcomes during animal testing [1, 2]. This approach not only raises ethical concerns but is also time-consuming and expensive. Additionally, there continues to be uncertainty regarding the extrapolation of animal data to humans. Thus, academia, industry, government and regulators are motivated to develop alternatives [3, 4] and are, consequently, investing in new approach methodologies (NAMs), enhancing the mechanistic understanding of a chemical toxicity mode of action (MOA), and thereby improving extrapolation to human hazard and risk [1, 2, 5-8].

The zebrafish embryo (ZE) model is a well-established vertebrate whole embryo tool for studying molecular mechanisms of developmental toxicity. This model is relatively high-throughput and inexpensive while also remaining a complete organism model [9-13]. The intact ZE embryo enables the study of developmental mechanisms at different levels of integration, from molecular changes to pattern and organ formation and organ function. It is also fast-developing, having completed gastrulation after 24 hours post-fertilization (hpf), with most organs fully functional at 120hpf [14]. Since it is not subjected to the EU Directive for animal protection 2010/63/EU before 120 hpf [15], this rapid developmental time frame can be used to study a large spectrum of developmental processes in an animal-free context. Additionally, the early embryology mechanism is highly conserved in the vertebrate taxon [16-20]. Thus, the early morphogenesis effects of chemicals in the zebrafish embryo can be considered relevant for human risk assessment.

The ZE has already been used for several decades as a safety assessment tool to study chemically-induced maldevelopment [21-24]. Although this was historically done by observing morphological effects, the desire to implement a new safety assessment approach has led to an increase in mechanistic based research in this model, which includes protein, epigenetic, and gene expression analysis. Such research has revealed gene expression profiles specific to toxicity mechanisms [25], which may aid interspecies extrapolation. Thus, gene expression regulation offers significant information about mechanisms of action and is a useful tool for studying human developmental toxicity.

Since it is one of the central developmental pathways in the vertebrate, the gene expression regulation of the retinoic acid signaling pathway (RA-SP) is actively studied when researching maldevelopment [26-30]. For decades, the scientific community has been aware of the importance of its main actor, all-trans retinoic acid (ATRA), in development [31-37]. ATRA concentration is regulated by a balance between two families of enzymes, the *raldh* family that metabolizes precursor into ATRA and the *cyp26* family that degrades it; together, they help create local ATRA concentration gradients throughout the embryo [38]. These gradients control a plethora of developmental processes, including craniofacial formation, heart and kidney development, and the formation of early somites [33, 38-44]. Mining this pathway could therefore help to identify teratogenicity biomarker genes [37, 45].

In this study, we explore the perturbation of RA-SP gene expression in the ZE after exposition to three reference compounds: the two teratogens ATRA and valproic acid (VPA), and a non-teratogenic reference compound folic acid (FA).

VPA is known to cause maldevelopment in experimental models [46] and in humans, up to 30% of infants exposed in utero to high dosages, experience intellectual disabilities and/or congenital malformations such as heart defects, urogenital abnormalities, and skeletal defects [28, 47-53]. Although these adverse outcomes have been well defined, their underlying mechanisms remain unclear [49]. The consensus is that maldevelopment is due to VPA-induced HDAC (histone deacetylase) inhibition, which perturbs gene expression. This perturbation includes the RA-SP since VPA regulates ATRA metabolism enzymes (e.g., *cyp26a1* and *aldh1a2*), and the gene expression of developmental regulators known to be transcriptional targets of the RA-SP (e.g., *cdx1* and *hoxa1*) [50, 54]. Thus, studying the VPA-induced perturbation of this signaling pathway could help uncover maldevelopment mechanisms related to RA-SP.

The non-teratogen FA is given during pregnancy to help reduce the chance of congenital malformation [55]. In animal models, FA has been shown to rescue VPA-induced cardiotoxicity and malformation due to an excess of ATRA [55-58]. This protective action is believed to be due to the role of FA as source of one carbon group used to methylate DNA during early development [69]. For these reasons, it has been chosen as the non-teratogenic reference compound in the present study.

Shortly after fertilization, we exposed groups of zebrafish embryos to FA (FA 70 μ M) and each teratogen (ATRA 10nM; VPA 1000 μ M) for 4 hours. This time point was shown in a previous study to be optimal for observing significant transcriptomic changes in the RA-SP [59]. Samrani et al. (Toxicology Letters, 2023 Aug, 384; 96-104, DOI: 10.1016/j.toxlet.2023.07.005) explored the RA-SP-related gene expression changes to find potential neurodevelopmental toxicity biomarkers. As VPA and ATRA treatment causes principally heart and skeletal (scoliosis) development in the ZE model, we here used the same strategy as described by Samrani et al (Toxicology Letters, 2023 Aug, 384; 96-104, DOI: 10.1016/j.toxlet.2023.07.005) to search for additional maldevelopment biomarkers in mesoderm-derivatives.

Methods

Zebrafish maintenance and embryo generation

The AB strain of zebrafish were bred and handled in-house (RIVM laboratory) according to Dutch regulations under permit NVWA-32600. In the 8L Tecniplast ZebTech tanks the temperature, pH, and conductivity conditions were maintained between $27.5 \pm 0.5^{\circ}\text{C}$, 7.5 ± 0.5 , and $500 \pm 100\mu\text{S}$, with a light/dark cycle of 14/10hrs, respectively. Embryo collection was performed in Petri dishes with Dutch Standard Water (DSW; demineralized water supplemented with 100mg/l NaHCO_3 , 20mg/l KHCO_3 , 200mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 180mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and then aerated for 24 h at 27°C) 30 minutes after spawning, and good quality was confirmed by microscopy.

Compounds

All the compounds were purchased from Sigma-Aldrich, Zwijndrecht, Netherlands, including dimethyl sulfoxide (DMSO, CAS# 67-68-5), all-trans retinoic acid (ATRA, CAS# 302-79-4), valproic acid (VPA, CAS# 1069-66-5), and folic acid (FA, CAS# 59-30-3). All teratogenic compounds were used at an equipotent concentration equal to their CED20 (critical effect dose 20%), defined from a dose-response analysis as the concentration at which the general morphology scoring (GMS) is reduced by 20% [60]. These concentrations were established as 10 nM ATRA and 1000 μ M VPA using PROAST (version 69) software. The FA concentration (70 μ M) was selected based on publicly

available literature [56, 61, 62], then verified by dose-response analysis using the Hermesen scoring system to ensure FA did not induce developmental delay, malformation, or toxicity at the concentration selected.

Exposure

Each sample was composed of 30 good quality eggs separated in two wells with 5 ml test medium. The test medium contained at least DSW plus 0.1% DMSO for control and treated samples, for the treated samples the different chemicals at the concentrations mentioned above were added to the test medium. The plates were incubated at 27.5°C for 4 hours. Two samples per condition were collected in 2 ml low-binding DNA tubes for each of the four independent experiments. Thirty eggs (2 wells) were used for one sample. After collection, the samples were placed at -80°C before RNA isolation. For each experiment, an additional 5 to 10 eggs per condition were allowed to develop for 72 hours post fertilization (hpf) in parallel to confirm the effect of the compound on development.

RNA isolation

The samples were processed according to the manufacturer's protocol using the RNeasy Mini kit (Qiagen, Cat# 74104). The samples were homogenized in 700µM Qiazol Lysis Reagent (Qiagen, Cat# 79306) using a prospective autoclave blender, then 300 µl of Qiazol was added. A DNase step was done using an RNase-Free DNase set (Qiagen, Cat # 79254). The sample RNA quantity and quality were assessed, using the Qubit3 (Invitrogen, Carlsbad, CA, USA) and 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). All samples scored greater than 7.5 on the RIN (RNA Integrity Number) scale.

RNA sequencing

RNA sequencing

GenomeScan (Leiden, The Netherlands) processed and analyzed the RNA samples, following the same protocol as Mennen et al. 2022 [63]. The gene expression transcripts were mapped and annotated using the Ensembl zebrafish genome assembly GRCz11 + corresponding GTF file.

Statistical analysis

Genes with a read count mean for all samples lower than 10 were discarded for downstream analysis. Statistical analysis was done using a read data count matrix for 32 samples and 15710 Ensembl genes. Data were first linearized using the voom function from the Limma R package [64], then a quantile normalization, and log2 transformation was applied. Then, differentially expressed genes (DEGs) were found by applying a two-way analysis of variance of MUTATION and BATCH factors and by performing a pairwise Tukey post hoc test between groups. DEGs were obtained by filtering results for $p < 0.05$, fold-change > 1.1 for upregulation, and fold-change < -1.1 for downregulation. Enrichment analysis was performed using the MSigDB v7.5 [65] as a gene-set database and a Fisher exact test with false discovery rate (FDR) correction 5%. Interaction networks were generated using STITCH interaction networks of chemicals [66], and Cytoscape software (version 3.9.1) were used for visualization of the interaction network.

All processed data have been submitted to GEO NCBI database with the accession number GSE226367.

Mesoderm tissue GO-term selection

Tissues derived from the mesoderm layer (discovery.lifemapsc.com) were researched using associated keywords. The tissue names were entered into Quickgo website, where representative keywords emerged after studying the “ancestor chart” and “child terms” sections. These keywords were then used in our Gene Ontology (GO)-term list, to find the GO-terms related to the mesoderm tissues. The genes associated with the select GO-terms were used to generate a network in STITCH and create heatmaps.

Results

Analysis of mesodermal tissues perturbed by both teratogen treatments

Prior to this analysis, a gene set of 248 differentially expressed genes (DEG) were selected corresponding to the genes commonly regulated by both teratogenic compounds (ATRA and VPA) but not by the non-teratogenic compound (FA) ($p < 0.05$, $FC > 1.1$ and $FC < -1.1$). A gene ontology analysis was then performed on this gene set, leading to 533 related GO-terms (Toxicology Letters, 2023 Aug, 384; 96-104, DOI: 10.1016/j.toxlet.2023.07.005).

To investigate ATRA and VPA induced perturbation of gene expression related to mesodermal derived tissues, we first studied the responsive gene ontology (Figure 1BC). Out of the 533 initial GO-terms, 54 were

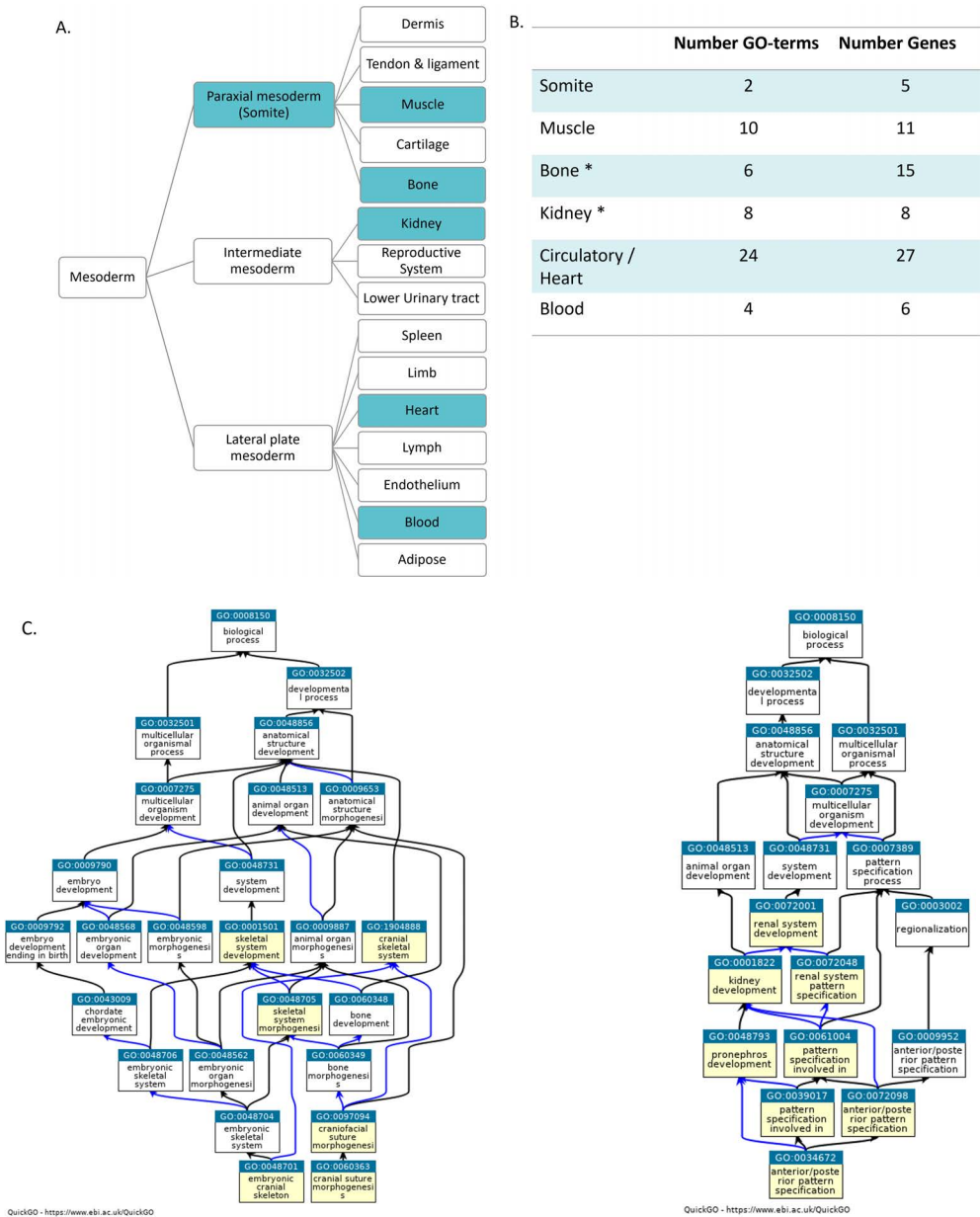
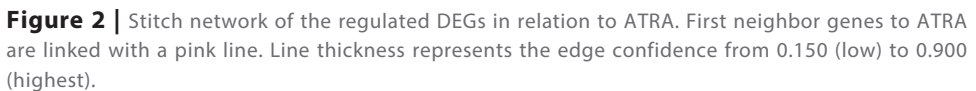


Figure 1 | A Mesoderm derived tissues summary figure inspired by discovery.lifemaps.com. The tissues with regulated DEG are in blue; B. Table of the number of GO-terms and DEG regulated in each tissue, the * indicates the tissues selected to show C. ancestor charts of GO-terms of the selected example tissues. In yellow are the regulated GO-terms amongst the hierarchy of GO-terms for each of the tissues.

associated with the development of mesodermal-derived tissue (figure 1) (Supplementary Data table 1-5). For example, heart development is related to 24 responsive GO-terms and 27 responsive DEGs, the highest numbers among the organ systems that showed related gene expression changes after exposure. Overall, these GO-terms were associated with the development of six tissues which originate from different mesodermal sections (Figure 1A). The somite, and skeletal system (including skeletal muscle and bones) derive from the paraxial mesoderm, the kidney system and the urogenital system derive from the intermediate mesoderm and the hematopoiesis (blood), the heart and circulatory system derive from the lateral plate mesoderm.

Analysis of DEGs implicated in mesodermal development related to the RA-SP

We created an ATRA-related gene network using the STITCH database and the DEG regulated in these tissues (Figure 2). We observed the presence of genes involved in retinoic acid metabolism (e.g., *cyp26a1*, *cyp26c1*), of genes involved in other major developmental pathways known to have cross-regulation with the RA-SP (e.g., *bmp1a*, *fgf8a*, and *fzd4*) and of well-known patterning gene targets of ATRA regulation (e.g., *hoxb5*, *meis1*, *pax8*). Thus, 47 genes in this study are potential biomarkers for mesodermal maldevelopment.



We then investigated these biomarker candidate genes by mesoderm sections. In the paraxial, intermediate, and lateral plate mesoderm, there are 24, 8, and 30 genes, respectively, regulated by both ATRA and VPA (Figure 3). Although these potential gene biomarkers are regulated by both compounds, each treatment leads to a specific-gene signature profile. Thus, 4 clusters are observed on each of the heatmaps (Figure 3ACD). In two of the clusters, the DEGs are regulated in opposite directions: 14, 5, 17 DEGs up-regulated by VPA and down-regulated by ATRA; and 2, 1, 5 DEGs down-regulated by VPA and up-regulated by ATRA. In the other two clusters, the DEGs are regulated in the same direction: 2, 1, 4 DEGs commonly up-regulated; and 5, 1, 5 DEGs commonly down-regulated.

A comparison can be made between the DEGs of the different sections. In both the lateral and paraxial mesoderm sections, 11 common DEGs are regulated: these include RA-SP related genes such as *meis1b*, *klf2b*, *nr2f1a*, *cyp26c1*, and *fgf8a*. For the lateral plate and intermediate mesoderm sections, the *atp6v0d1* is commonly regulated. Yet for the three studied sections of the mesoderm, only one DEG is commonly regulated: *cyp26a1* (Figure 2BCD). The presence and regulation of this major actor in the RA-SP metabolism highlights the importance of this pathway in the development of the mesoderm and its derived tissues and organs.

Analysis of DEGs by mesodermal tissues

We then investigated each tissue, comparing the DEG list between these different tissues. We observed that some DEGs are unique to one specific tissue: for example, *josed2*, *myt1a*, and *adarb1a*, are implicated only in skeletal development (Figure 4A); *fgfr4*, *tph1b*, *klf6a*, and *bmi1a* are specific to hematopoiesis (Figure 4E); and *hnf1ba*, *ift172*, *mpped2*, *myo1cb*, *pax8*, and *ptgs2a* are specific to kidney development (Figure 3D). In contrast, in one mesoderm section some DEGs are implicated in several tissues. For example, *inpp5ka* is implicated in somite formation and muscle development (Figure 4BC), and *sox17* is implicated in hematopoiesis and heart development (Figure 4DE). Thus, the 47 potential gene expression biomarkers could be used to study maldevelopment at a different level of specificity, from the mesoderm level to each specific organ.

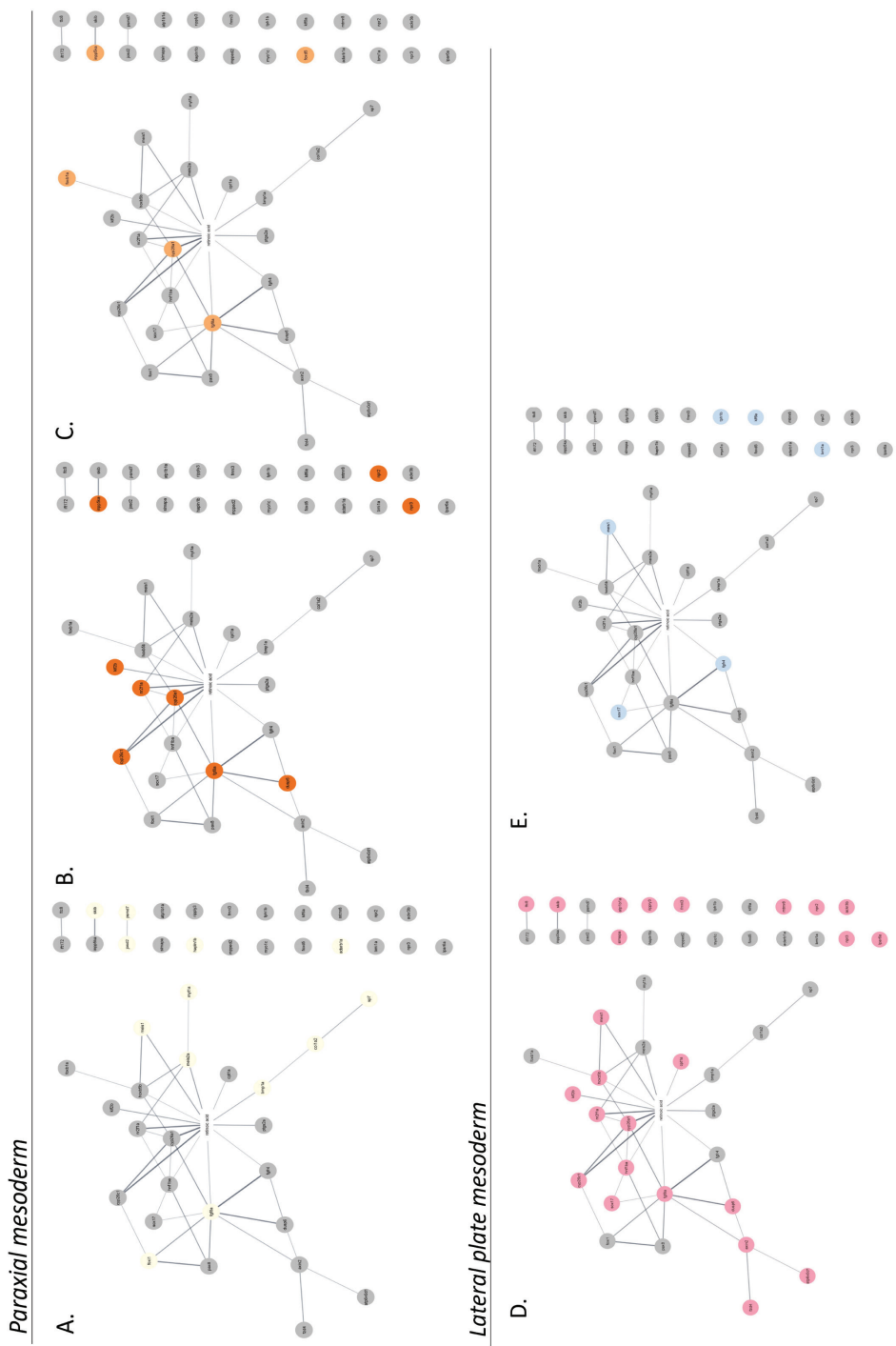


Figure 4 | Stitch network of the 47 DEGs regulated (network identical to figure 2) related to A. bone/skeletal tissue in light yellow, B. muscle in orange, C. somite in light orange, D. circulatory/heart development in pink, E. blood cell development in light blue.

Discussion

In this study, we determined the regulation of RA-SP related developmental genes associated with tissues from a mesodermal origin in the ZE, and thus identified possible early maldevelopment gene expression biomarkers of the mesoderm layer. This was done by further exploring NGS data after exposure to known teratogens ATRA and VPA, and a non-teratogenic reference compound FA.

The mesoderm layer is responsible for the formation of critical structures within the developing embryo, including the circulatory, skeletal, muscular, excretory, and reproductive systems [67]. These systems are highly sensitive to some xenobiotics due to the precise regulation necessary for their establishment and axis formation. RA-SP plays a crucial role in both mesodermal organ development, and in axis formation. For example, ATRA is responsible for left-right patterning during heart development and somitogenesis, and for the antero-posterior patterning during presomitic mesoderm and digit formation [31, 68-70]. ATRA and *fgf8* antiparallel gradients are known to define the boundaries of the heart field, and to regulate chamber size and identity. Thus, RA-SP perturbation may lead to cardiotoxicity, urogenital tract malformation, and skeletal defects [71, 72]. These particular congenital anomalies are known to be caused by ATRA and VPA treatment. Due to the adverse outcomes caused by exposure to these compounds and their well-studied MOA, which includes RA-SP genes, these teratogens were used as example compounds in this study. We observed that both compounds induced a perturbation of the RA-SP; however, this perturbation diverges depending on the compound. For example, the following DEGs were found to be up-regulated by ATRA and down-regulated by VPA in our study : *cyp26*, the crucial gene family responsible for the metabolism of ATRA ; the morphogens *fgf8a* and *bmp1a* [31]; the morphogen receptors *fzd4*, and *fgfr4*; and the direct effectors of RA-SP, *meis1*, *meis2*, and *pax8*. These findings are consistent with other studies using zebrafish and other models [33, 59, 73].

To our knowledge, this is the only toxicity study done after a short exposure duration of 4 hrs, focusing on early development (7 hpf), whereas most research has focused on the 24 – 120 hpf time points [74-77]. Most existing transcriptomic studies in the ZE use a curated gene list, whereas the present whole-genome-scale strategy [78, 79] has enabled us to study all

regulated genes, thereby offering a more comprehensive view towards the understanding of compound MOAs and a more unbiased early biomarker selection. To study the toxicity MOA related to the RA-SP and to identify these biomarkers, we used the gene list extracted from Samrani et al. 2023. This list contained DEGs regulated by both teratogenic compounds but not by FA. From this list, we then focused on the DEGs implicated in tissue development of mesodermal origin by GO-term selection (Figure 1C, Supplementary data Figure 1). These 54 GO-terms were related to mesodermal system development such as skeletal system morphogenesis, muscle tissue development, renal system pattern specification, and heart growth. This analysis left 47 possible biomarker candidates, with different levels of specificity. Our research found that the DEGs present in several mesoderm sections are subject to ATRA regulation (Figure 2) and play a crucial role in development; for example, ATRA metabolism genes *cyp26a1* and *cyp26c1*, ATRA effectors *meis1b*, *klf2b*, and *nr2f1a*, and ATRA regulator gene *fgf8a* [80]. Other genes found to be related to processes driven by the RA-SP include *atp6v0d1* implicated in the left-right patterning [81] and *skib* implicated in dorso-ventral patterning [82, 83].

Tissue specific genes are also valuable as they are good candidates for tissue-specific developmental biomarkers. For example, in the circulatory system, the human orthologue of the DEGs *atp1b1a* and *fzd4* are, respectively, associated with essential hypertension [84], and with vitreoretinopathy 1, an incomplete development of the retina vasculature [85, 86]. In the renal system, the human orthologue of the DEG *hnf1ba* is associated with maturity-onset diabetes of the young type 5, an abnormal renal development resulting in non-diabetic kidney disease [87]. Although existing publications have not highlighted these genes as potential biomarkers yet [76, 88, 89], these genes are involved in crucial developmental processes, and their mutations are associated with human developmental anomalies. Such characteristics demonstrate the suitability of these candidates as early developmental biomarkers.

It should be noted that in the study we elected to look at the RA-SP as a source of biomarkers; this implicates focusing on morphogen and morphogen regulators, focusing on other developmental signaling pathways, for example *fgf*, could be interesting next steps. In this study we used whole embryo samples for analysis, in order to have sufficient sample sizes, which precluded the localization within the embryo of the

gene expression changes. An example of a more specific method, single-cell RNA-sequencing, which may aid resolve this challenge was mentioned by Huang and al. [90, 91]. The model chosen may have also brought some limitations. First, although the early embryology is highly conserved through the taxon, some divergence can occur; for example, the human gene SKI has two paralogues in the zebrafish, *skia* and *skib*, each having a specific role in development [83]. Second, the information available on gene identification and human homologies in the ZE model is limited as it is relatively young compared to the mouse or rat, which are the more historical models in toxicology.

In conclusion, our investigation advances the knowledge of the mesodermal tissue toxicity MOA related to the perturbation of the RA-SP, highlighting 47 potential gene expression biomarkers for developmental toxicity. This included 13 DEGs implicated in several mesodermal derived tissues which are considered as potential early biomarkers of mesodermal maldevelopment (Figure 4 and 5) and some novel potential biomarkers for specific mesoderm organs, pointing to the central role of the RA-SP in a plethora of developmental processes. Gene expression biomarkers are thus an especially attractive tool for potentially detecting the possible developmental impact of compounds. To determine their robustness as biomarkers, these candidate genes should be more broadly studied using different *in vitro* models and chemical exposures. Thus, they could contribute to predictive tools in animal-free chemical hazard and risk assessment.

References

1. Ball, N., et al., *A framework for chemical safety assessment incorporating new approach methodologies within REACH*. Arch Toxicol, 2022. **96**(3): p. 743-766.
2. Doke, S.K. and S.C. Dhawale, *Alternatives to animal testing: A review*. Saudi Pharm J, 2015. **23**(3): p. 223-9.
3. Leenaars, C.H.C., et al., *Animal to human translation: a systematic scoping review of reported concordance rates*. J Transl Med, 2019. **17**(1): p. 223.
4. Shanks, N., R. Greek, and J. Greek, *Are animal models predictive for humans?* Philos Ethics Humanit Med, 2009. **4**: p. 2.
5. Balls, M., *Future improvements: replacement in vitro methods*. ILAR J, 2002. **43** Suppl: p. S69-73.
6. Bal-Price, A., et al., *Strategies to improve the regulatory assessment of developmental neurotoxicity (DNT) using in vitro methods*. Toxicol Appl Pharmacol, 2018. **354**: p. 7-18.
7. Pastoor, T.P., et al., *A 21st century roadmap for human health risk assessment*. Crit Rev Toxicol, 2014. **44** Suppl 3: p. 1-5.
8. Mone, M.J., et al., *Setting the stage for next-generation risk assessment with non-animal approaches: the EU-ToxRisk project experience*. Arch Toxicol, 2020. **94**(10): p. 3581-3592.
9. Hansen, J.M., *In vivo models of developmental toxicology*. Methods Mol Biol, 2012. **889**: p. 7-13.
10. Lee, H.Y., et al., *Alternative models in developmental toxicology*. Syst Biol Reprod Med, 2012. **58**(1): p. 10-22.
11. Wixon, J., *Featured organism: Danio rerio, the zebrafish*. Yeast, 2000. **17**(3): p. 225-31.
12. Hill, A.J., et al., *Zebrafish as a model vertebrate for investigating chemical toxicity*. Toxicol Sci, 2005. **86**(1): p. 6-19.
13. Teraoka, H., W. Dong, and T. Hiraga, *Zebrafish as a novel experimental model for developmental toxicology*. Congenit Anom (Kyoto), 2003. **43**(2): p. 123-32.
14. Lawson, N.D., et al., *An improved zebrafish transcriptome annotation for sensitive and comprehensive detection of cell type-specific genes*. Elife, 2020. **9**.
15. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance. 2010; Available from: <http://data.europa.eu/eli/dir/2010/63/oj>.
16. Ali, S., et al., *Zebrafish embryos and larvae: a new generation of disease models and drug screens*. Birth Defects Res C Embryo Today, 2011. **93**(2): p. 115-33.
17. Ali, S., H.G. van Mil, and M.K. Richardson, *Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing*. PLoS One, 2011. **6**(6): p. e21076.
18. Collazo, A., *Developmental variation, homology, and the pharyngula stage*. Syst Biol, 2000. **49**(1): p. 3-18.
19. Irie, N. and S. Kuratani, *Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis*. Nat Commun, 2011. **2**: p. 248.
20. Falfushynska, H., et al., *Toxic effects and mechanisms of common pesticides (Roundup and chlorpyrifos) and their mixtures in a zebrafish model (Danio rerio)*. Sci Total Environ, 2022. **833**: p. 155236.
21. Nishimura, Y., et al., *Using zebrafish in systems toxicology for developmental toxicity testing*. Congenit Anom (Kyoto), 2016. **56**(1): p. 18-27.
22. Teixido, E., et al., *Assessment of developmental delay in the zebrafish embryo teratogenicity assay*. Toxicol In Vitro, 2013. **27**(1): p. 469-78.
23. Nagel, R., *DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology*. ALTEX, 2002. **19** Suppl 1: p. 38-48.

24. Selderslaghs, I.W., et al., *Locomotor activity in zebrafish embryos: a new method to assess developmental neurotoxicity*. *Neurotoxicol Teratol*, 2010. **32**(4): p. 460-71.
25. Daston, G.P., *Gene expression, dose-response, and phenotypic anchoring: applications for toxicogenomics in risk assessment*. *Toxicol Sci*, 2008. **105**(2): p. 233-4.
26. Niederreither, K., et al., *Differential expression of retinoic acid-synthesizing (RALDH) enzymes during fetal development and organ differentiation in the mouse*. *Mech Dev*, 2002. **110**(1-2): p. 165-71.
27. Kamm, J.J., *Toxicology, carcinogenicity, and teratogenicity of some orally administered retinoids*. *J Am Acad Dermatol*, 1982. **6**(4 Pt 2 Suppl): p. 652-9.
28. Lee, L.M., et al., *A paradoxical teratogenic mechanism for retinoic acid*. *Proc Natl Acad Sci U S A*, 2012. **109**(34): p. 13668-73.
29. Dolk, H.M., et al., *Dietary vitamin A and teratogenic risk: European Teratology Society discussion paper*. *Eur J Obstet Gynecol Reprod Biol*, 1999. **83**(1): p. 31-6.
30. Wilson, J.G. and J. Warkany, *Malformations in the genito-urinary tract induced by maternal vitamin A deficiency in the rat*. *Am J Anat*, 1948. **83**(3): p. 357-407.
31. Bernheim, S. and S.M. Meilhac, *Mesoderm patterning by a dynamic gradient of retinoic acid signalling*. *Philos Trans R Soc Lond B Biol Sci*, 2020. **375**(1809): p. 20190556.
32. Shimoazono, S., et al., *Visualization of an endogenous retinoic acid gradient across embryonic development*. *Nature*, 2013. **496**(7445): p. 363-6.
33. Duester, G., *Retinoic acid synthesis and signaling during early organogenesis*. *Cell*, 2008. **134**(6): p. 921-31.
34. White, R.J., et al., *Complex regulation of cyp26a1 creates a robust retinoic acid gradient in the zebrafish embryo*. *PLoS Biol*, 2007. **5**(11): p. e304.
35. Schilling, T.F., Q. Nie, and A.D. Lander, *Dynamics and precision in retinoic acid morphogen gradients*. *Curr Opin Genet Dev*, 2012. **22**(6): p. 562-9.
36. Samarut, E., et al., *ZebRA: An overview of retinoic acid signaling during zebrafish development*. *Biochim Biophys Acta*, 2015. **1849**(2): p. 73-83.
37. Piersma, A.H., E.V. Hessel, and Y.C. Staal, *Retinoic acid in developmental toxicology: Teratogen, morphogen and biomarker*. *Reprod Toxicol*, 2017. **72**: p. 53-61.
38. Dubey, A., et al., *Generating retinoic acid gradients by local degradation during craniofacial development: One cell's cue is another cell's poison*. *Genesis*, 2018. **56**(2).
39. Zile, M.H., et al., *Retinoid signaling is required to complete the vertebrate cardiac left/right asymmetry pathway*. *Dev Biol*, 2000. **223**(2): p. 323-38.
40. Stefanovic, S. and S. Zaffran, *Mechanisms of retinoic acid signaling during cardiogenesis*. *Mech Dev*, 2017. **143**: p. 9-19.
41. Merlet-Benichou, C., et al., *Role of retinoids in renal development: pathophysiological implication*. *Curr Opin Nephrol Hypertens*, 1999. **8**(1): p. 39-43.
42. Mendelsohn, C., et al., *Stromal cells mediate retinoid-dependent functions essential for renal development*. *Development*, 1999. **126**(6): p. 1139-48.
43. Batourina, E., et al., *Vitamin A controls epithelial/mesenchymal interactions through Ret expression*. *Nat Genet*, 2001. **27**(1): p. 74-8.
44. Bollig, F., et al., *A highly conserved retinoic acid responsive element controls wt1a expression in the zebrafish pronephros*. *Development*, 2009. **136**(17): p. 2883-92.
45. Tonk, E.C., J.L. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis*. *Reprod Toxicol*, 2015. **55**: p. 104-13.
46. Massa, V., et al., *Valproic acid-induced skeletal malformations: associated gene expression cascades*. *Pharmacogenet Genomics*, 2005. **15**(11): p. 787-800.
47. Goyal, M., et al., *Fetal Valproate Syndrome with Limb Defects: An Indian Case Report*. *Case Rep Pediatr*, 2016. **2016**: p. 3495910.
48. Rodriguez-Pinilla, E., et al., *Prenatal exposure to valproic acid during pregnancy and limb deficiencies: a case-control study*. *Am J Med Genet*, 2000. **90**(5): p. 376-81.

49. Kowalski, T.W., et al., *Transcriptome meta-analysis of valproic acid exposure in human embryonic stem cells*. Eur Neuropsychopharmacol, 2022. **60**: p. 76-88.
50. Beker van Woudenberg, A., et al., *Zebrafish embryotoxicity test for developmental (neuro) toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs*. Reprod Toxicol, 2014. **49**: p. 101-16.
51. Landrigan, P.J., *What causes autism? Exploring the environmental contribution*. Curr Opin Pediatr, 2010. **22**(2): p. 219-25.
52. Pierro, J.D., et al., *Computational model for fetal skeletal defects potentially linked to disruption of retinoic acid signaling*. Front Pharmacol, 2022. **13**: p. 971296.
53. Bryant, S.L., et al., *Sex specific retinoic acid signaling is required for the initiation of urogenital sinus bud development*. Dev Biol, 2014. **395**(2): p. 209-17.
54. Li, A.S. and Y. Marikawa, *Adverse effect of valproic acid on an in vitro gastrulation model entails activation of retinoic acid signaling*. Reprod Toxicol, 2016. **66**: p. 68-83.
55. Lee, M.S., et al., *Disruption of the folate pathway in zebrafish causes developmental defects*. BMC Dev Biol, 2012. **12**: p. 12.
56. Muhsen, M., et al., *Folic acid supplementation rescues valproic acid-induced developmental neurotoxicity and behavioral alterations in zebrafish embryos*. Epilepsia, 2021. **62**(7): p. 1689-1700.
57. Padmanabhan, R. and M.M. Shafiullah, *Amelioration of sodium valproate-induced neural tube defects in mouse fetuses by maternal folic acid supplementation during gestation*. Congenit Anom (Kyoto), 2003. **43**(1): p. 29-40.
58. Zhang, X.Z., et al., *Folic Acid Rescues Valproic Acid-Induced Morphogenesis Inhibition in Neural Rosettes Derived From Human Pluripotent Stem Cells*. Front Cell Neurosci, 2022. **16**: p. 888152.
59. Samrani, L.M.M., et al., *Dynamic regulation of gene expression and morphogenesis in the zebrafish embryo test after exposure to all-trans retinoic acid*. Reprod Toxicol, 2022.
60. Hermesen, S.A., et al., *Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies*. Toxicol In Vitro, 2011. **25**(3): p. 745-53.
61. Han, X., et al., *Precise Dose of Folic Acid Supplementation Is Essential for Embryonic Heart Development in Zebrafish*. Biology (Basel), 2021. **11**(1).
62. Jiang, Q., et al., *Folic acid supplement rescues ethanol-induced developmental defects in the zebrafish embryos*. Acta Biochim Biophys Sin (Shanghai), 2020. **52**(5): p. 536-545.
63. Mennen, R.H., et al., *Genome-wide expression screening in the cardiac embryonic stem cell test shows additional differentiation routes that are regulated by morpholines and piperidines*. Curr Res Toxicol, 2022. **3**: p. 100086.
64. Ritchie, M.E., et al., *limma powers differential expression analyses for RNA-sequencing and microarray studies*. Nucleic Acids Res, 2015. **43**(7): p. e47.
65. Liberzon, A., et al., *Molecular signatures database (MSigDB) 3.0*. Bioinformatics, 2011. **27**(12): p. 1739-40.
66. Szklarczyk, D., et al., *STITCH 5: augmenting protein-chemical interaction networks with tissue and affinity data*. Nucleic Acids Res, 2016. **44**(D1): p. D380-4.
67. Stewart, K. and M. Bouchard, *Kidney and urinary tract development: an apoptotic balancing act*. Pediatr Nephrol, 2011. **26**(9): p. 1419-25.
68. Rankin, S.A., et al., *A Retinoic Acid-Hedgehog Cascade Coordinates Mesoderm-Inducing Signals and Endoderm Competence during Lung Specification*. Cell Rep, 2016. **16**(1): p. 66-78.
69. Keegan, B.R., et al., *Retinoic acid signaling restricts the cardiac progenitor pool*. Science, 2005. **307**(5707): p. 247-9.
70. Abu-Abed, S., et al., *The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures*. Genes Dev, 2001. **15**(2): p. 226-40.

71. Song, H.M., et al., *High-dose retinoic acid modulates rat calvarial osteoblast biology*. J Cell Physiol, 2005. **202**(1): p. 255-62.
72. Michaelsson, K., et al., *Serum retinol levels and the risk of fracture*. N Engl J Med, 2003. **348**(4): p. 287-94.
73. Mennen, R.H.G., J. Pennings, and A.H.A. Piersma, *Neural crest related gene transcript regulation by valproic acid analogues in the cardiac embryonic stem cell test*. Reprod Toxicol, 2019. **90**: p. 44-52.
74. Ducharme, N.A., et al., *Meta-analysis of toxicity and teratogenicity of 133 chemicals from zebrafish developmental toxicity studies*. Reprod Toxicol, 2013. **41**: p. 98-108.
75. Schuttler, A., et al., *The Transcriptome of the Zebrafish Embryo After Chemical Exposure: A Meta-Analysis*. Toxicol Sci, 2017. **157**(2): p. 291-304.
76. Qiu, W., et al., *Transcriptomic Responses of Bisphenol S Predict Involvement of Immune Function in the Cardiotoxicity of Early Life-Stage Zebrafish (Danio rerio)*. Environ Sci Technol, 2020. **54**(5): p. 2869-2877.
77. Vasamsetti, B.M.K., et al., *Transcriptome-Based Identification of Genes Responding to the Organophosphate Pesticide Phosmet in Danio rerio*. Genes (Basel), 2021. **12**(11).
78. Liu, M.Y., W.C. Hsieh, and B.C. Yang, *In vitro aberrant gene expression as the indicator of lead-induced neurotoxicity in U-373MG cells*. Toxicology, 2000. **147**(1): p. 59-64.
79. Fan, C.Y., et al., *Gene expression changes in developing zebrafish as potential markers for rapid developmental neurotoxicity screening*. Neurotoxicol Teratol, 2010. **32**(1): p. 91-8.
80. Knudsen, T.B., J.D. Pierro, and N.C. Baker, *Retinoid signaling in skeletal development: Scoping the system for predictive toxicology*. Reprod Toxicol, 2021. **99**: p. 109-130.
81. Chen, Y., et al., *A SNX10/V-ATPase pathway regulates ciliogenesis in vitro and in vivo*. Cell Res, 2012. **22**(2): p. 333-45.
82. Cui, Z., et al., *Inhibition of skiA and skiB gene expression ventralizes zebrafish embryos*. Genesis, 2001. **30**(3): p. 149-53.
83. Kaufman, C.D., G. Martinez-Rodriguez, and P.B. Hackett, Jr., *Ectopic expression of c-ski disrupts gastrulation and neural patterning in zebrafish*. Mech Dev, 2000. **95**(1-2): p. 147-62.
84. Chang, Y.P., et al., *Multiple genes for essential-hypertension susceptibility on chromosome 1q*. Am J Hum Genet, 2007. **80**(2): p. 253-64.
85. Caceres, L., et al., *Frizzled 4 regulates ventral blood vessel remodeling in the zebrafish retina*. Dev Dyn, 2019. **248**(12): p. 1243-1256.
86. Mao, J., et al., *Clinical characteristics and mutation spectrum in 33 Chinese families with familial exudative vitreoretinopathy*. Ann Med, 2022. **54**(1): p. 3286-3298.
87. Nakayama, M., et al., *HNF1B alterations associated with congenital anomalies of the kidney and urinary tract*. Pediatr Nephrol, 2010. **25**(6): p. 1073-9.
88. Wang, Y., et al., *Cardiac developmental toxicity and transcriptome analyses of zebrafish (Danio rerio) embryos exposed to Mancozeb*. Ecotoxicol Environ Saf, 2021. **226**: p. 112798.
89. Nishimura, Y., et al., *Genomic biomarkers for cardiotoxicity in rats as a sensitive tool in preclinical studies*. J Appl Toxicol, 2013. **33**(10): p. 1120-30.
90. Gu, W., et al., *Single-Cell RNA Sequencing Reveals Size-Dependent Effects of Polystyrene Microplastics on Immune and Secretory Cell Populations from Zebrafish Intestines*. Environ Sci Technol, 2020. **54**(6): p. 3417-3427.
91. Huang, W., et al., *A transcriptomics-based analysis of toxicity mechanisms of zebrafish embryos and larvae following parental Bisphenol A exposure*. Ecotoxicol Environ Saf, 2020. **205**: p. 111165.

Supplementary data

Supplementary data Table 1 | Child terms of GO-term developmental process related to the circulatory system.

GOID	GOTerm	Annotated	Significant	Expected	pvalFisher
GO:0055011	atrial cardiac muscle cell differentiation	6	3	0.07	3.00E-05
GO:0048738	cardiac muscle tissue development	103	7	1.2	2.00E-04
GO:0003007	heart morphogenesis	241	10	2.8	0.00054
GO:0072359	circulatory system development	1014	24	11.8	0.00075
GO:0055014	atrial cardiac muscle cell development	4	2	0.05	8.00E-04
GO:0007507	heart development	568	16	6.61	0.00103
GO:0003151	outflow tract morphogenesis	5	2	0.06	0.00132
GO:0035051	cardiocyte differentiation	70	5	0.81	0.00133
GO:0060419	heart growth	22	3	0.26	0.00203
GO:0055007	cardiac muscle cell differentiation	52	4	0.61	0.00312
GO:0003206	cardiac chamber morphogenesis	29	3	0.34	0.00454
GO:0010002	cardioblast differentiation	11	2	0.13	0.00692
GO:0001944	vasculature development	535	13	6.23	0.01005
GO:0003205	cardiac chamber development	46	3	0.54	0.01633
GO:0060038	cardiac muscle cell proliferation	17	2	0.2	0.01634
GO:0086001	cardiac muscle cell action potential	18	2	0.21	0.01824
GO:0003208	cardiac ventricle morphogenesis	19	2	0.22	0.02023
GO:0055017	cardiac muscle tissue growth	19	2	0.22	0.02023
GO:0003223	ventricular compact myocardium morphogenesis	2	1	0.02	0.02314
GO:0086012	membrane depolarization during cardiac muscle cell action potential	2	1	0.02	0.02314
GO:1900825	regulation of membrane depolarization during cardiac muscle cell action potential	2	1	0.02	0.02314
GO:1905178	regulation of cardiac muscle tissue regeneration	2	1	0.02	0.02314
GO:0061299	retina vasculature morphogenesis in camera-type eye	3	1	0.03	0.03451
GO:0003231	cardiac ventricle development	27	2	0.31	0.03909
GO:0003139	secondary heart field specification	4	1	0.05	0.04574

Supplementary data Table 2 | Child terms of GO-term developmental process related to the kidney/urinary system development.

GOLD	GOTerm	Annotated	Significant	Expected	pvalFisher
GO:0034672	anterior/posterior pattern specification involved in pronephros development	6	3	0.07	3.00E-05
GO:0072098	anterior/posterior pattern specification involved in kidney development	6	3	0.07	3.00E-05
GO:0039017	pattern specification involved in pronephros development	8	3	0.09	8.30E-05
GO:0061004	pattern specification involved in kidney development	8	3	0.09	8.30E-05
GO:0072048	renal system pattern specification	8	3	0.09	8.30E-05
GO:0001822	kidney development	193	8	2.25	0.00193
GO:0072001	renal system development	194	8	2.26	0.002
GO:0048793	pronephros development	132	6	1.54	0.0045

Supplementary data Table 3 | Child terms of GO-term developmental process related to the hematopoietic system development.

GOLD	GOTerm	Annotated	Significant	Expected	pvalFisher
GO:0035701	hematopoietic stem cell migration	12	2	0.14	0.00824
GO:1902033	regulation of hematopoietic stem cell proliferation	2	1	0.02	0.02314
GO:0060215	primitive hemopoiesis	58	3	0.67	0.03001
GO:2000471	regulation of hematopoietic stem cell migration	3	1	0.03	0.03451
GO:1902036	regulation of hematopoietic stem cell differentiation	30	2	0.35	0.04736

Supplementary data Table 4 | Child terms of GO-term developmental process related to the skeletal system development.

GOLD	GOTerm	Annotated	Significant	Expected	pvalFisher
GO:1904888	cranial skeletal system development	224	9	2.61	0.00127
GO:0060363	cranial suture morphogenesis	2	1	0.02	0.02314
GO:0048705	skeletal system morphogenesis	205	6	2.39	0.03302
GO:0097094	craniofacial suture morphogenesis	3	1	0.03	0.03451
GO:0098529	neuromuscular junction development, skeletal muscle fiber	3	1	0.03	0.03451
GO:0048701	embryonic cranial skeleton morphogenesis	164	5	1.91	0.04298

Supplementary data Table 5 | Child terms of GO-term developmental process related to somite development.

GOID	GOTerm	Annotated	Significant	Expected	pvalFisher
GO:0061053	somite development	150	5	1.75	0.03108
GO:0001756	somitogenesis	103	4	1.2	0.03229

Supplementary data Table 6 | Child terms of GO-term developmental process related to muscle development.

GOID	GOTerm	Annotated	Significant	Expected	pvalFisher
GO:0055011	atrial cardiac muscle cell differentiation	6	3	0.07	3.00E-05
GO:0048738	cardiac muscle tissue development	103	7	1.2	2.00E-04
GO:0014706	striated muscle tissue development	106	7	1.23	0.00024
GO:0055014	atrial cardiac muscle cell development	4	2	0.05	8.00E-04
GO:0055007	cardiac muscle cell differentiation	52	4	0.61	0.00312
GO:0060537	muscle tissue development	232	8	2.7	0.0059
GO:0060038	cardiac muscle cell proliferation	17	2	0.2	0.01634
GO:0055017	cardiac muscle tissue growth	19	2	0.22	0.02023
GO:0014855	striated muscle cell proliferation	20	2	0.23	0.02231
GO:0033002	muscle cell proliferation	26	2	0.3	0.03647

Chapter 5

Summary and General discussion



Summary of main findings

This dissertation falls within the context of the paradigm shift in regulatory toxicology testing which promotes using a mechanistic-based approach based on *in vitro* tests instead of traditional animal testing to predict chemical hazards to human health such as developmental toxicity (**chapter 1**). The novel research expands the understanding of developmental toxicity pathways by studying chemically-induced gene expression changes related to the perturbation of the retinoic acid signaling pathway (RA-SP) in a vertebrate embryo model. By using the zebrafish embryo (ZE) model it was possible to take advantage of the conservation of this biological pathway across vertebrate taxa, to predict potential human developmental toxicity.

The ZE model is not new, however it has been primarily used and optimized for its morphology readout due to the transparent eggs enabling morphological observations during chemical exposure. However, improvements and harmonization are necessary to utilize this model with a reliable molecular level readout, to reveal relevant changes in gene expression. In **chapter 2**, the protocol design was refined to identify gene expression (GE) changes in the ZE [1]. This was done by investigating the optimal exposure duration to study such changes due to the perturbation of the RA-SP. An exposure of ZE to the RA-SP agonist all-trans retinoic acid (ATRA) was performed using 6 different exposure durations, ranging from 2-117 hrs. The morphological and GE readouts were analyzed for each exposure duration. The highest magnitudes of GE regulation were observed after shorter exposure (2-24 hours), in contrast to the morphological readout where longer exposure durations (24-117 hours) were necessary to induce observable changes. These results identified that 4h exposure was the optimal exposure duration to study chemically-induced GE regulation specifically related to the RA-SP perturbation, thereby optimizing the ZE protocol for GE analysis.

In **chapters 3** and **4**, the optimized ZE-GE protocol was employed to identify GE biomarker candidates for maldevelopment. After exposing ZE to two teratogenic compounds known to perturb the RA-SP (ATRA and Valproic Acid, VPA) and one non-teratogenic control compound (Folic Acid, FA), the chemically-induced perturbation of the RA-SP was explored using a whole genome scale GE analysis approach referred to as RNA-sequencing (RNAseq). The 3 test compounds each showed a specific mRNA expression profile, with 248 genes commonly regulated by both teratogenic compounds (ATRA and VPA) but not by FA. These 248 genes

were implicated in several developmental processes including the nervous, circulatory, and skeletal systems. 62 differentially expressed genes (DEGs) were associated with nervous system development and were further examined in **Chapter 3** (Toxicology Letters, 2023 Aug, 384; 96-104, DOI: 10.1016/j.toxlet.2023.07.005). These 62 genes were identified as potential biomarkers of early neurodevelopmental toxicity.

In **chapter 4**, the perturbation of RA-SP on the GE associated with development of mesoderm derived tissues was further investigated using bioinformatics methods [2]. The investigation identified 54 gene ontology (GO)-terms related to 47 DEGs. Literature indicates that these genes were normally expressed among 3 mesodermal sections (paraxial, intermediate, and lateral plate section) and 6 mesodermal tissues (somites, striated muscle, bone, kidney, circulatory system, and blood). These 47 DEGs were identified as potential biomarkers of early mesodermal maldevelopment or novel potential biomarkers for specific mesodermal organs.

These proposed biomarker candidates advance the knowledge on the retinoic acid mediated developmental toxicity mechanism. As their responses become even more broadly characterized by exploring different exposure regimes and the profiles of different chemicals, these biomarkers could contribute to predictive tools in animal-free chemical hazard and risk assessment. Potential consequences of these observations are discussed below.

The zebrafish embryo model for transcriptomic analysis

The ZE model is being increasingly used for chemical hazard assessment, particularly for developmental toxicity. The morphological readout is widely used as a stand-alone test to screen for a chemical's potential developmental toxicity properties [3-5]. Although this readout gives crucial information on the overall teratogenicity status of a chemical, it gives little to no information on the underlying toxicity mechanism leading to these changes. This absence of mechanistic information increases the level of uncertainty regarding the human relevance of the findings. To elucidate chemical toxicity mode of actions (MOAs), it is crucial to integrate a mechanistic-based molecular readout, such as gene expression in the testing strategy. The assay predictivity for humans could then be increased by ensuring that the toxicity mechanism identified is related to a relevant, likely well-conserved developmental mechanism [6].

GE analysis in zebrafish embryos is a useful molecular readout to add to the testing strategy for chemical hazard assessment, to facilitate the extrapolation to the human situation. It is believed that GE perturbation occurs after virtually all chemical exposures [7-9]. GE analysis has been shown to detect sub-morphological effects and to discriminate between chemical classes when the morphological assessment could not, and this in a highly sensitive and specific manner [10-12]. However, additional knowledge is needed to efficiently utilize the ZE-GE readout in a testing strategy. Currently there is a gap in the scientific knowledge regarding toxicity mechanisms and a need for optimization/harmonization of the experimental protocol for ZE-GE analysis [13]. The current work addresses both points by proposing an optimum exposure duration to study the gene expression changes specifically for the RA-SP and then using this optimized protocol to study RA-SP-mediated toxicity mechanisms [1, 2].

Optimization for the zebrafish embryo model for transcriptomic analysis

Chemically-induced GE perturbation in the ZE has been studied using many protocols using different concentrations, time windows, and exposure durations [13, 14]. These conditions influence GE responses [10, 15-17], including the RA-SP GE (chapter 2, [11, 18, 19]). The differences in GE response highlight the importance of optimizing and harmonizing the ZE-GE analysis assay to enable confident interpretations of findings for future chemical safety assessment [13]. Similar harmonization occurred for the apical morphology readout of acute fish toxicity testing during its regulatory validation (OECD TG236 [20]). Additionally, significant steps have been taken to harmonize the same morphology readout for developmental toxicity research [21, 22]. Lessons from this previous work can be applied to this ZE-GE readout optimization and harmonization. These include:

1. Exposure protocol design (duration, age, etc.): These parameters are known to influence the morphological readout and are also likely to influence gene expression readout. Therefore, studying the impact of each parameter on GE analysis is crucial. This first lesson is essential because a difference in signal optimum exists between the two readouts; thus, simply adopting the morphology analysis conditions for GE analysis could lead to a significant signal loss, as described in chapter 2 [1].

2. Parameter Information: A lot of information is necessary for an international regulatory body such as OECD to negotiate and agree the optimal protocol, including negative results; thereby, publishing unsuccessful results is scientifically valuable and even crucial.
3. Endpoint selection: Different morphology endpoints in scoring systems lead to different teratogenicity conclusions (chapter 1); thereby, choosing the GE assay's endpoint is crucial.

Gene expression predictive biomarkers of RA-SP mediated toxicity

As chemical hazard assessment moves toward a more mechanistic-based assessment approach, developing new predictive tests includes identifying and validating objective and quantitative assay endpoints [23]. These endpoints can be measured accurately and reproducibly through the use of biomarkers. Several studies highlight the usefulness of gene expression and pathway regulation analysis as starting points for biomarker definition [19, 24-27].

In the current work, biomarkers of teratogenesis in the RA-SP were explored since this pathway is described as a well-conserved mechanism [28-30], is crucial for vertebrate development, and when perturbed leads to abnormal cellular proliferation, differentiation, apoptosis, migration, and ultimately maldevelopment (chapter 1). In recent years, gene expression analysis helped uncover the perturbation of this pathway with ill-understood teratogen toxicity MOA. For example, a dysregulation of ATRA direct effectors (ATRA metabolic enzymes and receptors) was observed in the toxicity MOAs of chemicals such as triazole derivatives [31, 32]; ethanol [33, 34]; polybrominated diphenyl ethers [35] and VPA [36-38]. In addition to direct effectors, ATRA indirect effectors could be central to RA-SP-mediated toxicity, and as such fundamental to predict chemical dysmorphogenesis potential. However, not all of these indirect target genes are known, as they are often regulated through transcriptional intermediaries [39, 40]. Toxicogenomic approaches, such as the RNAseq used in chapters 3 and 4, may help refine toxicity profile signatures by removing potential gene selection bias, putting the "usual suspect" genes into context and uncovering crucial indirect effectors.

The RA-SP has been described as a good source of embryotoxicity biomarkers; due to its extensive role in embryogenesis (chapter 1) and because malformation is often accompanied by its perturbation. Previous findings confirm the utility of assessing the gene expression changes in this pathway for biomarker identification [19, 41-44]. In Chapter 3, the definition of the RA-SP effector genes correlated with early developmental processes was advanced. In chapters 3 and 4, by further studying specific developmental processes related to malformation caused by RA-SP perturbation, the definition of RA-SP toxicity MOA was advanced, and potential gene expression signature profiles of adverse developmental outcomes were identified.

Some of the most well-known genes in the biomarker lists identified in the current work were also identified as potential biomarkers in other studies, as discussed in chapter 3. Furthermore, human orthologues of genes in the list are known to cause malformation when mutated, as discussed in chapter 4. These characteristics emphasize the potential of the current findings to be used as a starting point for identifying predictive and biologically relevant signature profiles for human teratogenicity. However, additional work is needed to validate the usefulness of these potential biomarker lists in an assay for screening purposes. For example, testing more compounds (teratogens and non-teratogens) against these gene lists will help evaluate the predictive potential of each gene and reduce these lists to the most relevant genes. As the developmental process is time- and localization-dependent additional information on the gene expression changes of the potential biomarker list such as gene expression localization (using in situ hybridization/ transgenic ZF with fluorescent markers) and cell type expression (using single-cell RNAseq) may help deepen the understanding of toxicity MOA [45]. These refinement steps may help to better define the relationship between the potential biomarkers of neurodevelopmental toxicity (chapter 3), and mesodermal-tissues toxicity (chapter 4) and their respective teratogenic effects.

Human relevance and zebrafish gene expression

The RA-SP is a highly conserved pathway in vertebrate embryogenesis, regulating cell proliferation and differentiation in a time- and location-specific manner in the developing embryo [46-48]. Its role and associated

pathway are highly conserved between developing zebrafish and humans [49]. As the ZE is a non-amniotic vertebrate, retinol is stored in its yolk sac instead of being transferred across the placenta; however, its biosynthesis is conserved through the vertebrates (chapter 1). ATRA acts as a regulator of other developmental signaling pathways such as *fgf8* and as a morphogen during the development of both zebrafish and humans. Across species, its tightly regulated gradient is involved in positional identity by, for example, directly regulating the *hox* gene family. These genes are responsible for positional identity along the body axis in all bilateral animals and have a similar organization in multiple clusters throughout vertebrates [49, 50].

ATRA metabolism is also conserved through vertebrates, as the same enzyme with similar isoforms is responsible for its gradient-tight regulation. For example, the *cyp26* family responsible for ATRA metabolism is composed of 3 isoforms in both species with relatively the same level of homology between the isoforms [48]. In humans, three RARs exist (α , β , γ) while in zebrafish 4 exist: two RAR α and two RAR β [49, 51]. Additionally, the zebrafish has 2 isoforms of RALH (*aldh1a2* and *aldh1a3*), whereas humans have 3; the last one, *aldh1a1*, is not associated with development [49]. Despite some interspecies differences in the RA-SP as described above, cross-species research shows that impairment of the RA-SP balance leads to similar malformations, through similar mechanisms [1]. Consequently, the ZE-GE readout remains a useful tool in the ZE assay to study developmental toxicity.

The ZE model is less time consuming, faster, and when using as part of an integrated approach including mechanistic information could be considered as having a higher relevance for the human situation as compared to traditional apical studies using mammalian models. It is interesting to note that the zebrafish embryo model is also used in pharmacology to study human diseases related to RA-SP, such as cancer (e.g. adenoid cystic carcinoma,[52]) [53, 54].

Model limitations

Although the zebrafish embryo is a useful model for studying developmental toxicity due to its many practical advantages and biological relevance (chapter 1), some of its practical limitations must be considered too. For example, some characteristics such as the presence of a chorion until 48

hpf, the possible accumulation of compounds on the petri dish plastic due to low water solubility and the possible accumulation of chemicals in the yolk sac could influence bioavailability, however quantifying the internal and external concentrations may help to overcome this challenge.

Biological differences should also be taken into consideration. For example, zebrafish are exothermic which can influence chemical reaction rates. Determining a chemical's toxicokinetics, in short, its ADME (absorption, distribution, metabolism, and excretion) in the ZE may help extrapolate between species[55]. As the ZE is oviparous, in the assays described in this work they lack maternal biotransformation, therefore, the possible effects of metabolites have to be taken into consideration separately during the testing strategy for human prediction.

Additionally, extrapolating from an *in vitro* assay to an effective dose in humans can be challenging. Tools such as QVIVE (quantitative *in vitro* - *in vivo* extrapolation) modelling and PBK (Physiologically based pharmacokinetic modeling) are emerging to overcome this challenge [56, 57].

Future perspectives

Dependence on laboratory animal testing for human hazard identification of chemicals is widely recognized as sub-optimal by the scientific community, and as ethical concerns of such animal uses grows in society, the need for alternative testing and assessment methods is growing [58]. In the past decades, due to the boom in biotechnology using new approach methodology (NAMs) has become more available, especially for predictive testing strategies and potentially for regulatory decision making too. However, additional knowledge is necessary to exclusively use NAMs in regulatory testing. Developing assays to evaluate a chemical's potential to induce RA-SP perturbation is part of it, as emphasized in several OECD reports for endpoints such as neurodevelopmental toxicity and endocrine disruption [46, 47, 59, 60]. In the future, the integration of gene expression data in regulatory testing may be used in assays to detect developmental toxicants. A predictive gene expression biomarker assay has already been implemented in another toxicology field: the GARD assay relies on a genomic prediction signature and machine learning to classify chemical skin sensitizers (OECD #442E [61]) [62-64]. However, using gene expression biomarkers remains in its infancy, and learning from the establishment of

the GARD prediction signature may help to accelerate the development of gene expression assay for other endpoints.

The current work identifies biomarker gene lists as potential genomic prediction signatures for RA-SP imbalance-specific mechanisms. It is a starting point toward developing RA-SP perturbation-mediated developmental toxicity assays. Additional steps are necessary to further develop these findings into a validated predictive classification assay. For example, knowledge about quantification and directionality of pathway perturbation should be elaborated e.g., by studying other compounds with known MOA (teratogen or not, perturbing the RA-SP or not), performing dose-response analyses, and cross-linking the gene expression data with apical response test data to create logistic regression models. These models could then help the quantitative prediction of the probability/or severity of an adverse outcome in a quantitative AOP (qAOP) [65, 66].

A thorough understanding of ZE-GE assays is also key for implementing such an assay in the testing strategy [55]. For example, further knowledge about the biological and chemical applicability domains is crucial e.g., respectively, which human mechanisms are represented by this assay and which chemical structures could it be (non-)relevant for.

All *in vitro* assays have a restricted biological and chemical applicability domain. Consequently, no stand-alone assay can comprehensively predict the safety of every chemical [67]. This needs to be taken into consideration for the testing strategy as it has consequences on the integrated approach to testing and assessment (IATA) strategy for regulatory decision-making [68, 69]. The ZE-GE allows the study of specific AO toxicity mechanisms in an intact vertebrate organism setting, which is crucial, especially as patterning disturbance often leads to AO. The experimental design established in this work has the potential to inform on various AO in rapid experimental settings contrary to animal testing and other *in vitro* tests. This intact vertebrate embryo development model in which both molecular and morphogenetic parameters can be monitored overtime and linked to one another, inform about mechanism of action and allows the study of AOP from MIE to adverse outcome, which may eventually contribute to improve chemical regulation in the field of DART.

References

1. Samrani, L.M.M., et al., *Dynamic regulation of gene expression and morphogenesis in the zebrafish embryo test after exposure to all-trans retinoic acid*. Reprod Toxicol, 2022.
2. Samrani, L.M.M., et al., *Retinoic acid signaling pathway perturbation impacts mesodermal-tissue development in the zebrafish embryo: biomarker candidate identification using transcriptomics*. Reprod Toxicol, 2023: p. 108404.
3. Augustine-Rauch, K., C.X. Zhang, and J.M. Panzica-Kelly, *In vitro developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays*. Birth Defects Res C Embryo Today, 2010. **90**(2): p. 87-98.
4. Chahardehi, A.M., H. Arsad, and V. Lim, *Zebrafish as a Successful Animal Model for Screening Toxicity of Medicinal Plants*. Plants (Basel), 2020. **9**(10).
5. Van den Bulck, K., et al., *Zebrafish developmental toxicity assay: A fishy solution to reproductive toxicity screening, or just a red herring?* Reprod Toxicol, 2011. **32**(2): p. 213-9.
6. Beker van Woudenberg, A., et al., *Zebrafish embryotoxicity test for developmental (neuro) toxicity: Demo case of an integrated screening approach system using anti-epileptic drugs*. Reprod Toxicol, 2014. **49**: p. 101-16.
7. Pennie, W.D., et al., *The principles and practice of toxigenomics: applications and opportunities*. Toxicol Sci, 2000. **54**(2): p. 277-83.
8. Corvi, R., *Genomics: an in vitro toxicology point of view*. Altern Lab Anim, 2002. **30 Suppl 2**: p. 129-31.
9. Hill, A.J., et al., *Zebrafish as a model vertebrate for investigating chemical toxicity*. Toxicol Sci, 2005. **86**(1): p. 6-19.
10. Yang, L., et al., *Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo*. Genome Biol, 2007. **8**(10): p. R227.
11. Hermesen, S.A., et al., *Chemical class-specific gene expression changes in the zebrafish embryo after exposure to glycol ether alkoxy acids and 1,2,4-triazole antifungals*. Reprod Toxicol, 2011. **32**(2): p. 245-52.
12. Wang, R., et al., *Toxicogenomic signatures associated with methylmercury induced developmental toxicity in the zebrafish embryos*. Chemosphere, 2023. **313**: p. 137380.
13. Schuttler, A., et al., *The Transcriptome of the Zebrafish Embryo After Chemical Exposure: A Meta-Analysis*. Toxicol Sci, 2017. **157**(2): p. 291-304.
14. Padilla, S., *Chapter 5 - Biomarkers of toxicity in zebrafish*, in *Biomarkers in Toxicology*, R.C. Gupta, Editor. 2014, Academic Press: Boston. p. 103-112.
15. Muncke, J. and R.I. Eggen, *Vitellogenin 1 mRNA as an early molecular biomarker for endocrine disruption in developing zebrafish (Danio rerio)*. Environ Toxicol Chem, 2006. **25**(10): p. 2734-41.
16. Gong, S., et al., *Toxicity assessment of hexafluoropropylene oxide-dimer acid on morphology, heart physiology, and gene expression during zebrafish (Danio rerio) development*. Environ Sci Pollut Res Int, 2023. **30**(12): p. 32320-32336.
17. Felix, L.M., et al., *MS-222 induces biochemical and transcriptional changes related to oxidative stress, cell proliferation and apoptosis in zebrafish embryos*. Comp Biochem Physiol C Toxicol Pharmacol, 2020. **237**: p. 108834.
18. Fan, Z., et al., *Molecular mechanism of ethylparaben on zebrafish embryo cardiotoxicity based on transcriptome analyses*. Sci Total Environ, 2022. **842**: p. 156785.
19. Hermesen, S.A., et al., *Transcriptomic analysis in the developing zebrafish embryo after compound exposure: individual gene expression and pathway regulation*. Toxicol Appl Pharmacol, 2013. **272**(1): p. 161-71.
20. OECD, *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*. 2013.

21. Beekhuijzen, M., et al., *From cutting edge to guideline: A first step in harmonization of the zebrafish embryotoxicity test (ZET) by describing the most optimal test conditions and morphology scoring system*. *Reprod Toxicol*, 2015. **56**: p. 64-76.
22. Pype, C., et al., *Incubation at 32.5 degrees C and above causes malformations in the zebrafish embryo*. *Reprod Toxicol*, 2015. **56**: p. 56-63.
23. Huuskonen, H., *New models and molecular markers in evaluation of developmental toxicity*. *Toxicol Appl Pharmacol*, 2005. **207**(2 Suppl): p. 495-500.
24. Gatzidou, E.T., A.N. Zira, and S.E. Theocharis, *Toxicogenomics: a pivotal piece in the puzzle of toxicological research*. *J Appl Toxicol*, 2007. **27**(4): p. 302-9.
25. Song, Y., et al., *Whole-Organism Transcriptomic Analysis Provides Mechanistic Insight into the Acute Toxicity of Emamectin Benzoate in Daphnia magna*. *Environ Sci Technol*, 2016. **50**(21): p. 11994-12003.
26. Brockmeier, E.K., et al., *The Role of Omics in the Application of Adverse Outcome Pathways for Chemical Risk Assessment*. *Toxicol Sci*, 2017. **158**(2): p. 252-262.
27. Robinson, J.F. and A.H. Piersma, *Toxicogenomic approaches in developmental toxicology testing*. *Methods Mol Biol*, 2013. **947**: p. 451-73.
28. Balmer, J.E. and R. Blomhoff, *A robust characterization of retinoic acid response elements based on a comparison of sites in three species*. *J Steroid Biochem Mol Biol*, 2005. **96**(5): p. 347-54.
29. Huang, P., V. Chandra, and F. Rastinejad, *Retinoic acid actions through mammalian nuclear receptors*. *Chem Rev*, 2014. **114**(1): p. 233-54.
30. Dubey, A., et al., *Generating retinoic acid gradients by local degradation during craniofacial development: One cell's cue is another cell's poison*. *Genesis*, 2018. **56**(2).
31. Menegola, E., et al., *Study on the common teratogenic pathway elicited by the fungicides triazole-derivatives*. *Toxicol In Vitro*, 2005. **19**(6): p. 737-48.
32. Hermesen, S.A., et al., *Triazole-induced gene expression changes in the zebrafish embryo*. *Reprod Toxicol*, 2012. **34**(2): p. 216-24.
33. Kane, M.A., et al., *Ethanol elevates physiological all-trans-retinoic acid levels in select loci through altering retinoid metabolism in multiple loci: a potential mechanism of ethanol toxicity*. *FASEB J*, 2010. **24**(3): p. 823-32.
34. Zhang, C., A. Anderson, and G.J. Cole, *Analysis of crosstalk between retinoic acid and sonic hedgehog pathways following ethanol exposure in embryonic zebrafish*. *Birth Defects Res A Clin Mol Teratol*, 2015. **103**(12): p. 1046-57.
35. Haggard, D.E., S.R. Das, and R.L. Tanguay, *Comparative Toxicogenomic Responses to the Flame Retardant mITP in Developing Zebrafish*. *Chem Res Toxicol*, 2017. **30**(2): p. 508-515.
36. Jergil, M., et al., *Valproic acid-induced deregulation in vitro of genes associated in vivo with neural tube defects*. *Toxicol Sci*, 2009. **108**(1): p. 132-48.
37. Kultima, K., et al., *Early transcriptional responses in mouse embryos as a basis for selection of molecular markers predictive of valproic acid teratogenicity*. *Reprod Toxicol*, 2010. **30**(3): p. 457-68.
38. Li, A.S. and Y. Marikawa, *Adverse effect of valproic acid on an in vitro gastrulation model entails activation of retinoic acid signaling*. *Reprod Toxicol*, 2016. **66**: p. 68-83.
39. Balmer, J.E. and R. Blomhoff, *Gene expression regulation by retinoic acid*. *J Lipid Res*, 2002. **43**(11): p. 1773-808.
40. Savory, J.G., et al., *Identification of novel retinoic acid target genes*. *Dev Biol*, 2014. **395**(2): p. 199-208.
41. Tonk, E.C., J.L. Pennings, and A.H. Piersma, *An adverse outcome pathway framework for neural tube and axial defects mediated by modulation of retinoic acid homeostasis*. *Reprod Toxicol*, 2015. **55**: p. 104-13.
42. Robinson, J.F., M.J. Gormley, and S.J. Fisher, *A genomics-based framework for identifying biomarkers of human neurodevelopmental toxicity*. *Reprod Toxicol*, 2016. **60**: p. 1-10.

43. Chen, H., M.A. Chidboy, and J.F. Robinson, *Retinoids and developmental neurotoxicity: Utilizing toxicogenomics to enhance adverse outcome pathways and testing strategies*. *Reprod Toxicol*, 2020. **96**: p. 102-113.
44. Zurlinden, T.J., et al., *Profiling the ToxCast Library With a Pluripotent Human (H9) Stem Cell Line-Based Biomarker Assay for Developmental Toxicity*. *Toxicol Sci*, 2020. **174**(2): p. 189-209.
45. Hermesen, S., *Implementation of transcriptomics for developmental toxicity assessment in the zebrafish embryo model*. 2013.
46. Baker, N.C., et al., *Identifying candidate reference chemicals for in vitro testing of the retinoid pathway for predictive developmental toxicity*. *ALTEX*, 2023. **40**(2): p. 217-236.
47. Pierro, J.D., et al., *Computational model for fetal skeletal defects potentially linked to disruption of retinoic acid signaling*. *Front Pharmacol*, 2022. **13**: p. 971296.
48. Nelson, D.R., *A second CYP26 P450 in humans and zebrafish: CYP26B1*. *Arch Biochem Biophys*, 1999. **371**(2): p. 345-7.
49. Hawkins, M.R. and R.A. Wingert, *Zebrafish as a Model to Study Retinoic Acid Signaling in Development and Disease*. *Biomedicines*, 2023. **11**(4).
50. Yamada, K., et al., *An atlas of seven zebrafish hox cluster mutants provides insights into sub/neofunctionalization of vertebrate Hox clusters*. *Development*, 2021. **148**(11).
51. Hale, L.A., et al., *Characterization of the retinoic acid receptor genes *raraa*, *rarab* and *rarg* during zebrafish development*. *Gene Expression Patterns*, 2006. **6**(5): p. 546-555.
52. Mandelbaum, J., et al., *Zebrafish blastomere screen identifies retinoic acid suppression of MYB in adenoid cystic carcinoma*. *J Exp Med*, 2018. **215**(10): p. 2673-2685.
53. Weiss, F.U., et al., *Retinoic acid receptor antagonists inhibit miR-10a expression and block metastatic behavior of pancreatic cancer*. *Gastroenterology*, 2009. **137**(6): p. 2136-45 e1-7.
54. Kobar, K., et al., *Zebrafish Cancer Predisposition Models*. *Front Cell Dev Biol*, 2021. **9**: p. 660069.
55. Sipes, N.S., S. Padilla, and T.B. Knudsen, *Zebrafish: as an integrative model for twenty-first century toxicity testing*. *Birth Defects Res C Embryo Today*, 2011. **93**(3): p. 256-67.
56. Chang, X., et al., *IVIVE: Facilitating the Use of In Vitro Toxicity Data in Risk Assessment and Decision Making*. *Toxics*, 2022. **10**(5).
57. Dayana Lancheros, K., I. Alves, and M. Aragon, *PBPK modeling as an alternative method of extrapolation interspecies that reduce the use of animals: a systematic review*. *Curr Med Chem*, 2023.
58. Akhtar, A., *The Flaws and Human Harms of Animal Experimentation*. *Cambridge Quarterly of Healthcare Ethics*, 2015. **24**(4): p. 407-419.
59. Robinson, J.F., *Retinoids and Developmental Neurotoxicity In Vivo and In Vitro*.
60. Development, o.F.E.C.-O.A., *Detailed review paper on the state of the science on novel in vitro and in vivo screening and testing methods and endpoints for evaluating endocrine disruptors no. 178*. 2012.
61. OECD, *Test No. 442E: In Vitro Skin Sensitisation*. 2022.
62. OECD, *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins*. 2014.
63. Gradin, R., et al., *The GARDpotency Assay for Potency-Associated Subclassification of Chemical Skin Sensitizers-Rationale, Method Development, and Ring Trial Results of Predictive Performance and Reproducibility*. *Toxicol Sci*, 2020. **176**(2): p. 423-432.
64. DIRECTORATE, E. and C.A.B. COMMITTEE, *Supporting document to the Genomic Allergen Rapid Detection Test Method for Skin Sensitisation (GARD™skin), described in Test Guideline 442E*, 357, Editor. 2022.
65. Paini, A., et al., *Towards a qAOP framework for predictive toxicology - Linking data to decisions*. *Comput Toxicol*, 2022. **21**: p. 100195.

66. Conolly, R.B., et al., *Quantitative Adverse Outcome Pathways and Their Application to Predictive Toxicology*. Environ Sci Technol, 2017. **51**(8): p. 4661-4672.
67. Piersma, A.H., et al., *Evaluation of an alternative in vitro test battery for detecting reproductive toxicants*. Reproductive Toxicology, 2013. **38**: p. 53-64.
68. Brescia, S., et al., *Risk assessment in the 21st century: where are we heading?* Toxicology Research, 2023. **12**(1): p. 1-11.
69. Council, N.R., *Toxicity Testing in the 21st Century: A Vision and a Strategy*. 2007, Washington, DC: The National Academies Press. 216.

Appendix

Summaries

List of publication

Acknowledgements

CV



Résumé Vulgarisé (Français)

Comment tester les produits chimiques pour prévoir leurs effets sur la santé humaine ? La réponse à cette question a évolué ces dernières années. Au début, l'expérimentation animale était la seule solution, mais les progrès technologiques permettent maintenant d'utiliser des méthodes alternatives à l'expérimentation animale.

L'objectif de ce travail est de développer de nouveaux tests pour prédire les effets des produits chimiques sur le développement embryonnaire. Nous avons utilisé un organisme modèle, le poisson zèbre. Comme les premières étapes du développement sont similaires entre les vertébrés, nous avons exposé des embryons de poisson zèbre à des produits chimiques afin de prédire les effets potentiels sur le développement embryonnaire humain. Les résultats obtenus ont permis d'identifier des gènes indiquant si un produit chimique nuit au développement de l'embryon. Ces résultats pourront être utilisés pour développer de nouveaux tests alternatifs à l'expérimentation animale.

Résumé Scientifique (Francais)

Cette thèse s'inscrit dans le contexte du changement de paradigme des tests toxicologiques pour l'évaluation des risques des produits chimiques sur la santé humaine tels que la toxicité sur le développement. Cette nouvelle approche encourage l'utilisation de tests basés sur la compréhension des mécanismes d'action des produits chimiques plutôt que sur les conséquences pathologiques de l'exposition à ces produits traditionnellement testés *in vivo* chez l'animal.

Ces travaux ont abordé la compréhension des voies impliquées dans la toxicité de développement, grâce à une étude des changements d'expression génique induits par l'exposition à des produits chimiques dans un modèle d'embryon vertébré. En utilisant l'embryon de poisson zèbre (ZE), il a été possible de tirer parti de la conservation de la voie de signalisation de l'acide rétinoïque (RA-SP) à travers les taxons vertébrés, pour prédire la toxicité potentielle des produits chimiques sur le développement de l'embryon chez l'humain.

Dans ce travail, la conception d'un protocole d'étude a été affinée afin d'identifier les changements d'expression génique (GE) chez le ZE. Nous avons analysé la durée d'exposition optimale pour étudier les changements dus à la perturbation de la voie de RA-SP connue pour jouer un rôle central dans le développement embryonnaire. Une exposition des ZE à un agoniste de la RA-SP, l'acide tout-trans rétinoïque (ATRA), a été réalisée en utilisant six durées d'exposition différentes, allant de 2 heures à 117 heures. Les données morphologiques et l'expression des gènes ont été analysées pour chaque durée d'exposition. Ces résultats ont permis d'identifier qu'une durée d'exposition de 4 heures était optimale pour étudier la régulation des gènes liée à la perturbation de la voie RA-SP, optimisant ainsi le protocole ZE pour l'analyse GE.

Ce protocole ZE-GE optimisé a ensuite été employé pour identifier des gènes candidats biomarqueurs d'une atteinte du développement et responsable d'un effet tératogène. Après avoir exposé ZE à deux composés tératogènes connus pour perturber la voie RA-SP (ATRA et acide valproïque, VPA) et à un composé de référence non tératogène (acide folique, FA), la perturbation de la voie RA-SP induite par les produits chimiques a été explorée par une approche de RNAseq. Les trois composés testés ont chacun montré un profil d'expression d'ARNm spécifique, avec 248 gènes

communément régulés par les deux composés tératogènes (ATRA et VPA) mais pas par le FA. Ces 248 gènes sont impliqués dans plusieurs processus de développement, notamment le système nerveux, le compartiment sanguin et le squelette. Un nombre de 62 gènes différentiellement exprimés (DEG) ont été associés au développement du système nerveux et ont fait l'objet d'un examen plus approfondi. Ces 62 gènes ont été identifiés comme des biomarqueurs potentiels de la toxicité neurodéveloppementale précoce des produits chimiques.

La perturbation de la voie RA-SP sur les GE associée au développement des tissus dérivés du mésoderme a été étudiée à l'aide de méthodes bio-informatiques. L'étude a permis d'identifier 54 termes de *gene ontology* liés à 47 DEG. Ces 47 DEG ont été identifiés comme des biomarqueurs potentiels d'une atteinte du développement mésodermique précoce ou comme de nouveaux biomarqueurs potentiels pour des organes mésodermiques spécifiques.

Les biomarqueurs candidats proposés dans ces travaux ont permis d'acquérir des connaissances sur le mécanisme de toxicité développementale médiée par l'acide rétinoïque. Après une plus ample caractérisation de ces candidats, ces derniers pourraient être utilisés comme outils prédictifs pour l'évaluation de la sécurité chimique.

Laymans Summary (English)

The methods to assess chemical safety for humans have evolved greatly in the past decades. Initially, animal testing in mammals was the approach of choice, but technological advances and molecular approaches now allow the use of more refined methods.

The objective of this thesis was to use zebrafish embryos to predict chemical effects on humans by studying how chemicals can affect the expression of genes that control embryo development. The zebrafish is relevant as early development is conserved between vertebrate species. First, we identified the optimal time point for assessing gene expression changes after exposure, focusing on the retinoic acid pathway as a crucial developmental regulatory mechanism. Then, using this timing, we identified genes that could be markers for maldevelopment of the nervous system and other organs. These findings help to understand how chemicals can affect development and could be used to improve the testing chemicals using fewer animals.

Scientific Summary (English)

This dissertation falls within the context of the paradigm shift in regulatory toxicology testing which promotes using a mechanistic-based approach based on *in vitro* tests instead of traditional animal testing to predict chemical hazards to human health such as developmental toxicity (**chapter 1**). The novel research expands the understanding of developmental toxicity pathways by studying chemically-induced gene expression changes related to the perturbation of the retinoic acid signaling pathway (RA-SP) in a vertebrate embryo model. By using the zebrafish embryo (ZE) model it was possible to take advantage of the conservation of this biological pathway across vertebrate taxa, to predict potential human developmental toxicity.

The ZE model is not new, however it has been primarily used and optimized for its morphology readout due to the transparent eggs enabling morphological observations during chemical exposure. However, improvements and harmonization are necessary to utilize this model with a reliable molecular level readout, to reveal relevant changes in gene expression. In **chapter 2**, the protocol design was refined to identify gene expression (GE) changes in the ZE [1]. This was done by investigating the optimal exposure duration to study such changes due to the perturbation of the RA-SP. An exposure of ZE to the RA-SP agonist all-trans retinoic acid (ATRA) was performed using 6 different exposure durations, ranging from 2-117 hrs. The morphological and GE readouts were analyzed for each exposure duration. The highest magnitudes of GE regulation were observed after shorter exposure (2-24 hours), in contrast to the morphological readout where longer exposure durations (24-117 hours) were necessary to induce observable changes. These results identified that 4h exposure was the optimal exposure duration to study chemically-induced GE regulation specifically related to the RA-SP perturbation, thereby optimizing the ZE protocol for GE analysis.

In **chapters 3 and 4**, the optimized ZE-GE protocol was employed to identify GE biomarker candidates for maldevelopment. After exposing ZE to two teratogenic compounds known to perturb the RA-SP (ATRA and Valproic Acid, VPA) and one non-teratogenic control compound (Folic Acid, FA), the chemically-induced perturbation of the RA-SP was explored using a whole genome scale GE analysis approach referred to as RNA-sequencing (RNAseq). The 3 test compounds each showed a specific mRNA expression profile, with 248 genes commonly regulated by both teratogenic

compounds (ATRA and VPA) but not by FA. These 248 genes were implicated in several developmental processes including the nervous, circulatory, and skeletal systems. 62 differentially expressed genes (DEGs) were associated with nervous system development and were further examined in **Chapter 3** (manuscript submitted). These 62 genes were identified as potential biomarkers of early neurodevelopmental toxicity.

In **chapter 4**, the perturbation of RA-SP on the GE associated with development of mesoderm derived tissues was further investigated using bioinformatics methods [2]. The investigation identified 54 gene ontology (GO)-terms related to 47 DEGs. Literature indicates that these genes were normally expressed among 3 mesodermal sections (paraxial, intermediate, and lateral plate section) and 6 mesodermal tissues (somites, striated muscle, bone, kidney, circulatory system, and blood). These 47 DEGs were identified as potential biomarkers of early mesodermal maldevelopment or novel potential biomarkers for specific mesodermal organs.

These proposed biomarker candidates advance the knowledge on the retinoic acid mediated developmental toxicity mechanism. As their responses become even more broadly characterized by exploring different exposure regimes and the profiles of different chemicals, these biomarkers could contribute to predictive tools in animal-free chemical hazard and risk assessment. Potential consequences of these observations are discussed below.

Laymans Samenvatting (Nederlands)

De methoden voor veiligheidsevaluatie van chemische stoffen hebben zich in de afgelopen decennia sterk ontwikkeld. Dierstudies zijn lang eerste keus geweest, maar technologische ontwikkelingen en moleculaire methoden maken nu meer verfijnde benaderingen mogelijk.

Doel van dit proefschrift was het gebruik van zebravis embryo's voor de veiligheidsevaluatie van stoffen voor de embryogenese bij de mens. De zebravis is relevant vanwege de sterke conservering van de vroege embryogenese bij vertebraten. Allereerst zochten we het optimale tijdstip voor het vaststellen van genexpressieveranderingen na blootstelling, met de focus op retinolzuur metabolisme als een cruciale regulator van de embryogenese. Zo werden genen gevonden die kunnen dienen als markers voor ontwikkelingsschade in het centraal zenuwstelsel en andere organen. Deze bevindingen helpen ons te begrijpen hoe chemische stoffen de embryogenese kunnen verstoren, en kunnen gebruikt worden om beter te testen met minder proefdieren.

Wetenschappelijke Samenvatting (Nederlands)

Dit proefschrift past in de context van de paradigmaverschuiving in de testprocedures toegepast in de regulatoire toxicologie. Deze vernieuwing staat een mechanistische benadering voor, gebaseerd op in vitro testen in plaats van de traditionele dierstudies, om de gevaren van chemische stoffen vast te stellen voor de gezondheid van de mens. Een van die gevaren betreft de mogelijke effecten op de embryonale ontwikkeling (Hoofdstuk 1). Dit onderzoek vergroot de kennis van metabole routes die bij verstoring kunnen leiden tot ontwikkelingsschade. Dit is uitgevoerd door het bestuderen van chemisch-geïnduceerde veranderingen in genexpressie, gerelateerd aan het retinolzuur-metabolisme in de zebravis als diermodel voor gewervelde dieren. Het zebravismodel is evolutionair zodanig geconserveerd dat het de embryotoxiciteit van stoffen in de mens in hoge mate kan voorspellen.

Het zebravismodel is niet nieuw, maar is vooral toegepast en geoptimaliseerd voor wat betreft morfologische effecten, omdat effecten van stoffen vanwege de transparante eieren goed geobserveerd kunnen worden. Echter, verbeteringen en harmonisatie zijn nodig om dit model toe te passen voor mechanistisch onderzoek, onder meer door relevante veranderingen in genexpressie vast te stellen. In Hoofdstuk 2 werd het testprotocol geoptimaliseerd voor het meten van genexpressie veranderingen in het zebravis embryo model. Hierbij werd de optimale blootstellingsduur vastgesteld voor het meten van veranderingen in de expressie van genen gerelateerd aan het retinolzuur-metabolisme. Zebravis embryo's werden blootgesteld aan al-trans retinolzuur gedurende 6 verschillende tijdsduren, van 2-117 uur. Morfologische en genexpressie parameters werden geanalyseerd voor elke blootstellingsduur. De grootste effecten op genexpressie werden na kortere blootstelling gezien (2-24 uur), terwijl de morfologische effecten bij langere blootstellingsduur zichtbaar werden (24-117 uur). Deze resultaten lieten zien dat 4 uur de optimale blootstellingsduur is voor het vaststellen van effecten van chemische stoffen op genen betrokken bij het retinolzuur-metabolisme.

In Hoofdstuk 3 & 4 werd het geoptimaliseerde protocol gebruikt om met behulp van genexpressie biomarker kandidaten voor ontwikkelingstoxiciteit te identificeren. Effecten van twee teratogene stoffen waarvan bekend is dat ze het retinolzuur-metabolisme verstoren (al-trans retinolzuur en valproïnezuur), en een niet-teratogene controle stof (foliumzuur) werden

bestudeerd met een genoom-brede analyse met RNA-sequencing. De drie teststoffen vertoonden elk een specifiek mRNA expressieprofiel, waarbij 248 genen gereguleerd werden door beide teratogene stoffen maar niet door foliumzuur. Deze genen zijn van belang bij verschillende ontwikkelingsprocessen waaronder die van het zenuwstelsel, hart en vaten, en het skelet. Van 62 genen werd een rol bij de vroege ontwikkeling van het zenuwstelsel gevonden, en deze zijn verder onderzocht in Hoofdstuk 3.

In Hoofdstuk 4 werden gereguleerde genen in het retinolzuur metabolisme bestudeerd die een rol spelen bij de ontwikkeling van mesodermale weefsels. Gebruik makend van verschillende methoden in de bioinformatica werden 54 gereguleerde Gene Ontology termen gevonden, gerelateerd aan 47 differentieel gereguleerde genen. Deze genen komen tot expressie in paraxiaal, internediair, dan wel laterale plaat mesoderm, en in 6 daarvan afgeleide weefsels. Dit betreft somieten, gestreept spierweefsel, bot, nier, hart en vaten, en bloed. Deze 47 genen werden aangemerkt als mogelijke biomarkers van verstoring van de vroege mesodermale ontwikkeling, of potentiële biomarkers voor de ontwikkeling van specifieke mesodermale organen.

Deze voorgestelde biomarker kandidaten dragen bij aan de kennis van ontwikkelingsschade door verstoring van het retinolzuur metabolisme. Naarmate de respons van deze genen breder gekarakteriseerd wordt op basis van meerdere blootstellingsregimes en de effecten van meerdere stoffen, kunnen deze biomarkers bijdragen als voorspellers van ontwikkelingsschade in proefdiervrije gevaars- en risicobeoordeling van stoffen. Mogelijke consequenties van deze bevindingen werden bediscussieerd.

List of publications

Peer reviewed publications

Samrani LMM, Pennings JLA, Hallmark N, Bars R, Tinwell H, Pallardy M, Piersma AH. Dynamic regulation of gene expression and morphogenesis in the zebrafish embryo test after exposure to all-trans retinoic acid. *Reprod Toxicol*. 2023 Jan;115:8-16. doi: 10.1016/j.reprotox.2022.11.001. Epub 2022 Nov 12. PMID: 36375755.

Samrani LMM, Dumont F, Hallmark N, Bars R, Tinwell H, Pallardy M, Piersma AH. Nervous system development related gene expression regulation in the zebrafish embryo after exposure to valproic acid and retinoic acid: A genome wide approach. *Toxicol Lett*. 2023 Aug 1;384:96-104. doi: 10.1016/j.toxlet.2023.07.005. Epub 2023 Jul 13. PMID: 37451652.

Samrani LMM, Dumont F, Hallmark N, Bars R, Tinwell H, Pallardy M, Piersma AH. Retinoic acid signaling pathway perturbation impacts mesodermal-tissue development in the zebrafish embryo: Biomarker candidate identification using transcriptomics. *Reprod Toxicol*. 2023 Aug;119:108404. doi: 10.1016/j.reprotox.2023.108404. Epub 2023 May 18. PMID: 37207909.

Oral presentations

Laura M.M. Samrani, Jeroen L.A. Pennings, Marc Pallardy, Helen Tinwell, Aldert H. Piersma.

The retinoic acid pathway as a source of gene expression biomarkers of developmental toxicity in the zebrafish embryotoxicity assay.

European Teratology Society (ETS) Cologne 17-20 September 2019

DOI: 10.1016/j.reprotox.2019.07.073

Samrani, L., Dumont, F., Hallmark, N., Bars, R., Pallardy, M., Piersma, A.

Gene expression responses in the zebrafish embryo model after exposures to retinoic acid pathway disruptors.

Dutch Society for Toxicology (NVT) Ede 25-26 May 2022

Gene expression responses in the zebrafish embryo model after exposures to retinoic acid pathway disruptors: all-trans-retinoic acid, Valproic acid

Journée de l'école doctoral (JED) Paris 28 June 2022

Poster presentations

Laura M.M. Samrani, Jeroen L.A. Pennings, Marc Pallardy, Helen Tinwell, Aldert H. Piersma.

Are we too late? What exposure duration is optimal for transcriptomics assessment?

European Teratology Society (ETS) Cologne 17-20 September 2019

Laura M.M. Samrani, Jeroen L.A. Pennings, Edwin P. Zwart, Marc Pallardy, Helen Tinwell, Aldert H. Piersma.

Transcriptomic and morphological study of the influence of all-trans retinoic acid's duration exposure on the zebrafish developmental mechanisms
Society of toxicology (SOT) online 12-16 March 2021

Laura M.M. Samrani, Jeroen L.A. Pennings, Edwin P. Zwart, Nina Hallmark, Remi Bars, Marc Pallardy, Helen Tinwell, Aldert H. Piersma.

Mining retinoic acid pathway related biomarkers of vertebrate developmental toxicity in the zebrafish embryo model.

World conference (WC11) online 22-26 August 2021

L.M.-E.M. Samrani, J.Pennings, E.Zwart, N.Hallmark, R.Bars, M.Pallardy, H.Tinwell, A.Piersma

Transcriptomic and morphological study of the influence of all-trans retinoic acid's duration exposure on the zebrafish developmental mechanisms.

Eurotox online 26 September- 1 october 2021

DOI: 10.1016/S0378-4274(21)00581-6

Samrani, L., Dumont, F., Hallmark, N., Bars, R., Pallardy, M., Piersma, A.

Gene expression responses in the zebrafish embryo model after exposures to retinoic acid pathway disruptors.

Dutch Society for Toxicology (NVT) Ede 25-26 May 2022

L.M.M. Samrani, F. Dumont, N. Hallmark, R. Bars, H. Tinwell, A.H. Piersma, M. Pallardy
Gene expression responses in the zebrafish embryo model after exposures to retinoic acid pathway disruptors: all-trans-retinoic acid, Valproic acid
Journée de l'école doctorale (JED) Paris 28 June 2022

L.M.M. Samrani, F. Dumont, N. Hallmark, R. Bars, H. Tinwell, A.H. Piersma, M. Pallard
Retinoic acid signaling pathway perturbation impacts the nervous system and the mesodermal-tissue development in the zebrafish embryo: biomarker candidate identification using transcriptomics.

Dutch Society for Toxicology (NVT) Ede 7-8 June 2023

Acknowledgements

The journey towards completing this PhD thesis was filled with challenges, triumphs, and countless moments of growth and learning. I want to express my deepest gratitude to all those who have supported and guided me throughout this project. Their invaluable insights and unwavering encouragement have been instrumental in its success. I would also like to extend my appreciation to the countless individuals who have directly or indirectly contributed to this endeavor.

I would like to express my sincere appreciation to my esteemed supervisors for their guidance and expertise throughout my research journey. Their insightful feedback has been invaluable in shaping my thesis and enhancing my skills as an independent researcher.

Aldert Piersma and **Remi Bars**, thank you for initiating of this project, and for consequently giving me the opportunity to discover and love an entire new field of biology.

To **Aldert Piersma** thank you for your invaluable supervision, scientific enthusiasm and support during the course of my PhD degree.

To **Marc Pallardy** thank you for asking the right questions at the right time and for training me to give articulate, scientifically sound answers.

To **Remi Bars** thank you for helping me see the big picture, and have practical thinking about what my project will bring to the scientific community.

To **Helen Tinwell** and **Nina Hallmark**, thank you for mentoring, sometimes even mothering me into the young female professional that I am today. No matter how busy you were, you always took the time to help me with my questions or hurdles. I can only wish to collaborate with such kind and knowledgeable people in the future.

I want to extend my thanks to **Janine Ezendam** and **Jan van Benthem** for enabling the realization of this PhD project within their Department (VTS-RIVM), along with the entire **VTS group**, for this nice period.

I would like to show my appreciation specifically to a number of people who took part in this work and played an important role in its completion: **Jeroen Pennings** for helping me with the bioinformatics and for being present for each of my coding questions, **Harm Heusinkveld** for the enlightening conversations about the value of the zebrafish as a model and its possible application in regulatory testing, **Eric Gremmer** and **Edwin Zwart** for training me in the handling of zebrafish and **Florent Dumont** for helping in the analysis of the NGS data. Thanks to all the people from Sophia-Antipolis for being so welcoming and for facilitating my experiments, especially, **Marie-Pierre, Jenifer, Isabelle, and Marie-Frantz**.

I want to thank the Zebrafish team, **Harm, Eric, Edwin, Leo van der Ven**, and all the interns for the fruitful discussion on our shared model and the laughter during the zebrafish meetings, in the lab and during our “uitje”.

A special thanks to **Eric** for creating such incredible pieces of art for my cover and the chapter heads; I could not imagine a more beautiful and relevant cover for this thesis. You are an incredible artist.

I would like to thank the **Writing Center** for helping me improve my articles. **Melissa Ann Thomas**, you taught me valuable lessons, that have influenced my writing style for the better. I will miss our conversations, especially the ones about our cats.

Anastasia, thank you for your advice on how to structure a PhD.

I would like to thank all my friends who supported me morally during the day-to-day hardship that is required for surviving a PhD. My fellow PhDs **Stella, Victoria, Gina, Astrid, Coen, Kim, Erna, Christy, Charlotte, Christina, Marcia, Nienke, Anniek, Els, Hannah, Job, Kirsten, Julia, Ina, Natalie**, and **Jente** and my roommates **Pegah, Bea, Nathalie, Nina**, and **Sini**; and my favorite buddy and first friend in this country, **Saskia**.

Especially thank you to what I call the “second generation of PhDs” you started during weird times but we still managed to build a friendship, I hope the payday drinks will stay a tradition between us.

To all the friends I met in Clemont over the years, the silicates, the granite, and all the others: **Anne-Pierre, Camille, Malivanh, Audrey, Marlene, Marie, Hélène, Jeanne, Laura, Magali, Thomas, Pierre, Celine** thank you for traveling and growing with me all these years.

To my paranympths **Katia** and **Nienke**, thank you for sharing this moment with me, I could not imagine better people to calm me down before the defense and organize this defense with me.

Above all, I want to thank my family. Despite the geographical distance, you still managed to support and look after me during these years of dedicated work.

First, to my parents, thank you for raising me with unconditional love and for encouraging my curiosity and love of science.

To my mom, **Claudine**, thanks for taking the time to help me with all the extra work I needed, I know the countless “dictées” were probably as frustrating for you as for me (I remember some times when it was even dangerous ^^). I would not have arrived there without you. So, thank you for making me understand that being dyslexic is an additional challenge but not a deal breaker in education.

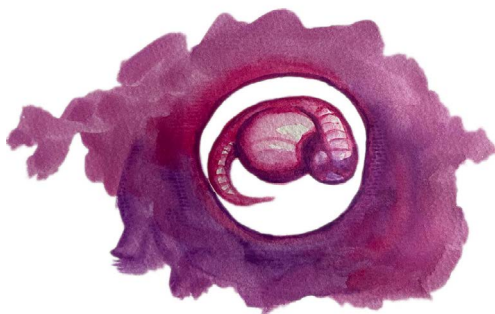
Katia thanks for your morning voice messages that helped me start my days, for making sure my texts in French were clear and without spelling mistakes, and for reminding me that doing a PhD abroad is awesome when I was doubting.

To my brother, **Nicolas** thanks for the statistics conversations and most of all for caring for me in your own way by making sure I was in good health during all these years.

A special thanks to my cousin, **Lea**, who made me appreciate the English language and lighted the first spark for a life abroad.

To my extended family, my aunts, uncles and cousins, thank you for supporting me and visiting me.

Alex, thank you for being there for me in so many ways: by cooking incredible meals when I did not even have the time to make myself a sandwich, being a shoulder to cry on, and making me laugh; you are a true partner. Additionally, thanks to your family for making me feel so welcome and supported in the Netherlands.



Curriculum vitae

Laura Marie-Elise Mathilde Samrani was born on the 23 of July 1991 in Clermont-Ferrand, France. She studied Cellular Biology in Blaise-Pascal University (Clermont-Ferrand) and she has an MSc in Physiology and Genetic (Blaise-Pascal University, Clermont-Ferrand, the Netherlands). Her first Master internship was in Pharmacology under the supervision of Prof. Dr. Gareth Sanger at the Blizzard institute of cell and molecular science in



London about protocol optimization of antibodies for immunofluorescence in human colon. Her second internship was on Antihyperalgetic effect of glutamate agonist's molecules on pain due to prostate cancer's metastasis at Neuro-dol lab in Clermont-Ferrand, France under the supervision of Dr. Jérôme Busserolles. She graduated in 2017. After spending one year working as biologist at BAAC SA center for integrative medicine in Montreux, Switzerland; she received a CIFRE grant and started her PhD project in 2018 under the supervision of Prof. Dr. Aldert Piersma and Prof. Dr Marc Pallardy. Her project unified different sectors; governmental institute (RIVM, the national institute for the health and the environment, Bilthoven, the Netherlands), industry (Bayer crop science, Sophia-Antipolis, France), and universities (Utrecht University, Utrecht, the Netherlands and Paris Saclay University, Paris, France) toward a better identification of teratogenic chemicals for human development. During her research, she was actively engaged in science community building creating news letters, organizing workshops, and social activities for PhD candidates through her board position in PRONERI networking association.

