Advancing the mechanistic understanding of the toxicological effects of non-steroidal anti-inflammatory drugs (NSAIDs) using integrated *in silico* and *in vivo* approaches

A thesis submitted for the degree of

Doctor of Philosophy (PhD)

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June 2021

Acknowledgements

I would firstly like to acknowledge and thank my primary supervisor, Luigi Marigotta-Casaluci, for his continuous and dedicated support throughout the course of my research, and without whom this thesis simply would not have been possible.

I would also like to acknowledge the remainder of my supervisory team, including Stewart Owen, Mariann Rand-Weaver, and John Sumpter for their encouragement, guidance, and support throughout the course of my PhD.

I would like to acknowledge the technical teams at Brunel University London for their dedicated and critical practical support including Julie Walker, Neil Brodigan, Giang Duong, Marta Straszkiewicz, and Nicola Beresford.

I would like to acknowledge my family and friends who were instrumental in providing the encouragement and the motivation required to complete my PhD.

Finally, I would like to dedicate this thesis to Jessica Mairi Turnbull for her unwavering support, encouragement, compassion, and love which made it all possible. All that I am, I owe to her.

Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have been available on the market for over 100 years, and they are some of the most widely and highly consumed pharmaceuticals worldwide. Everyday millions of people take NSAIDs to effectively relieve conditions involving inflammation and pain, however, their long-term therapeutic use is associated with many adverse effects which are characterised by a wide range of severity. Moreover, due to their high volume of consumption, NSAIDs excreted by patients are often detected in the aquatic environment raising concerns about possible adverse effects in aquatic wildlife. These multi-species safety concerns led to various regulatory actions aimed at protecting both human and environmental health over the last 20 years. Nonetheless, after decades of research our understanding of NSAIDs toxicological effects in multiple organs and systems remains incomplete. Here, we describe the development and integration of *in silico* and *in vivo* approaches to help further our mechanistic understanding of NSAIDs-mediated effects and to support NSAIDs safety assessment. Novel data mining, integration, and modelling of existing multi-dimensional datasets (Chapter 2) was used to generate accurate *in silico* predictions of the hazards and risks associated with exposure to both individual NSAIDs, and their mixtures. This computational approach led to the development of an innovative and predictive pharmacology-informed framework able to support the environmental risk assessment of NSAIDs mixtures in the environment, removing the need for additional *in vivo* testing (Chapter 3). This computational workflow also led to the identification of immunomodulation as a key mode-of-action for NSAIDs warranting further investigation. The *in vivo* characterisation of NSAIDs immunomodulatory effects in the gastrointestinal tract of zebrafish larvae revealed several novel findings (Chapter 4). Firstly, the activation and transendothelial migration of neutrophils into the intestine was identified as a probable key event in the pathogenesis of NSAIDs-induced enteropathy. Secondly, inhibition of the resolution of inflammation was identified as a novel putative mechanism of toxicity for the non-selective NSAID diclofenac. These effects may significantly hamper the ability of the gastrointestinal mucosa to resolve NSAIDs-induced neutrophilic inflammation, and ultimately lead to tissue damage. Furthermore, significant differences between selective and non-selective NSAIDs were revealed at both cellular and transcriptomic levels. Overall, the experimental results presented in this thesis support the notion that the mechanisms driving NSAIDs effects extend well beyond their primary mode-of-action (cyclooxygenase inhibition), and demonstrate the significance of immunomodulatory processes in mediating these effects within the gastrointestinal tract.

Statement of originality

I declare that this thesis has been composed by myself, and that the work has not been submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included, or where stated otherwise by reference or acknowledgment. My contribution, and those of the other authors, to this work has been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 3 was previously published in the journal *Environment International* as "*Pharmacology-informed prediction of the risk posed to fish by mixtures of non-steroidal anti-inflammatory drugs (NSAIDs) in the environment"* by myself - Philip Marmon, Dr Stewart F. Owen (industry supervisor), and Luigi Margiotta-Casaluci (first supervisor). This study was conceived by myself under the supervision of Dr Luigi Margiotta-Casaluci. I led all phases of the work, and the detailed author contributions are as follows:

Philip Marmon: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Conceptualization, Validation, Writing - review & editing.
Stewart F. Owen: Funding acquisition, Supervision, Conceptualization, Validation, Writing - review & editing.
Luigi Margiotta-Casaluci: Project administration, Resources, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Funding acquisition, Supervision, Conceptualization, Validation, Writing - review & editing.

One specific analysis presented in Chapter 4 (RNA sequencing) was carried out by an external service provider (Cambridge Genomics Services), which also carried out the initial data analysis workflow for the calculation of differential gene expression. I confirm that the interpretation of that data, the subsequent analysis, and all of the other experimental work described in Chapter 4 is my own work.

Contents

Chapter 1

Introduction

1.1 Project overview

The overall aim of this project is to advance the understanding of non-steroidal anti-inflammatory drugs (NSAIDs) pharmacology. These compounds exert their main therapeutic effect by inhibiting one or both isoforms of the enzyme cyclooxygenase. NSAIDs are used every day by millions of people around the world to treat conditions involving pain and inflammation. However, their use is also associated with a variety of adverse drug reactions that can hamper their clinical efficacy. This introductory chapter will provide an overview and perspective on the key elements of this scientific challenge, setting the scene for the detailed analyses and discussions that will be presented in the subsequent chapters of this thesis.

1.1.1 Cyclooxygenase and prostanoid biology

Prostanoids are eicosanoid lipids that are biosynthetically produced via the metabolism of free arachidonic acid (AA) in the cytosol. A variety of stimuli, such as cytokine signalling through the MAPK (mitogen-activated protein kinase) pathway or oxidative stress, are able to increase cytosolic levels of AA via the action of cytosolic phospholipase A_2 ($cPLA_2$) or phospholipase C (PLC). These enzymes act to release bound AA from phospholipids in the cell membrane. Prostaglandin G/H synthase, comprising a cyclooxygenase (COX) complex, transforms the AA into two intermediates; PGG_2 is produced initially, which is subsequently reduced to PGH_2 (Flower, 2003). Tissue-specific isomerases are then able to couple with COX to catalyse the conversion of this substrate into a variety of prostanoids including prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D2 (PGD_2), and thromboxane (TXA_2) (Flower, 2003). Each prostanoid acts on specific G-Protein coupled receptors (GPCRs) in order to exert a multitude of effects (Figure 1.1). The observed effects are likely to be spatially and temporally dependent as most prostanoids are able to exert numerous, and sometimes contrasting, effects (Ricciotti and FitzGerald, 2011).

COX exists as a dimeric enzyme complex which elicits hydroperoxidase and cyclooxygenase activity and can be subdivided into the variant isozymes COX-1 and COX-2. A third variant, COX-3, has been discovered more recently; however, its functional role in humans and other species is still largely unknown (Flower, 2003). COX-1 is expressed constitutively in a large majority of cell types whilst COX-2 is considered an inducible isoform produced in response

to various stimuli including cytokines, hormones, tumour promoters, and shear stress (Ricciotti and FitzGerald, 2011; Smyth *et al.,* 2009). Both isoforms have the capacity to catalyse the synthesis of any prostanoid, however, they each show a preferential coupling to certain isomerases. COX-1 displays preferential coupling with three isomerases: thromboxane synthase (TXS), the cytosolic form of prostaglandin E synthase, and prostaglandin F synthase (Ricciotti and FitzGerald, 2011). Whereas COX-2 tends to co-localise with the microsomal form of prostaglandin E synthase, and prostaglandin I synthase (Ricciotti and FitzGerald, 2011).

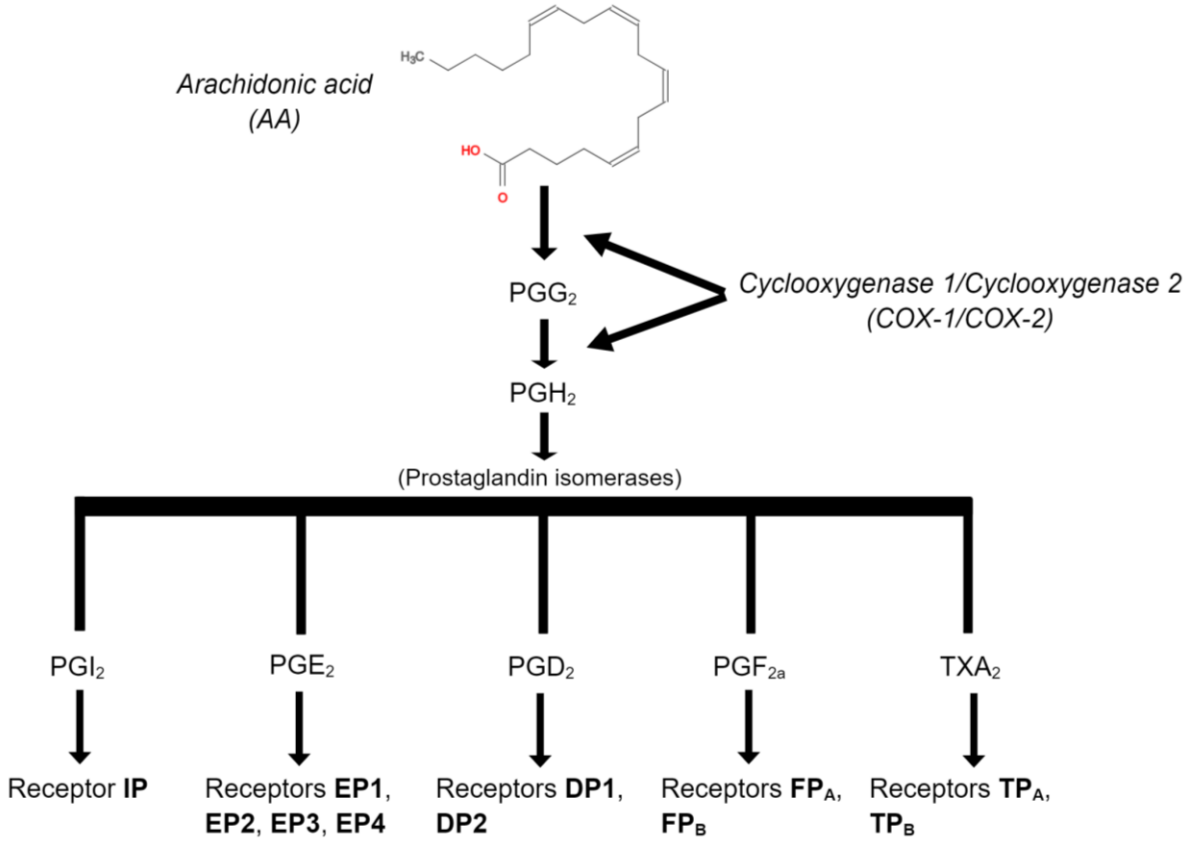

Figure 1.1. Prostanoid biosynthesis pathway, and their receptors.

1.1.2 Prostanoids in health and disease

In health, prostanoids are thought to function by maintaining homeostatic mechanisms such as regulating the gastrointestinal (GI) mucosa, vascular endothelium, blood pressure, and a multitude of other processes (Rouzer and Marnett, 2009). For example, PGE_2 is the most abundant prostaglandin and has been identified as one of the main components responsible for regulating blood pressure, maintaining the integrity of the GI mucosa, and mediating the immune response (Wallace, 2008). Disruption of PGE_2 physiology can lead to a vast range of pathological conditions, including intestinal epithelial cell damage through disruption of barrier

function, and vasoconstriction of mucosal vasculature (Legler *et al.,* 2010; Wallace, 2008). PGE_2 has also been found in high abundance in the synovial joints of patients with rheumatoid arthritis where it has been shown to downregulate the production of pro-inflammatory cytokines such as TNF-α (Akaogi *et al.,* 2006). Paradoxically, there is a wealth of evidence to suggest that NSAIDs, which are designed to reduce inflammation, can induce the upregulation of TNF-α synthesis as well as other pro-inflammatory molecules (Page *et al.,* 2010). This was demonstrated *in vitro* using human peripheral blood mononuclear cells (PBMCs) and rheumatoid synovial cultures exposed to celecoxib; expression of TNF- α was upregulated in these cultures (Page *et al.*, 2010).

Prostanoids are ubiquitous throughout the body, with specific levels of each variant depending on tissue/cell type and local homeostatic status. All prostanoids are synthesised from the PGH_2 substrate via tissue-specific isomerases which couple to the COX complex, so if one COX isoform and one tissue-specific isomerase happens to be more abundant in a particular tissue, or cell, it is likely that there will be higher levels of that respective prostanoid in that tissue or cell. In a non-diseased/non-inflamed state, most cells tend to produce mainly one or two PGs which act to regulate homeostasis locally (Ricciotti and FitzGerald, 2011). However, uncertainty still surrounds the significance of these endogenous prostanoids in uninflamed or healthy tissues, at homeostatic levels of abundance.

During inflammation, processes are activated by certain unspecific stimuli which evoke an increase in free cytosolic AA, and hence, an increase in COX expression/activation and prostanoid biosynthesis. COX-2 is the inducible form of the enzyme, expressed in response to inflammatory stimuli, and is the major source of prostanoid production during inflammation. COX-1, however, is also thought to contribute to the increased production of prostanoids during acute inflammation (Ricciotti and FitzGerald, 2011). PGE_2 and PGI_2 are the most potent pro-inflammatory prostanoids as they significantly increase levels of oedema, vascular permeability, and leukocyte infiltration as a result of increased blood flow (Smyth *et al.,* 2009). Higher levels of prostaglandins, and TXA_2, act to rapidly promote localised inflammation and pain via interaction with their respective G-Protein Coupled Receptors (GPCRs) (FitzGerald, 2003). Interestingly, there is also some evidence to suggest that COX-2 derived prostanoids can elicit anti-inflammatory effects and contribute to resolution of inflammation. Hence, they may not solely be mediators of pro-inflammatory pathways (Ricciotti and FitzGerald, 2011). In fact, COX-2 has been significantly implicated in both homeostatic and pro-resolving functions such as maintaining the integrity of the GI mucosa, mediating the resolution of GI inflammation, and regulating ulcer healing (Wallace and Devchand, 2005).

Prostanoids have a very diverse range of outputs which are dependent upon localised spatial and temporal elements. Different cell types and tissues will comprise variant patterns of expression and activity with regards to receptors, isomerases, and COX isoforms. Specific occurrences, such as receptor dimerization, can also result in differing outputs which can even be contrasting in some cases (Ricciotti and FitzGerald, 2011). The prostaglandin I_2 (prostacyclin) receptor (IP) and the thromboxane receptor (TP) have been found to interact with themselves to form homodimers (IP-IP or TP-TP), as well as with one another to form heterodimers (IP-TP). The heterodimer formation is able to promote cyclic adenosine monophosphate (cAMP) production via activation of the TP receptor. This increase in intracellular cAMP is usually mediated through IP receptor activation (Wilson *et al.,* 2004). TXA_2 is found in abundance in platelets, synthesised predominantly by COX-1, and acts as a potent vasoconstrictor and major factor contributing to platelet activation and aggregation. In contrast, PGI_2 is most abundant in the vasculature and acts as a vasodilator and inhibitor of platelet aggregation via the modulation of TXA_2 mechanisms *in vivo* (Wilson *et al.,* 2004). However, as previously mentioned, these actions of PGI_2 and TXA_2 are dependent upon their respective receptors, IP, and TP. They can have different, and even contrasting, modes of action depending on receptor status (Wilson *et al.,* 2004). These examples highlight some of the difficulties in making predictions about the specific signalling processes taking place at localised inflammatory sites during an acute response. Clearly there is still much to learn about the exact molecular mechanisms involved in prostanoid biology.

PGD_2 is produced in the central nervous system (CNS) where it is involved in the regulation of sleep and pain perception. It is also produced in peripheral tissues by mast cells which if activated lead to the initiation of IgE-mediated allergic responses (Ricciotti and FitzGerald, 2011). Dendritic cells (DCs) and Th2 cells are components of the immune system which also produce PGD_2, which suggests a potential role for PGD_2 in the regulation of antigen-specific immune responses (Urade *et al.,* 1989). PGD_2 produced by activated mast cells, and other immune cells, is capable of eliciting its pro-inflammatory effects via its receptors 'prostaglandin D_2 receptor 1' (DP1) and 'prostaglandin D_2 receptor 2' (DP2) (Ricciotti and FitzGerald, 2011). Contrastingly, PGD_2 is also capable of producing anti-inflammatory effects via its DP1 receptor expressed on DCs. Another potentially significant role of PGD_2 is its inhibitory effect on the progression of atherosclerosis. A knock-out of L-PGDS, the specific isomerase which transforms PGH_2 into PGD_2, in mice has been associated with an accelerated progression of atherosclerosis (Tanaka *et al.,* 2009). Thus, it is feasible that a depletion of both PGD_2 and PGI_2 may lead to significantly accelerated atherosclerotic phenotypes, combined with vasoconstriction and thrombus formation, which could be detrimental to patients with increased cardiovascular risk factors.

$PGF_{2\alpha}$ elicits its effects through the 'prostaglandin F receptor' (FP). When the receptor is bound by $PGF_{2\alpha}$, it couples with protein Gq in order to increase the intracellular concentrations of free calcium (Ricciotti and FitzGerald, 2011). As with other prostanoids, $PGF_{2\alpha}$ has been associated with many diverse biological roles which include vasoconstriction, pain perception, renal function, and tachycardia (Ricciotti and FitzGerald, 2011). In contrast to PGD_2, which displays cardio-protective properties, increases in $PGF_{2\alpha}$ have been associated with various cardiovascular toxicity risk factors including obesity, smoking, and thickening of the carotid artery wall (Ricciotti and FitzGerald, 2011). Studies in mice have shown that the deletion of the FP receptor leads to a decrease in blood pressure and halts the progression of atherosclerosis (Yu *et al.,* 2009). On the other hand, Smyth *et al.* (2009) suggest that prostanoids derived from COX-2 play a pivotal role in the normal functioning of cardiac tissue, and that deletion of murine cardiomyocyte COX-2 led to adverse cardiac events such as heart failure and fibrosis in the cardiac tissue.

The whole story is yet to be uncovered, with ambiguity surrounding the exact molecular mechanisms that underpin prostanoid biology in both health and disease states. It is critical for the development of new, safer, and more efficient anti-inflammatory drugs, to better understand the varied and significant actions of prostanoids.

1.1.3 Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of compounds with an overarching pharmacological feature: all NSAIDs are COX enzyme inhibitors. COX inhibitors or NSAIDs are some of the most highly consumed drugs worldwide. Several of these compounds are available over-the-counter and approximately 11.5 million NSAIDs prescriptions are dispensed in England alone each year (Machado *et al.,* 2021). They are used to treat conditions involving pain and inflammation, and in many cases, are prescribed in combination with other drugs to treat specific diseases. Treatment scenarios can range from localised shot-term inflammation at the site of a small cut, to headaches and chronic inflammatory conditions such as rheumatoid arthritis or osteoarthritis (Machado *et al.,* 2021). There is even evidence to suggest that they may aid in the treatment of Alzheimer's disease (Flower, 2003), and some of them have been proposed as preventive therapeutic intervention to reduce the risk of developing of cancer (Zhang, Chen, and Shang, 2018).

NSAIDs can be further subdivided into various categories: some are non-selective for either COX isozyme (COX1 and COX2) like ibuprofen, naproxen, and diclofenac, whereas others are COX-2 selective inhibitors such as celecoxib and rofecoxib. NSAIDs also differ in terms of inhibitory kinetics, such as competitive reversible inhibition *versus* competitive non-reversible

inhibition (Flower, 2003). Due to the heterogeneity between distinct NSAIDs, their pharmacokinetic and pharmacodynamic properties are likely to vary between compounds, and like most pharmaceuticals there is an array of potential off-target effects. A recent paper by Grosser and collaborators highlighted this issue in terms of the cardiovascular risk profiles of distinct NSAIDs, and the heterogeneity between these drugs (Grosser *et al.,* 2017). Currently there are over 25 NSAIDs available on the market with some drastic differences in effect and safety profiles. Some of the most prominent adverse effects associated with NSAIDs exposure include serious gastrointestinal (GI), cardiovascular, renal, and hepatic toxicity (Smyth *et al.*, 2009). In fact, after more than 120 years since aspirin was first introduced to the market, GI toxicity still represents one of the major clinical challenges associated with NSAIDs use (Wallace, 1997; Bindu, Mazumder, and Bandyopadhyay, 2020). Clearly even after decades of research there remains a significant health risk associated with NSAIDs that is yet to be solved. Nonetheless, levels of consumption are ever increasing regardless of the evident risks associated with their use, due to the lack of safe alternatives. Furthering our knowledge of the mechanisms involved in NSAIDs mode-of-action is critical to understanding their risk. Although we understand a great deal about prostanoid biology, the promiscuity of these drugs is such that mechanisms independent of COX inhibition are likely to be significant contributors to some of the adverse effects that we see in the clinical setting.

1.1.4 A brief history of NSAIDs

Aspirin was the first NSAID introduced to the market in 1897, followed by indomethacin and ibuprofen in 1964 and 1969, respectively (Conaghan, 2012). A common feature shared between all NSAIDs introduced into the market before 1998 is that they can inhibit both COX-1 and COX-2 isoforms, with varying degrees of selectivity and potency. Unfortunately, another commonality shared by all NSAIDs from this era is the GI toxicity attributed to their long-term therapeutic use, which has been evident for many decades, and remains a significant risk factor today (Wallace, 1997). Due to the fact that COX-1 is widespread and constitutively expressed and is thought to enact homeostatic functions, and that COX-2 is an inducible isoform upregulated in response to inflammatory stimuli, it was hypothesised that the adverse effects associated with NSAIDs could be attributed to the inhibition of COX-1 (Masferrer *et al.*, 1994). This theory in combination with some supportive evidence popularised the notion that COX-1 derived prostanoids are gastroprotective, whilst the inducible COX-2 derived prostanoids solely mediate a pro-inflammatory response (Griswold and Adams, 1996). These logical assumptions led to a race to develop the first COX-2 selective inhibitor which could specifically inhibit COX-2 mediated inflammation, without interfering with the regulatory or ‘protective’ properties of COX-1 derived prostanoids (Rouzer and Marnett, 2009). The search

for novel selective inhibitors of COX-2 was a success story as celecoxib (Celebrex) was developed by the Searle Monsanto programme, becoming the first of its kind to emerge in 1998, showing good *in vitro* and *in vivo* capabilities (Flower, 2003). Celecoxib with shortly followed by rofecoxib (Vioxx) which was introduced by Merck in 1999 (Krumholz *et al.*, 2007). Selective inhibitors of COX-2 such as celecoxib and rofecoxib have indeed been shown to reduce the incidence of adverse gastrointestinal events, compared with non-selective NSAIDs, whilst effectively providing anti-inflammatory and analgesic properties. These characteristics of COX-2 inhibitors added weight to the idea that COX-1 synthesised prostanoids play a pivotal role in the maintenance and protection of the gastric mucosa. What was not anticipated, however, was that a significant increase in adverse cardiovascular events is apparent with COX-2 selective inhibitors.

In the case of rofecoxib (Vioxx), mixed results about the cardiovascular safety of this drug came from a number of trials including the 'Vioxx Gastrointestinal Outcomes Research trial (VIGOR)', 'phase IIb/III osteoarthritis combined analysis study', and the 'Alzheimer's and mild cognitive impairment study' (Goldmann, 2001). However, it was the significant increase in serious adverse cardiac events in patients receiving Vioxx during the 'Adenomatous Polyp Prevention on Vioxx' (APPROVe) trial, which led Merck to implement the immediate withdrawal of this drug from the global market in 2004 (Cottor and Wooltorton, 2005). Subsequently, the U.S. Food and Drug Administration (FDA) released a study estimating that in the five years Vioxx was marketed for, it was responsible for around 56,000 cardiac-related deaths in the USA alone (O'Steen and O'Steen, 2006). The story of celecoxib (Celebrex) adds further weight to the hypothesis that selective COX-2 inhibitors increase the risk of adverse cardiac events in patients. However, initial indications looked positive for celecoxib during the 'Celecoxib Long-term Arthritis Safety Study' (CLASS) trial which began in 1998 and came to an end in early 2000. The aim of the study was to determine whether celecoxib, compared with non-selective NSAIDs, incurred significantly fewer upper gastrointestinal adverse events, as well as other endpoints. The overall conclusions drawn from this study determined that celecoxib, at dosages higher than therapeutic, was safer than non-selective NSAIDs at standard therapeutic levels (Silverstein *et al.,* 2000). Notably, they reported that "No difference was noted in the incidence of cardiovascular events between celecoxib and NSAIDs, irrespective of aspirin use" (Silverstein *et al.,* 2000) which does not fit with the hypothesis that COX-2 selective inhibitors increase the risk of cardiotoxicity. However, the story does not end there, as the 'Adenoma Prevention with Celecoxib (APC) trial' began in 1999 and ended in 2002, which was a longer-term placebo controlled study assessing the efficacy of celecoxib to reduce the occurrence of polyps in the colon and rectum (Solomon *et al.,* 2005). In light of the APPROVe trial with rofecoxib and the withdrawal of this drug from the market, a cardiovascular

safety committee was appointed to define and review end-points based on clinically relevant data and the previous data from the APPROVe trial (Solomon *et al.,* 2005). The trial concluded that, in terms of serious cardiovascular adverse events, long-term exposure to celecoxib put patients at a significantly increased risk. Hence, the clinical data generated for celecoxib are also in agreement with the theory that COX-2 selective inhibitors are associated with increased risk of serious adverse cardiac events. This led the FDA to determine that all prescribed and over-the-counter NSAIDs must include specific information on the potential cardiovascular, gastrointestinal, and other associated risks (Cottor and Wooltorton, 2005). A similar story became apparent with another COX-2 selective inhibitor called valdecoxib (Bextra). Following a safety trial Bextra also proved to significantly increase the risk of adverse cardiovascular events in patients, compared with placebo. The result of this trial led to the withdrawal of Bextra from the market in 2005, as requested by the FDA (Cottor and Wooltorton, 2005).

1.1.5 Clinical concerns and challenges

It is clear that long-term therapeutic use of NSAIDs is likely to result in a number of toxic or adverse phenotypes, depending on the specific NSAID administered and the specific risk factors of the individual. We know that NSAIDs-related toxicity can range from mild conditions of dry skin or rashes to more serious gastric, cardiac, and renal adverse events. In fact, 30% of all hospital admissions for adverse drug reactions (ADRs) in the UK can be attributed to NSAIDs use (Davis and Robson, 2016). This is a worrying statistic when you consider how widespread their use is, how easily available they are, and the lack of safe alternatives.

Contrary to popular opinion, there is evidence to suggest that the ‘constitutive COX-1 and inducible COX-2’ theory may not be as accurate as first thought (Zidar *et al.*, 2009). It seems that dependent upon the tissues in question, their expression patterns and roles may be very different, or even reversed. For example, COX-1 expression is induced in response to lipopolysaccharide (LPS) mediated inflammation, whereas COX-2 is constitutively expressed in the kidneys, female reproductive organs, and the brain (Rouzer and Marnett, 2009). Furthermore, even though COX-1 is constitutively expressed in the GI tract, and COX-2 is inducible, inhibiting their function may have more complex effects than initially thought. Studies using COX-1 knockout mice and selective COX-1 inhibitors have shown that a depletion of COX-1 does not increase the susceptibility of these mice to GI ulceration, and that selective inhibition of COX-1 is insufficient for inducing gastric lesions (Rouzer and Marnett, 2009). Moreover, genetic depletion of COX-1 has also revealed a role in mediating LPS-induced inflammation in mouse models (Choi *et al.*, 2008). The intricacies of the COX inhibition paradigm do not end there, however, as COX-2 has also been implicated in several other roles

besides solely initiating inflammation. Several studies have demonstrated crucial roles for COX-2 in regulating the defence of the GI mucosa, regulating ulcer healing, and significantly contributing to the resolution of GI inflammation (Wallace and Devchand, 2005). In fact, even if the risk of significant GI toxicity is ameliorated with the use of COX-2 selective inhibitors compared to non-selective NSAIDs, that risk is by no means negligible, as COX-2 selective inhibitors induce ulceration at around half the rate of non-selective NSAIDs (Bombardier *et al.*, 2000; Wallace and Devchand, 2005). Interestingly, although COX-2 selective inhibitors have been associated with increased prevalence of cardiovascular disease, a comparison between patients taking naproxen (non-selective) or rofecoxib (COX-2 selective) revealed that the overall mortality rate from cardiovascular complications was similar between each group (Bombardier *et al.*, 2000). Furthermore, it has been reported that all NSAIDs, regardless of selectivity for COX-1 or COX-2, increase the risk of hospitalisation due to heart failure by twofold (Davis and Robson, 2016). Clearly there is significant evidence which reveals roles for both COX-1 and COX-2 beyond the simplistic rationale which drove the development of COX-2 selective NSAIDs.

It is possible to hypothesise one plausible mechanism driving the cardiovascular toxicity associated with exposure to COX-2 selective NSAIDs. COX-2 is highly expressed in the vascular endothelium where it synthesises PGI_2, a potent vasodilator and regulator/inhibitor of platelet aggregation. Whereas COX-1 is highly expressed in platelets and is responsible for the synthesis of TXA_2, which is a potent vasoconstrictor and agonist of platelet aggregation (FitzGerald, 2003). Therefore, it is feasible that due to the reduced inhibition of platelet aggregation and vasodilation via PGI_2, and the unopposed action of TXA_2, activated platelet aggregation and vasoconstriction is able to occur unopposed. If these effects are induced through the selective inhibition of COX-2, then it would seem logical that adverse cardiovascular events such as thrombosis, atherosclerosis, and even myocardial infarction might occur in patients with increased risk factors for cardiovascular disease. Through enhancement, deletion, and antagonism experiments using the PGI_2 and TXA_2 receptors it has been shown that PGI_2 is responsible for the regulation of interactions between platelets and the vasculature, limiting the response to TXA_2 *in vivo* (Cheng *et al.,* 2002). Therefore, it is likely that COX-2 selective inhibitors can significantly disrupt these dynamics, leading to the clinically-relevant cardiovascular complications commonly associated with these drugs.

Significant renal toxicity has also been linked to COX-2 selective NSAIDs, including salt and water retention, and also hypertension in predisposed individuals (Catella-Lawson *et al.,* 1999). Studies in murine models have demonstrated these effects of COX-2 inhibitors on renal function, and in particular hypertension. Interestingly, it appears that in mice the relationship between COX-1 and COX-2 synthesised prostanoids in the kidney share similar

characteristics to their cardiovascular interactions (Qi *et al.,* 2002). For example, one study assessed the angiotensin-II hypertensive response in mice under a variety of conditions; reducing COX-1 activity by deletion or inhibition resulted in a reduced hypertensive response to angiotensin-II, which was exacerbated when the angiotensin-II receptor was also deleted. Alternatively, selective inhibition or deletion of COX-2 led to an increased hypertensive response to angiotensin-II, accompanied by a reduction in medullary blood flow (Qi *et al.,* 2002). If we consider the relationship between COX-1 derived TXA_2 and COX-2 derived PGI_2 (and even PGE_2) at the vascular endothelium-platelet interface to have a similar dynamic in the kidney, then this would provide a plausible explanation for the hypertensive effects of COX-2 selective NSAIDs seen in mice (FitzGerald, 2003).

Serious GI toxicity represents the major limitation to prolonged NSAIDs use. It is clear that the inhibition of COX-1 alone is not sufficient for the induction of clinically relevant GI complications. On the other hand, it is apparent that the selective inhibition of COX-2 does not completely abolish the prevalence of GI toxicity. Therefore, it appears that both COX-1 and COX-2 derived prostaglandins together are important mediators of mucosal homeostasis, defence, and inflammation (Wallace, 2008) and that inhibition of both isoforms is needed to induce GI toxicity (Wallace *et al.*, 2000). In addition to inhibiting the biosynthesis of prostanoids, NSAIDs are able to exert topical mechanisms of epithelial cell damage in the GI tract. Evidence suggests that NSAIDs can directly induce apoptosis in epithelial cells through either osmotic lysis or uncoupling of oxidative phosphorylation (Schoen and Vender, 1989; Somasundaram *et al.*, 1995; Wallace 2008). Moreover, NSAIDs have been shown to reduce bicarbonate and mucus secretion, disrupting the balance of pH at the epithelial membrane. NSAIDs also directly disrupt apical phospholipids on the mucosal surface leading to increased barrier permeability (Wallace, 2008). Although topical mechanisms are likely to contribute to the pathogenesis of NSAIDs-induced GI toxicity, they are not likely to be solely responsible, since injectable NSAIDs have even been shown to induce ulcer formation (Estes *et al.*, 1993), which implies that the systemic effects of NSAIDs are critical to the pathogenesis of GI toxicity. It is likely that systemic mechanisms, independent of COX inhibition, also significantly contribute to the manifestation of NSAIDs-induced enteropathy. The elucidation of some putative key events has been important in progressing our understanding of these mechanisms. In fact, some evidence suggests that neutrophilic adhesion to the vascular endothelium, and subsequent transendothelial migration into the mucosa, may represent an important event in the pathogenesis of NSAIDs-induced GI toxicity (Wallace, Keenan, and Granger, 1990). The potential significance of neutrophil contribution to GI injury may not be negligible, as one study found that attenuation of neutrophil function was shown to be protective against NSAIDs-induced gastric damage (Wallace, Arfors, and McKnight, 2008).

Although significant progress towards a better understanding of NSAIDs-mediated GI toxicity has been made, the mechanisms underpinning their pathogenesis are still not completely understood (Wallace, 2008). It is clear that the immunomodulatory effects of NSAIDs do indeed extend beyond COX inhibition, and that furthering our understanding of the mechanisms involved is critical to making better safety decisions concerning NSAIDs use.

1.1.6 Environmental health concerns

Pharmaceuticals entering into the environment has been a topic of concern for many years and remains a pressing issue to this day. Due to the very high usage of NSAIDs worldwide, low but sustained concentrations of these drugs are readily detectable in the aquatic environment (Lonappan *et al.*, 2016; Memmert *et al.,* 2013). Currently, wastewater treatment plants (WWTPs) do not have the capacity to completely extract all of the diverse types of contaminants from the wastewater which ultimately leads to a vast mixture of chemicals reaching the aquatic environment, albeit at low concentrations (Kern, 2014). In some instances, pharmaceutical contaminants have even been detected in drinking water due to incomplete elimination by WWTPs (McEachran et al., 2016). For instance, ibuprofen has been detected at concentrations of 1.35 µg/L in drinking water in California (Loraine and Pettigrove, 2006; Zhang *et al*., 2020). In the case of NSAIDs specifically, their detection in the environment may be somewhat unsurprising considering their global levels of production and consumption. For example, in the USA alone it is estimated that around 70 million NSAIDs prescriptions are given, and around 30 billion over-the-counter NSAIDs are sold each year (Green, 2001). In Spain, ibuprofen concentrations in WWTP effluents have been reported to be as high as 55 µg/L (Santos *et al.*, 2009; Zhang *et al.*, 2020). Similarly, in Germany diclofenac concentrations in surface waters have been detected at concentrations as high as 4.7 µg/L (Heberer, 2002; Zhang *et al.*, 2020).

Due to the significant health risks associated with prolonged NSAIDs exposure in humans, safety concerns were raised about the effects that chronic exposure to NSAIDs might have in wild fish populations. Thus, regulatory action regarding NSAIDs was sparked in 2015 when diclofenac was added to the European Watch List of emerging pollutants, under the European Water Framework Directive (European Commission, 2015). Although diclofenac has been detected in European rivers at low concentrations (ng/L - µg/L), which are below the NOEC (No Observed Effect Concentration) (Memmert *et al.,* 2013), there remain concerns from regulatory bodies about the potential toxicity associated with chronic exposure to pharmaceuticals such as NSAIDs. At least 26 studies between 2004 and 2018 have investigated the effects of long-term exposure to environmentally relevant concentrations of

NSAIDs on fish species, with a variety of adverse effects reported. These include changes in prostaglandin levels, effects on testosterone, immunomodulation, liver damage, and even effects on reproduction. In fact, a more recent publication, assessing the effects of diclofenac and ibuprofen on zebrafish, found that both NSAIDs were able to impair cardiovascular development at environmentally-relevant concentrations (Zhang *et al.*, 2020).

Although most NSAIDs were designed as human pharmaceuticals, due to the conservation of the cyclooxygenase enzymes and the prostanoid biosynthesis pathway in teleost fish (Grosser *et al.*, 2002), the environmental occurrence of these drugs represents a potentially significant risk to aquatic wildlife. In other words, as stipulated by the "read-across hypothesis", NSAIDs are likely to induce similar effects across species due to the conservation of their biological targets (Patel *et al.*, 2016). A study in rainbow trout, exposed to environmentally-relevant concentrations of diclofenac, ibuprofen, and naproxen, found their biotransformation to be efficient, with concentrations of each drug detectable in the plasma and the bile (Lahti *et al.*, 2011). When fish are exposed to pharmaceuticals in the environment, they will absorb them through their skin and gills, before eventually being metabolised predominantly in the liver (Lahti *et al.*, 2011). The level of uptake, however, can vary greatly depending on the pH of the surrounding water, as NSAIDs are ionisable compounds. Their ionisation, due to changes in pH, can markedly reduce their uptake due to a decrease in lipophilicity and therefore, membrane permeability (Tanoue *et al.*, 2017).

Despite our knowledge of the risks associated with NSAIDs use, it is evident that their usage remains significant with an ever increasing volume of NSAIDs prescriptions (Davis and Robson, 2016). This increasing volume of usage means that concentrations of NSAIDs entering into the aquatic environment are likely to increase also. Mechanistic knowledge surrounding NSAIDs toxicity in humans is not fully understood, however, our mechanistic knowledge of NSAIDs/COX biology in fish species is significantly limited by comparison. Hence, there is currently no mechanistic rationale to support the environmental risk assessment (ERA) of NSAIDs, which means that the potential for implementation of predictive toxicology approaches to support decision making is limited. Furthermore, over 25 different NSAIDs are currently available on the market all of which inhibit COX-1 and/or COX-2. This suggests that if all of these compounds are able to reach the aquatic environment then the risk of additive mixture effects is significantly increased, and diclofenac may not be the only NSAID to worry about. This scenario causes a significant problem for regulators, as there are currently no defined protocols for the mixture assessment of pharmaceuticals in the environment. Generating novel methods of risk assessment, which take mechanistic/mode-of-action considerations into account and incorporate mixture assessment, will be crucial to informing better decision making.

1.1.7 The zebrafish (*Danio rerio*) model organism

Zebrafish offer a powerful and tractable vertebrate model to effectively study the effects of human pharmaceuticals *in vivo*. The prostanoid biosynthesis pathway is highly conserved between vertebrates, and the zebrafish COX proteins represent functional orthologs of the human isozymes, with high genetic and pharmacological homology (Grosser *et al.*, 2002). Mapping of the zebrafish genome revealed large regions of chromosomes to be syntenic with human chromosomes, and moreover, both COX enzymes appear to fall within such a region (Prescott and Yost, 2002). The genetic, and functional, conservation of these proteins make the zebrafish a viable model organism for the study of NSAIDs effects *in vivo*. In fact, zebrafish have severed as a model organism in several thousands' of studies to date, as they offer an attractive alternative to other vertebrate models such as rodents. Practical advantages over rodent models include rapid development, transparent embryos, and the ability to produce hundreds of offspring with each egg lay (Langova *et al.*, 2020). Similar to human patterns of expression, zebrafish COX-1 appears to be widely expressed throughout the embryo, whereas the COX-2 isozyme appears to display a more tightly defined pattern of expression (Grosser *et al.*, 2002).

Due to the genetic and functional conservation of relevant pathway components, in addition to the practical advantages, the zebrafish represents a powerful tool to further study the mechanistic effects of NSAIDs. Moreover, due to the risks posed by NSAIDs to wild fish, the zebrafish offers a highly relevant model species to investigate these potential effects further.

1.2 Project hypotheses, aims, and methodological vision

The present project is based on two overarching hypotheses, one methodological and one biological. From a methodological perspective, the first hypothesis is that computationally driven mode-of-action profiling of NSAIDs can guide the generation of accurate and unbiased mechanistic testable hypotheses beyond the traditional COXs inhibition theory. On the other hand, this project hypothesises that NSAIDs-mediated immunomodulation may represent a major key event in the pathogenesis of NSAIDs-induced GI toxicity. Characterising NSAIDs-mediated immunomodulation may help to understand the safety profile of individual compounds, or of different sub-classes (i.e., non-selective NSAIDs *versus* COX-2 selective NSAIDs).

The overall goal of this project is to advance our current mechanistic understanding of NSAIDs-mediated effects both at system level and, specifically, in the gastrointestinal tract. As GI toxicity represents one of the major limiting factors to NSAIDs usage, and decades of

research has yet to underpin the exact mechanisms involved, there remains a need to better understand the effects of NSAIDs in the GI tract.

To address this ambitious goal, this thesis will firstly describe the generation of *in silico* predictions of NSAIDs-mediated effects in humans and fish. By data-mining existing mechanistic and phenotypic effect data, Chapter 2 will map and integrate the modes-of-action of NSAIDs at multiple levels of biological organisation. The newly generated integrated data platform will be used to guide the subsequent phases of the project. Chapter 3 will describe the application of the mechanistic profiling exercise to explain and predict the effects of NSAIDs (individually and in combination) in fish species, and to generate a pharmacology-informed environmental risk assessment. The results obtained in Chapter 2 and Chapter 3 will then be used to tailor hypothesis generation and design of the *in vivo* phases of the project aimed at characterising NSAIDs-mediated effects on innate immune cell trafficking in the GI tract in healthy fish (Chapter 4), and in fish displaying an inflamed GI tract (Chapter 5).

The zebrafish (*Danio rerio*) was selected as the experimental model for the *in vivo* phases of the project. The larval stages offer a tractable, cost-effective, and highly translational model to study the gastrointestinal and innate immune systems. Leveraging the latest advancements in the zebrafish genetic engineering, this project will use transgenic zebrafish lines expressing fluorescent protein markers on cells of the innate immune system, in order to evaluate the immunomodulatory effects of NSAIDs in the gastrointestinal tract. Performing fluorescent *in vivo* imaging of these transgenic zebrafish larvae will enable us to capture immune cell dynamics in the GI tract in real time. Moreover, RNA-Sequencing analysis will be used to characterise the molecular perturbations associated with NSAIDs exposure.

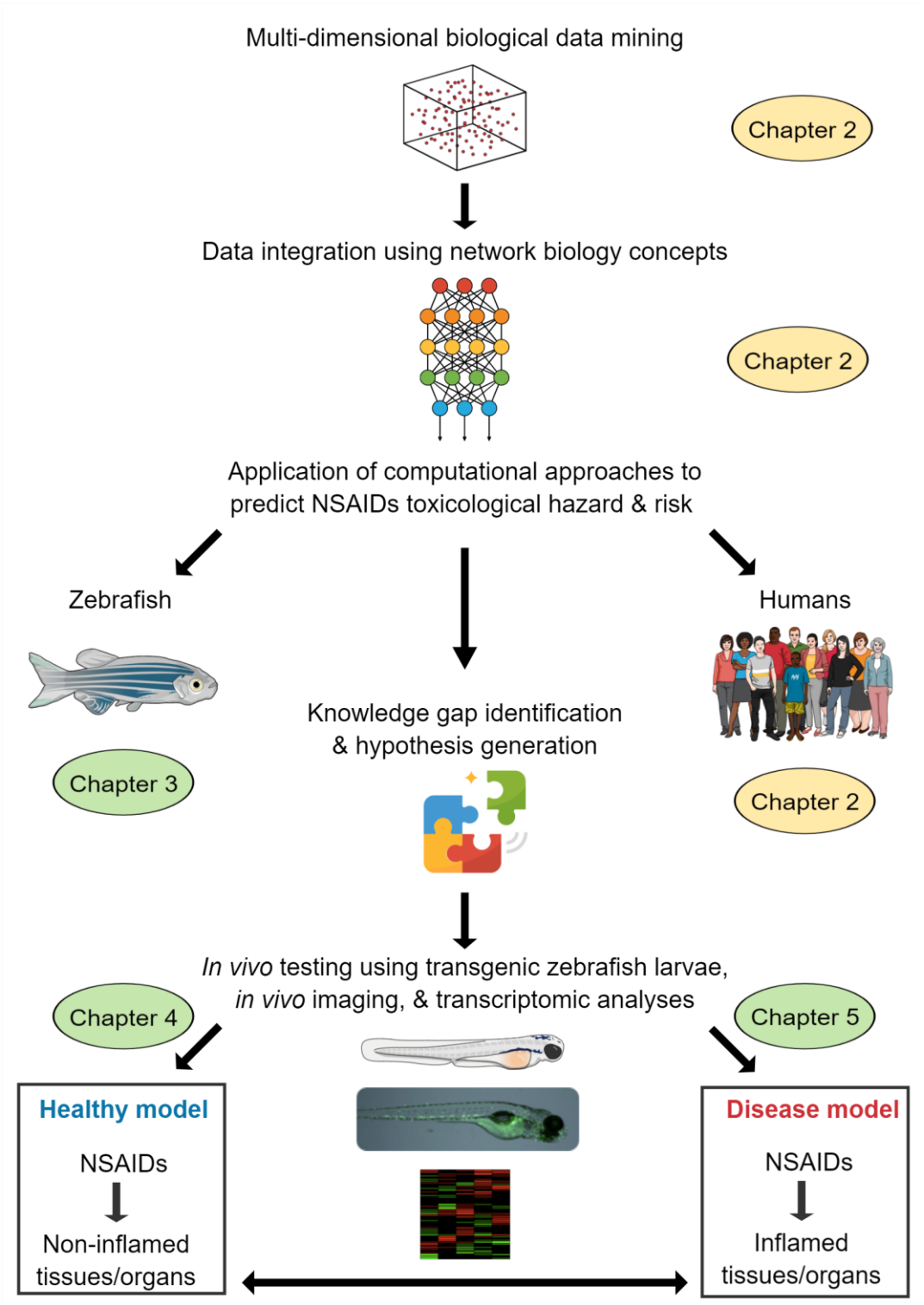

Figure 1.2. Methodological vision for the entire PhD project.

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Chapter 2

Multi-dimensional mode-of-action profiling of non-steroidal anti-inflammatory drugs (NSAIDs)

2.1 Abstract

The class of non-steroidal anti-inflammatory drugs (NSAIDs) is extensive with over 25 compounds currently available on the market. NSAIDs are also some of the most highly consumed drugs worldwide, used for their anti-inflammatory and analgesic properties. Although popular and relatively efficacious these drugs are associated with many adverse drug reactions, ranging from mild side effects to more serious adverse events, hospitalisations, and even mortality. A better understanding of NSAIDs mechanisms of toxicity is needed in order to improve their clinical efficacy, through either safer NSAIDs selection, or through guiding the development of novel therapeutic strategies to mitigate those mechanisms which lead to adversity. Here we describe the mapping and integration of NSAIDs modes-of-action at multiple levels of biological organisation. By using existing mechanistic data from a variety of sources, we were able to generate an integrated data platform which can be used to formulate specific testable hypotheses, and tailor the design of future *in vivo* studies. Our *in silico* analyses highlight immunomodulation as a key, but under-investigated, mode-of-action for NSAIDs-mediated effects. These analyses demonstrate the utility of mining existing experimental data for guiding the generation of accurate and unbiased mechanism-based testable hypotheses.

2.2 Introduction

According to the US NIH National Library of Medicine database PubMed, more than 200,000 scientific articles on non-steroidal anti-inflammatory drugs (NSAIDs) have been published in the last 50 years. In parallel, according to the US NIH and UK Research Innovation databases hundreds of research projects involving NSAIDs have been funded in the last two decades, such as the $18.5M grant awarded by the US NIH to “The Personalized NSAID Therapeutics Consortium (PENTACON)” in 2012 to advance the understanding of the mechanisms that underly NSAIDs efficacy and safety. Such high volume of research activity is justified by the critical therapeutic importance of NSAIDs at the global level for the treatment of numerous conditions involving inflammation and pain. This clinical importance is further highlighted by the inclusion of drugs such as ibuprofen, aspirin, and paracetamol in the World Health Organization (WHO) list of essential medicines. Despite such intensive research efforts, the full range of direct and indirect multi-scale mechanisms driving NSAIDs efficacy and safety remain elusive. This is demonstrated, for example, by the recent regulatory concerns related to the cardiovascular risk associated with NSAIDs administration that emerged after decades of clinical usage, which has led to regulatory action in both Europe and North America (e.g. UK Medicines and Healthcare products Regulatory Agency, 2007; UK Medicines and Healthcare products Regulatory Agency, 2012; US Food and Drug Administration, 2015).

The uncertainties surrounding NSAIDs pharmacology can hinder decision making concerning NSAIDs safety for both humans and wildlife. From a clinical standpoint, NSAIDs administration has been associated with numerous adverse drug reactions (ADRs) involving the cardiovascular, renal, and gastrointestinal systems (Conaghan, 2012; Fanelli *et al*., 2017). Risk mitigation strategies are often focused on long-term use; however, it has been reported that even from “the first day of use, all NSAIDs increase the risk of gastrointestinal (GI) bleeding, myocardial infarction, and stroke” (Davis and Robson, 2016). Although these ADRs are relatively well established in the clinic, the current mechanistic uncertainty prevents a personalised prescription strategy aimed at minimising potential ADRs in patients based on their specific risk factors. The UK National Institute for Health and Care Excellence (NICE) provides several Clinical Guidelines (CG) that include recommendations on NSAIDs administration to general practitioners (GPs) including CG177 Osteoarthritis care and management in adults, CG79 Rheumatoid arthritis, and CG88 Low back pain. These guidelines provide a series of general recommendations largely based on empirical evidence. For example, the NICE CG177 guideline recommends that for the treatment of osteoarthritis in adults GPs should favour paracetamol and/or topical NSAIDs over oral NSAIDs, COX-2 inhibitors, and opioids. It also suggests using “oral NSAIDs or COX-2 inhibitors at the lowest

effective dose for the shortest possible duration" and to consider individual risk factors. The guidelines also recommend that oral NSAID/COX-2 inhibitors should be co-prescribed with a proton pump inhibitor (PPI) (Davis and Robson, 2016). It is clear to see from these examples that there is a lack of an explicit mechanistic rationale to support these recommendations, and that there is a complete reliance on GPs' clinical experience alone. In turn, this can result in a wide variety of GP-specific prescription strategies (McDonald *et al.*, 2017). Decision making becomes even more complex if we consider that more than 25 NSAIDs are currently on the market, and that each patient can display a long list of specific risk factors that can influence prescription decisions including age, co-morbidities, and polypharmacy. These considerations suggest that mode-of-action-driven approaches may be highly valuable to support healthcare professionals to interpret complex clinical scenarios, and to support personalised prescription strategies.

In addition to the clinical challenges, the presence of low concentrations of NSAIDs in the aquatic environment has raised the concern that chronic exposure to these drugs may also cause adverse effects in wild fish populations (aus der Beek *et al.*, 2016; Lonappan *et al.*, 2016; Marmon, Owen, and Margiotta-Casaluci, 2021). In 2015, the NSAID diclofenac was added to the European Union Watch list of emerging pollutants (European Commission, 2015), and subsequently removed in 2018 (European Commission, 2018). However, the regulatory debate on the environmental safety of NSAIDs continues to date, with some stakeholders recommending tighter regulation of over-the-counter NSAIDs and others proposing the substitution of diclofenac with compounds that display lower environmental risk (OECD, 2019; Marmon, Owen, and Margiotta-Casaluci, 2021). Independent from the exact details of this issue (discussed in Chapter 3), a common feature of the regulatory discussions concerning environmental safety is their reliance on a set of regulatory-relevant apical adverse phenotypes (reproductive effects, organ damage, and mortality), and the complete lack of mode-of-action considerations. Similar to the clinical challenges, the lack of mechanistic understanding represents a striking limitation that hinders the implementation of modern predictive toxicology approaches to support the environmental safety assessment of NSAIDs. Improving our mechanistic understanding of NSAIDs toxicology could provide valuable support for the regulatory decision making process.

The high volume of research published on NSAIDs poses a significant challenge in terms of knowledge usability, as only a very small proportion of such knowledge is able to reach and influence the relevant stakeholders. In recent years, the scientific community has started to address this challenge in a more systematic manner using various strategies. For example, many important international initiatives and partnerships have been established to support evidence synthesis activities at the global level. These include 1) Cochrane Collaboration –

aimed at supporting health care interventions; 2) Collaboration for Environmental Evidence – aimed at supporting environmental management, sustainability, and conservation of biodiversity; 3) Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) – aimed at supporting decision making in translational medicine; and 4) CAMARADES/ NC3Rs Systematic Review Facility – aimed at supporting scientists to perform systematic review and meta-analysis of animal studies. A full perspective on this topic has been recently published by the partnership 'Evidence Synthesis International' (Gough *et al.*, 2020). In the last decade, a unique type of evidence synthesis initiative has gained significant traction in the toxicology field, supported by the Organisation for Economic Co-operation and Development (OECD), European Commission, and US Environmental Protection Agency (US EPA). This initiative is aimed at mapping the causally related multi-scale events that link specific molecular initiating events (chemical-target interactions) to the manifestation of adverse health effects. A conceptual multi-scale model, called the Adverse Outcome Pathway (AOP), has been proposed as the pragmatic tool that would enable this large-scale mapping exercise (Ankley *et al.*, 2011; Ankley and Edwards, 2018). The vision underlying the AOP development programme is that weight-of-evidence-based mechanistic knowledge, centralised using a Wiki format (https://aopwiki.org/), may improve the prediction of chemically-induced adverse effects, and facilitate the implementation of the 3R's (Replacement, Reduction, and Refinement) vision in current and future safety testing strategies. A third type of evidence synthesis strategy is represented by the development of computational databases. From these databases it is possible to extract experimental mechanistic data from heterogenous sources (scientific publications, data repositories), harmonise the ontological features, and explore these data via user-friendly interfaces. Some examples that are of particular relevance to the present work are the Comparative Toxicogenomic Database (CTD), ChEMBL, and the CompTox Chemicals Dashboard (ToxCast). However, it is important to highlight that the number of biological databases is expanding rapidly. For example, Rigden and Fernandez (2021) identified 189 biological databases in 2021, though this number is likely to be higher as none of the databases mentioned above are present in the list generated by this study. The CTD is a database supported by the US National Institute of Environmental Health Sciences that provides manually curated information about chemical–gene/protein interactions, chemical–disease, and gene–disease relationships. The ChEMBL database is maintained by the European Bioinformatics Institute (EBI) and contains >15 million bioactivity measurements for 1.8 million distinct chemicals, extracted from >67,000 papers and patents (Mendez *et al.*, 2019). Finally, the CompTox Chemicals Dashboard (ToxCast) integrates physicochemical, environmental fate and transport, exposure, usage, *in vivo* toxicity, and *in vitro* bioassay data for 883,000 chemicals (Williams *et al.*, 2017).

Generating accurate *in silico* models to predict potential toxicity from mechanistic data is becoming increasingly sought after. This PhD project is founded on the scientific vision that the mechanistic understanding of drug-mediated effects is essential to support modern regulatory and/or clinical decision making concerning drug safety assessment. Improving our mechanistic understanding will help drive the future implementation of predictive pharmacology and toxicology approaches into the regulatory arena. Moreover, there is an ever growing demand for reducing the volume of *in vivo* testing currently required to assess safety and risk, which is associated with high financial and ethical costs (Hartung, 2017; Burden, Sewell, and Chapman, 2015). All research groups currently working with animals in the UK must adhere to the principles of the 3R's at all stages of research, which coincide with the regulations and licensing laws set out by the Home Office (under the Animals (Scientific Procedures) Act 1986). In line with the 3R's vision, the present Chapter describes the use and maximisation of existing mechanistic knowledge to generate a multi-dimensional mode-of-action profile of NSAIDs. Specifically, we extracted and integrated data at three different levels – gene expression (molecular level), functional *in vitro* profiling (drug-target interaction), and predicted immunomodulation (cellular level).This valuable information was used to guide the hypothesis generation and experimental design of all the subsequent animal experiments conducted throughout this project.

2.3 Methods

2.3.1 Compound identification

To identify all available NSAIDs, we screened the DrugBank database (www.drugbank.ca; Wishart *et al.*, 2018) and selected all pharmaceuticals labelled as "COX-inhibitor" or "NSAID".

2.3.2 Extraction of NSAIDs-mediated gene expression data (CTD)

Drug-gene interaction, drug-pathway enrichment, and drug-disease association data for 24 different NSAIDs was downloaded from the Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/; Davis *et al.*, 2021). Due to the vast amount of data available, a list of only the top 20 interacting genes, pathways, and diseases associated with each NSAID was compiled into a single Excel data file which was uploaded onto the Brunel Data Repository System (FigShare), and can be retrieved from the following address: https://figshare.com/s/e442f3c3e47b80c97cd7. To enhance the interpretation of the biological significance of the drug-gene interaction data, the CTD provides a list of GO terms that are statistically enriched by the genes that interact with the chemical of interest. According to the

CTD, “the significance of the enrichment is calculated by the hypergeometric distribution and adjusted for multiple testing using the Bonferroni method. The hypergeometric distribution is used to calculate the probability that the fraction of interacting genes annotated to the GO term, or its descendants, is significantly higher than the fraction of all human genes annotated to that GO term, or its descendants in the genome”. To enhance the translational interpretation of drug-gene interaction data, the CTD also provides curated and inferred chemical–disease associations. According to the CTD, “curated associations are extracted from the published literature, whereas inferred associations are established via curated chemical–gene interactions (e.g., chemical A is associated with disease B because chemical A has a curated interaction with gene C, and gene C has a curated association with disease B)”. Each chemical-disease association is coupled with an ‘inference score’ that according to the CTD “reflects the degree of similarity between CTD chemical-gene-disease networks and a similar scale-free random network. The higher the score, the more likely the inference network has atypical connectivity. The inference score is calculated as the log-transformed product of two common-neighbour statistics used to assess the functional relationships between proteins in a protein-protein interaction network. The first statistic takes into account the connectivity of the chemical and disease along with the number of genes used to make the inference. The second statistic takes into the account the connectivity of each of the genes used to make the inference.”

2.3.3 Extraction of *in vitro* bioactivity data (ToxCast/ChEMBL)

In vitro bioactivity profiling data for 25 different NSAIDs was extracted, via download of csv. files, from two distinct sources: 1) the ‘US Environmental Protection Agency (US EPA) Toxicity Forecaster (ToxCast) database (U.S. EPA. 2015. ToxCast and Tox21 Summary Files from invitrodb_v3.2. Retrieved from https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data between May 2019 and October 2019. Data released May 2018) (Williams *et al.*, 2017), and 2) the European Bioinformatics institute (EBI) ChEMBL database (http://www.ebi.ac.uk/chembl; Gaulton *et al.*, 2017). The data downloaded from ToxCast included drug target identifier and drug concentration at 50% maximum activity (AC50). Data extraction was limited to the interactions labelled as ‘active’, hence, those labelled as ‘inactive’ were excluded from the analysis, via removal from the dataset. The data downloaded from ChEMBL included drug target identifier and half-maximum inhibitory concentrations (IC50). Similarly, in this case, data extraction was limited to the interactions labelled as ‘active’, whereas those labelled as ‘not active’ or ‘not determined’ were excluded from the analysis, via removal from the downloaded dataset. When data from multiple species were available, human data were used as the first choice, however if unavailable, rodent data were used

instead. This bioactivity data was uploaded onto the Brunel Data Repository System (FigShare), and can be retrieved from the following address: https://figshare.com/s/e8a7b2adeb285d6f1de8.

2.3.4 Harmonisation and analysis of ToxCast/ChEMBL data

Due to the differing nomenclature used by the two data sources, the combined data from ToxCast and ChEMBL was manually harmonised using the database GeneCards (GeneCards.org; Stelzer *et al.*, 2016) to provide consistent annotation of target identifiers (i.e., gene names). This manual process involved comparing the genes in these datasets with the GeneCards database, and aligning the nomenclature accordingly. Additionally, the AC50 values associated with each target were uniformly expressed as nanomolar (nM) concentrations to ensure inter-database comparability. Once the combined dataset was harmonised, if any duplicate targets were present, only the lowest AC50 values were retained.

2.3.5 Human therapeutic Cmax

A range of human therapeutic Cmax (peak plasma concentration) values for each NSAID were extracted from the scientific literature. Human Cmax values for 19 drugs were extracted from Schulz *et al.* (2020) and recorded in our dataset. The Cmax values for the remaining NSAIDs (amfenac, carprofen, flufenamic acid, rofecoxib, tolfenamic acid, and valdecoxib) were extracted from six distinct sources (*ema.europa.eu/en/documents/scientific-discussion/nevanac-epar-scientific-discussion_en.pdf*; *zoetisus.com/products/pages/rimadyl-injectable/pdf/tb.pdf*; Lentjes and Ginneken, 1987; Prescilla *et al.*, 2004; Niopas *et al.*, 1995; *accessdata.fda.gov/drugsatfda_docs/label/2004/21341lbl.pdf*), respectively, and recorded in our dataset.

2.3.6 Extraction of immunomodulatory data from existing datasets

In order to assess the immunomodulatory potential of NSAIDs, we data-mined, extracted, and processed a large dataset published by Kidd *et al*. (2016), who generated a large-scale prediction of the interactions between 1,309 drugs and 250 immune cell states. The authors generated immunological predictions through the computational analysis and comparison of transcriptomic signatures collected by two distinct projects: 1) the Immunological Genome project (ImmGen) (https://www.immgen.org/) (Aguilar *et al.*, 2020); and 2) The Connectivity Map (CMap) (https://clue.io/cmap) managed by the Broad Institute (Boston, USA) (Lamb, 2007). The ImmGen database contains transcriptomic signatures for over 250 distinct

immunological cell states in mice, 14 categories of immune cell types, collected from 25 tissue locations. On the other hand, the CMap database contains over 1.5 million transcriptomic signatures for ~5,000 small-molecule compounds tested in multiple cell types. Kidd *et al.* (2016) integrated the two data sources creating a system-wide interaction map between drugs and immune cells by matching 1,309 drug perturbation profiles to 304 immune cell state changes. They computed the similarity between the two transcriptional expression patterns by comparing the top and bottom ranked genes from both profiles. For each immune cell state change (e.g., A→B), this analysis led to the calculation of an 'immunomod score' based on the overlap of the top- and bottom-ranked genes in each profile. A positive immunomod score indicates that the specific drug directs the immune cell towards state 'B', whereas a negative score indicates that the drug shifts the cell towards state 'A'. Here we data-mined this large dataset to extract immunomod scores for all available NSAIDs (n = 34). This involved downloading the relevant dataset from the supplemental information provided by Kidd *et al*., filtering the data for NSAIDs, and removing all of the remaining data concerning other classes of pharmaceuticals. Due to the complexity of the 'immunological cell states' nomenclature, each immune cell state transition was manually replaced with a simplified unique code, to facilitate data handling and analysis. For example, the transition "Mo.6C-II-.Bl --> Mo.6C-II-.BM" was replaced with the code "Mo 1". A human-readable version of IMMGEN annotation codes is available at https://gist.github.com/nachocab/3d9f374e0ade031c475a. The example mentioned here would equate to: "Non-classical Monocytes MHCII-negative (blood) --> Non-classical Monocytes (bone marrow)". The full dataset used for this analysis was uploaded onto the Brunel Data Repository System (FigShare), and can be retrieved from the following address: https://figshare.com/s/f5e910433971a132eac2.

2.3.7 Analysis of predicted immunomodulation data

To generate a comparative assessment of the immunomodulatory activity of the 34 NSAIDs, immunomodulation scores were analysed by hierarchical clustering using the web tool ClustVis (https://biit.cs.ut.ee/clustvis/). The aim of this analysis was to identify clusters of NSAIDs displaying similar immunomodulatory activity. Hierarchical clustering facilitates the understanding of large multi-dimensional datasets, like the one considered here, by visualizing the data as a collection of progressively smaller clusters with different degree of granularity. The clustering starts with calculating all pairwise distances among the different elements in the dataset (i.e. immunomod scores). Elements with the smallest distance are merged in each step into clusters. On the other hand, defined clustering methods allow the calculation of the distance between two clusters. In the present analysis, Pearson correlation coefficients were used to determine the clustering distances, and the Ward linkage method (sum of squared

distances from points to centroids as the distance) to determine the inter-cluster linkages (Metsalu and Vilo, 2015). The results of the analysis were visualized using a heatmap plot integrated with two dendrograms which represented the clustering of 34 NSAIDs and 184 immune cell states, respectively.

2.4 Results

2.4.1 Selection of NSAIDs

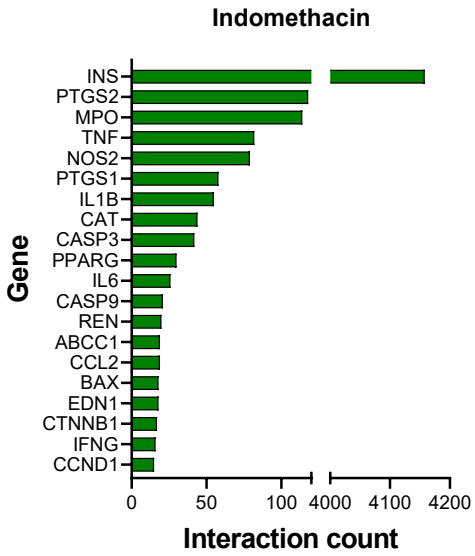
Searching the DrugBank database led to the identification of 25 NSAIDs: amfenac, aspirin, carprofen, celecoxib, diclofenac, etodolac, etoricoxib, flufenamic acid, flurbiprofen, ibuprofen, indomethacin, ketoprofen, ketorolac, mefenamic acid, meloxicam, naproxen, niflumic acid, nimesulide, oxaprozin, piroxicam, rofecoxib, sulindac, tenoxicam, tolfenamic acid, and valdecoxib. Four compounds were classified as COX-2 selective inhibitors (celecoxib, etoricoxib, rofecoxib, and valdecoxib), whereas 21 compounds were classified as non-selective COX inhibitors.

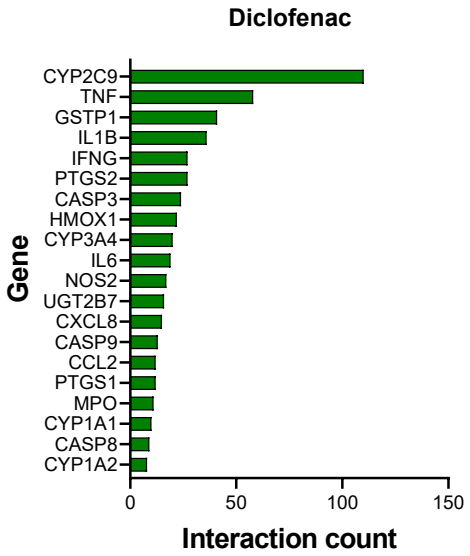
2.4.2 CTD: NSAID-gene, gene-pathway, and pathway-disease associations

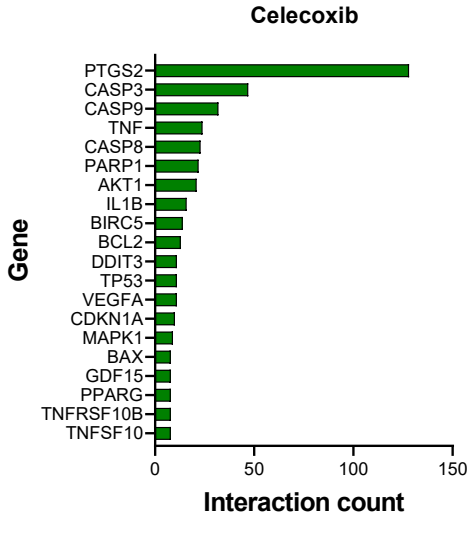
The CTD database contained information for all drugs, except amfenac. In addition, it did not contain pathways enrichment predictions for carprofen and tenoxicam. It is important to note that these analyses are dependent upon the amount of data recorded in the CTD for each NSAID (i.e., the number of interacting genes). Therefore, an NSAID with a higher number of recorded gene interactions may not necessarily indicate a higher level of biological activity compared to another NSAID which fewer interacting genes. The top 20 drug-gene interactions were plotted for each NSAID in the CTD (Figure 2.1). However, 20 drug-gene interactions were not available for four of the NSAIDs identified (carprofen, etoricoxib, oxaprozin, and tenoxicam). The interaction count (x-axis), which describes the number of times each drug-gene association has been detected in the literature, was plotted against the gene identifier (y-axis). These drug-gene interaction plots indicate the molecular perturbations that are likely to stem from exposure to each of the selected drugs. The interaction count for each gene can be interpreted as an indirect marker of reproducibility, as each count is linked to an independent study present in the scientific literature. The total number of observed drug-gene interactions ranged between one (carprofen and tenoxicam) and 4,722 (indomethacin). The three NSAIDs with the highest number of gene interactions were indomethacin, nimesulide, and diclofenac. The three NSAIDs with the lowest number of interactions were etoricoxib, carprofen, and tenoxicam.

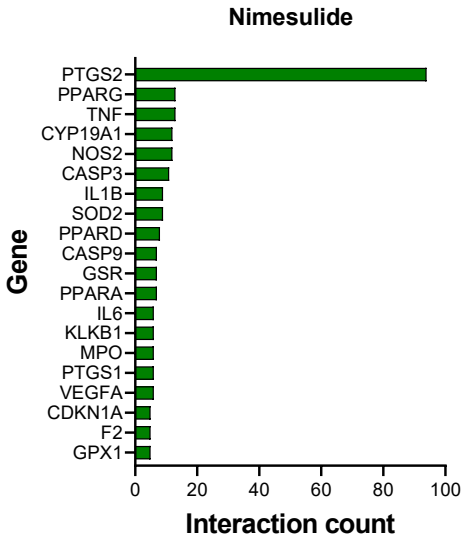
To support the interpretation of the biological significance of the drug-gene interaction data, the top 20 biological pathways significantly enriched by the genes associated with each NSAID are displayed in Figure 2.2. No pathway enrichment analysis is displayed for carprofen and tenoxicam due to the limited number of drug-gene interactions in the database. The number of statistically significant enriched pathways per NSAID ranged between 18 and 877. The three NSAIDs with the highest number of enriched pathways were indomethacin, diclofenac, and sulindac. Whereas the three drugs with lowest number of enriched pathways were mefenamic acid, oxaprozin, and etoricoxib. Out of all of the 'top 20 pathways' enriched by the 22 NSAIDs in the CTD around 30% (~131/438 pathways) are related to the immune system, highlighting the immunomodulatory potential of this class of drugs. It is important to note that in some instances 'Salmonella', 'Malaria', 'Allograft rejection', and 'Herpes simplex infection' appear amongst the drug-pathway interactions listed in Figure 2.2. Due to the nature of the CTD analysis, the reason these types of association may appear is the result of a number of the genes involved in these processes' being significantly enriched. The CTD software is unable to interpret the biological implausibility of these associations, hence why they appear in the analysis. For transparency, they remain part of the analysis and are listed in Figure 2.2.

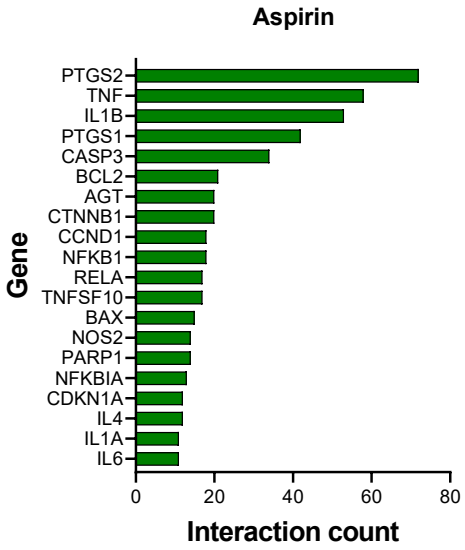
To support the interpretation of the adverse phenotypes that may be associated with each NSAID-gene interaction set, Figure 2.3 displays the top 20 diseases associated with each NSAID. These associations are either curated from the literature or inferred via the drug-gene interactions. Some of the curated associations include direct evidence from the literature, and so no inference score is recorded. Inferred associations are based upon the enrichment of interacting genes with several disease annotation sources, including the Gene Ontology (GO) database. The diseases associated with each NSAID seem to correlate relatively well with the types of NSAIDs-induced adverse effects reported in the clinic including renal, hepatic, cardiac, and gastrointestinal pathologies. Interestingly, 22 NSAIDs are associated with immune-related disease phenotypes, with the exception of flufenamic acid and tolfenamic acid.

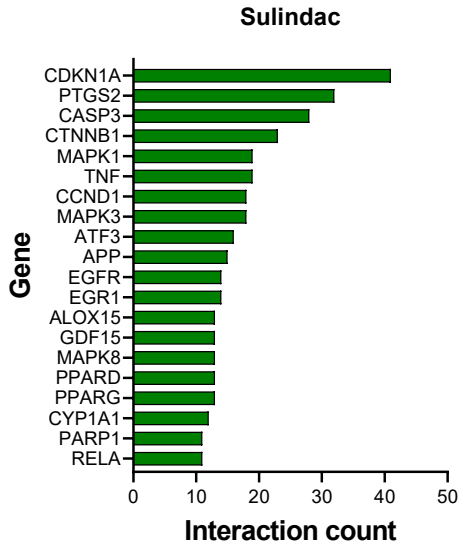












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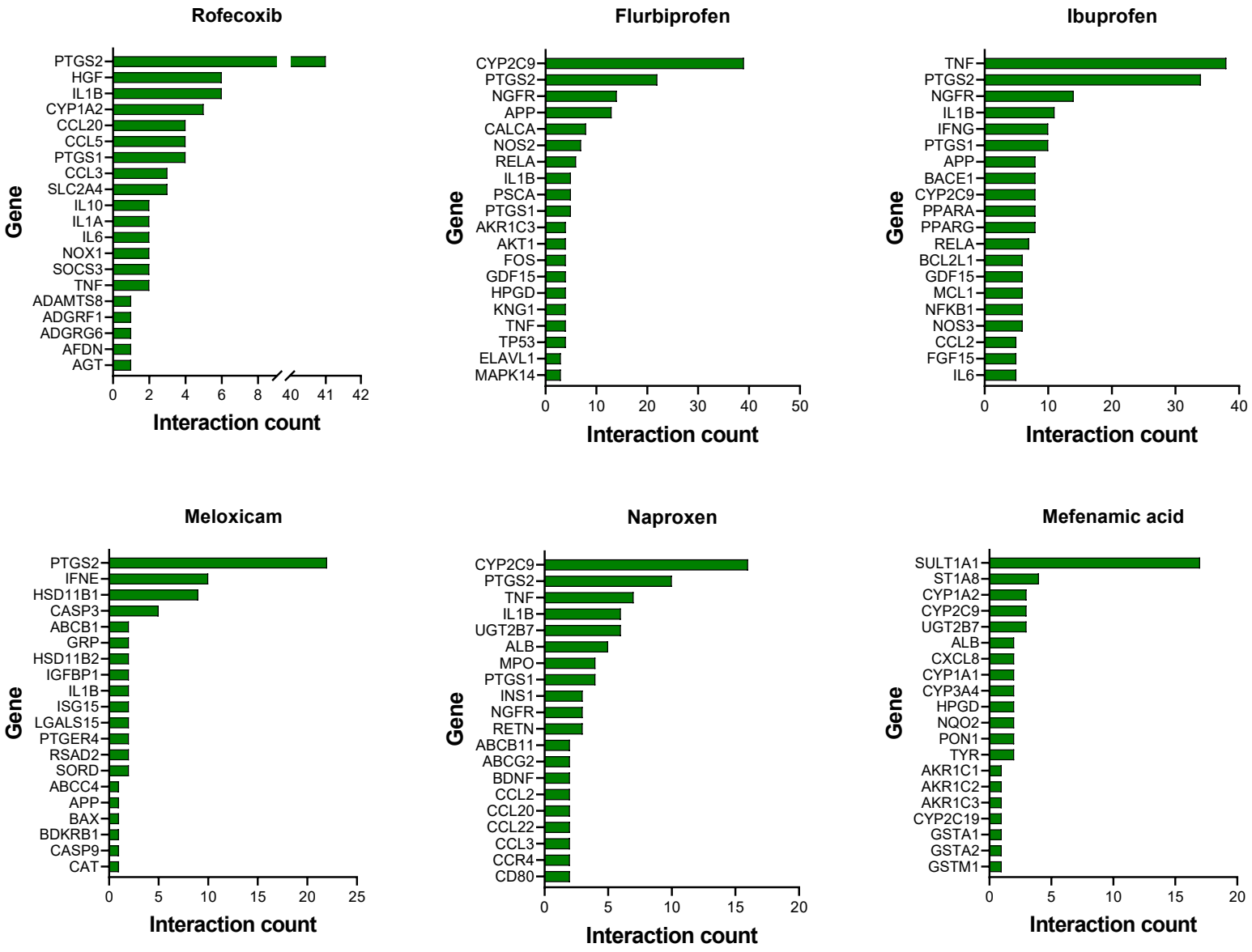

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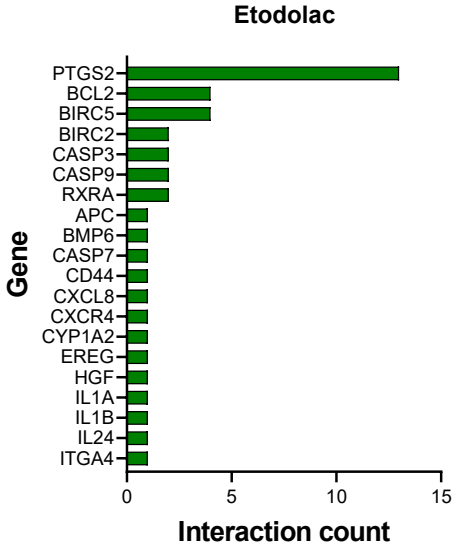

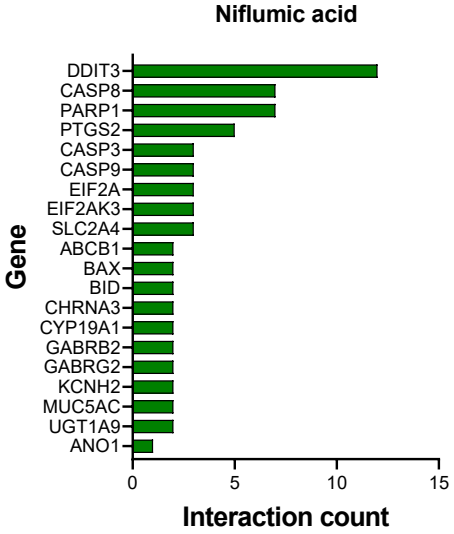

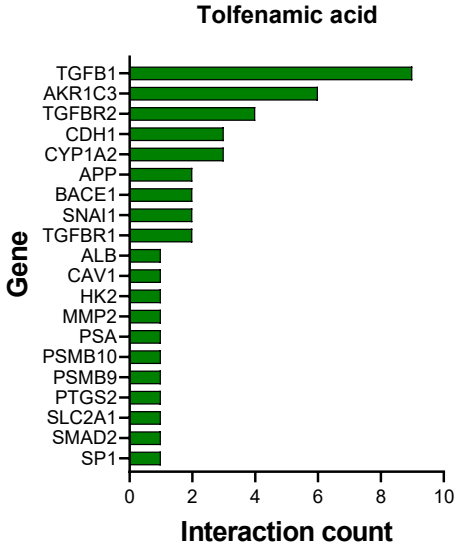

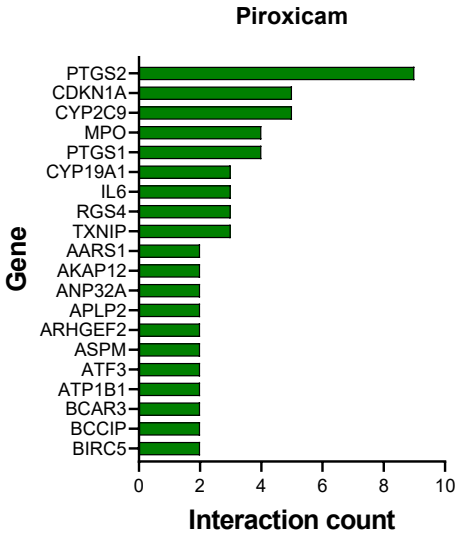

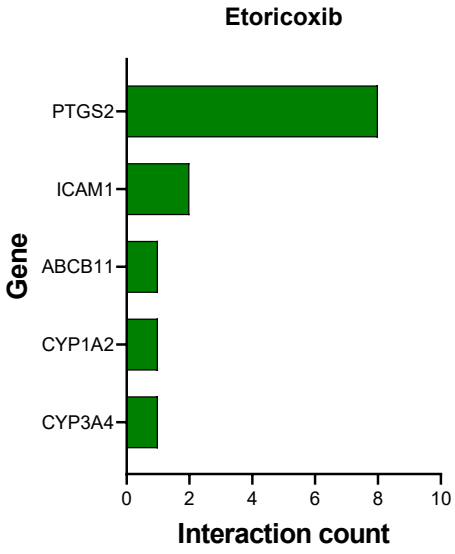

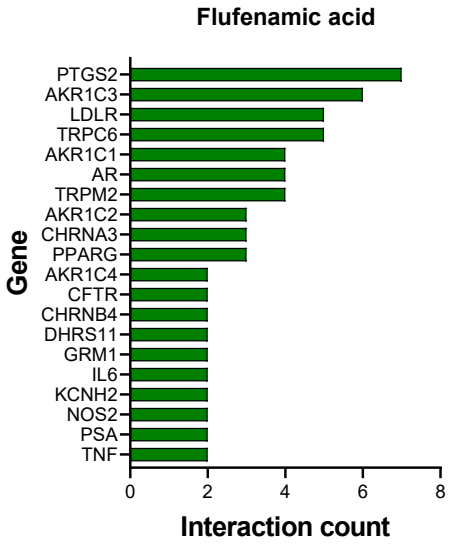

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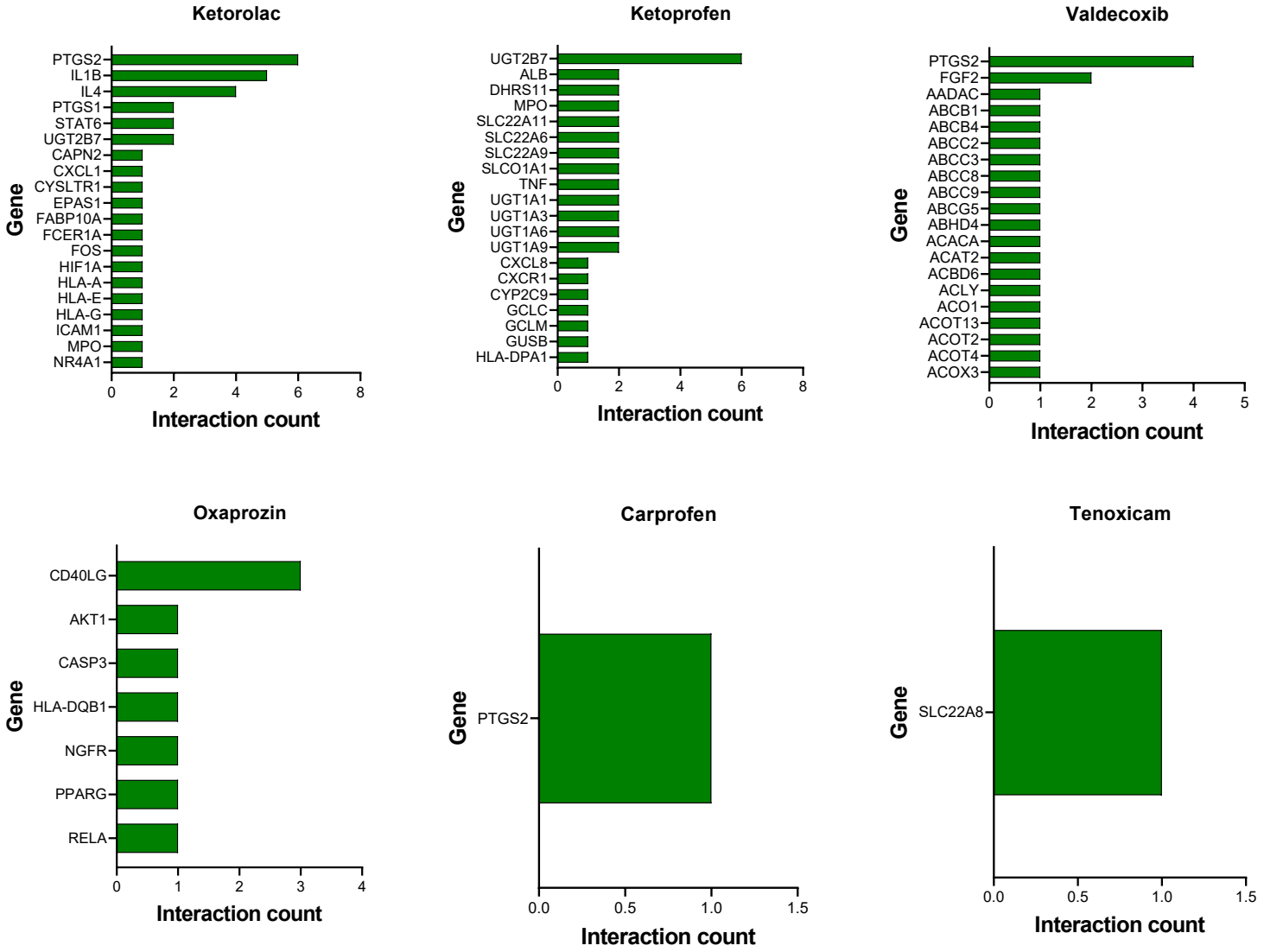
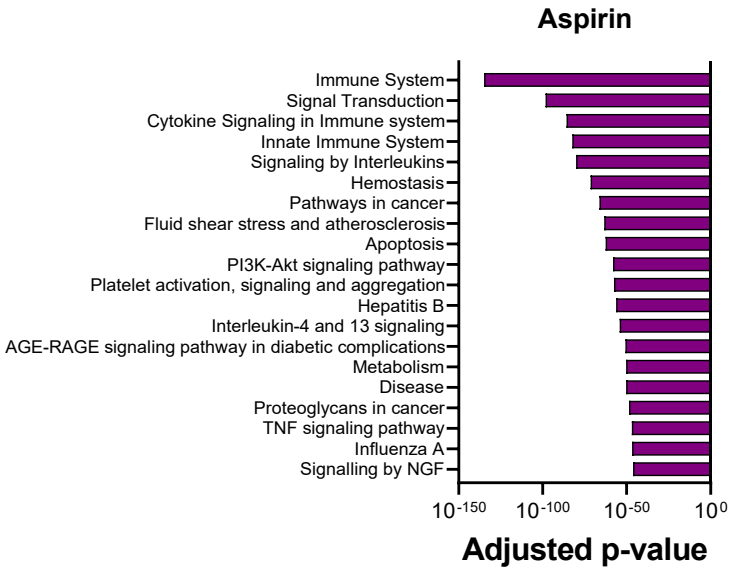
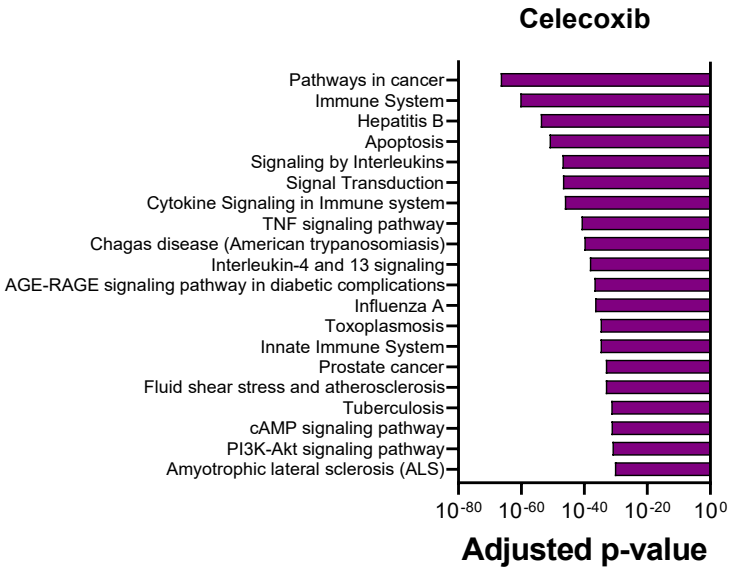
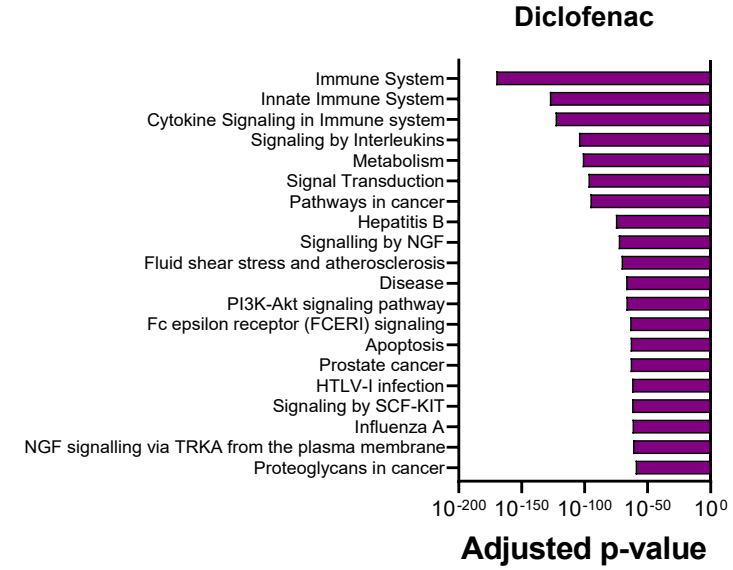
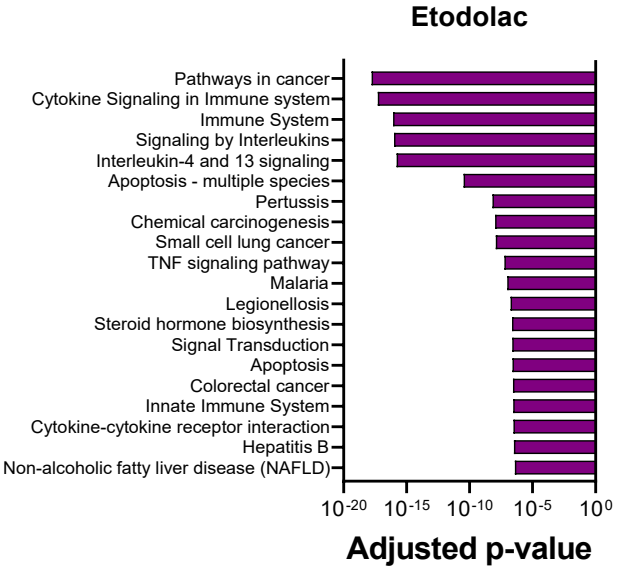


Figure 2.1. Top 20 CTD drug-gene interactions for each NSAID. *Drugs are ranked by interaction count from largest to smallest. The number of interactions does not necessarily indicate each drugs' potential for biological activity, as the number of interactions is dependent upon the curation process, and the amount of available data in the literature; 24 figures total.*









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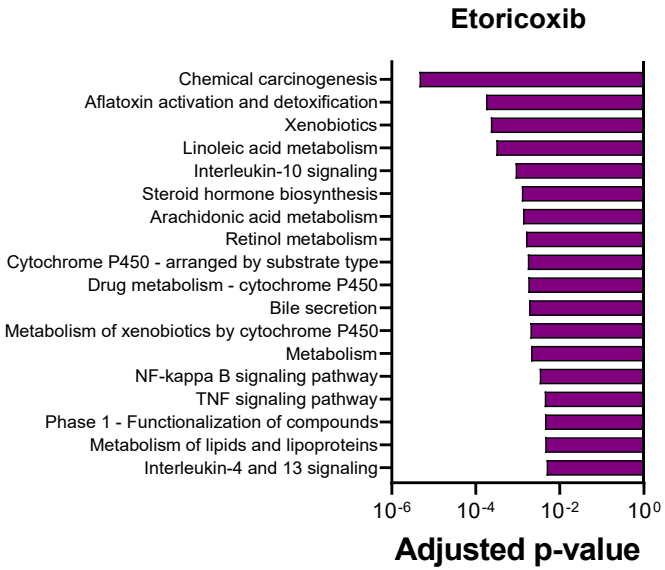

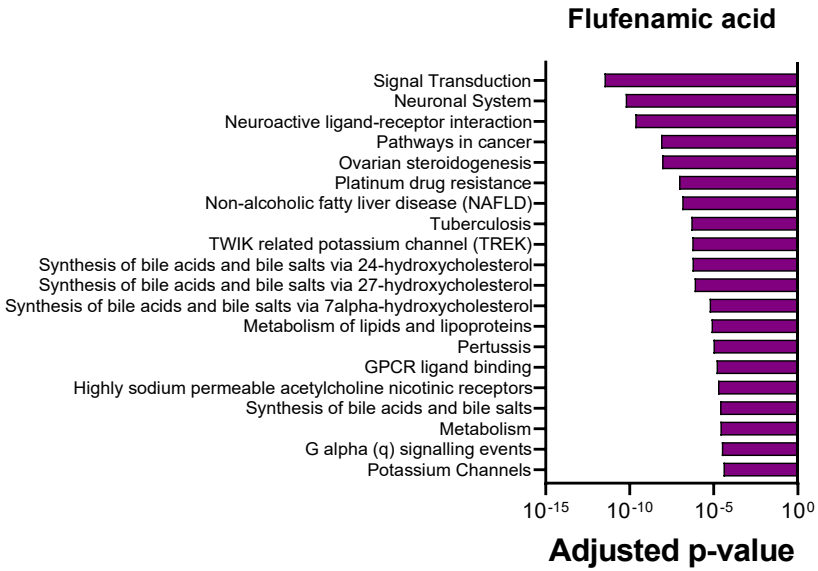

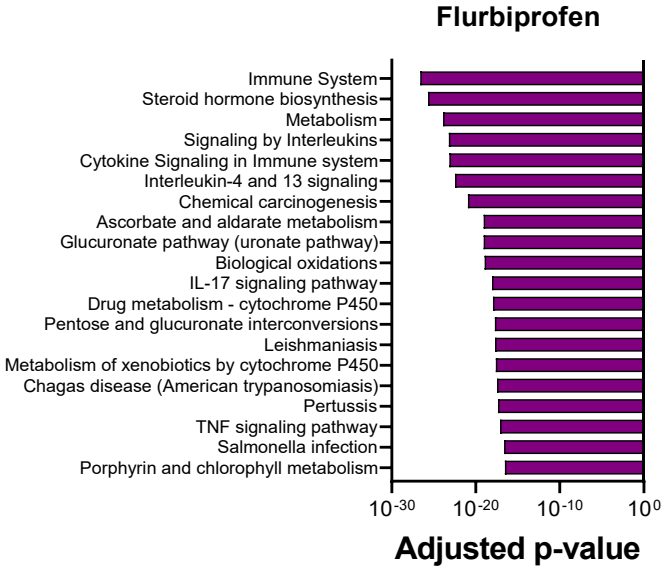

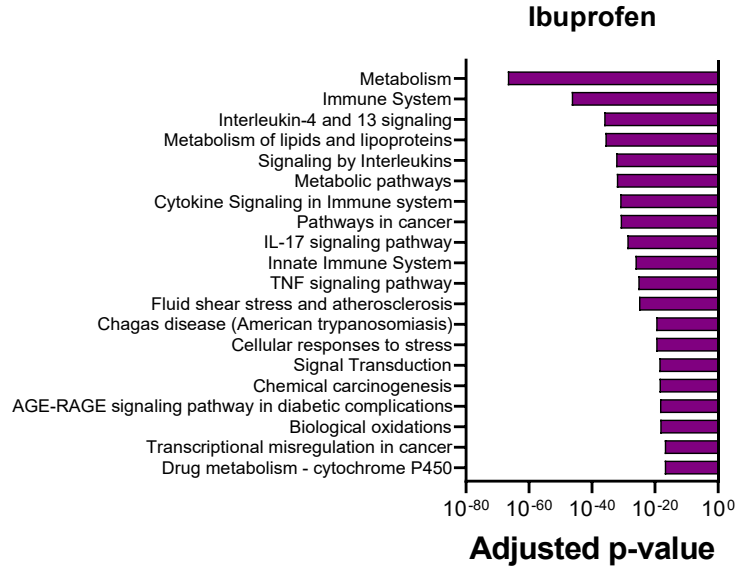

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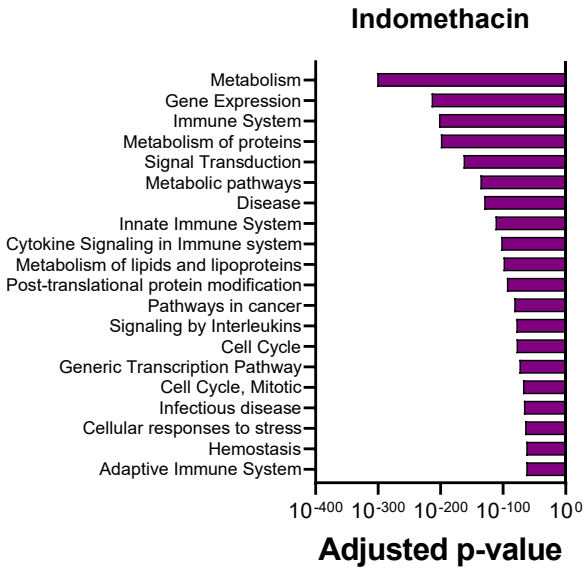

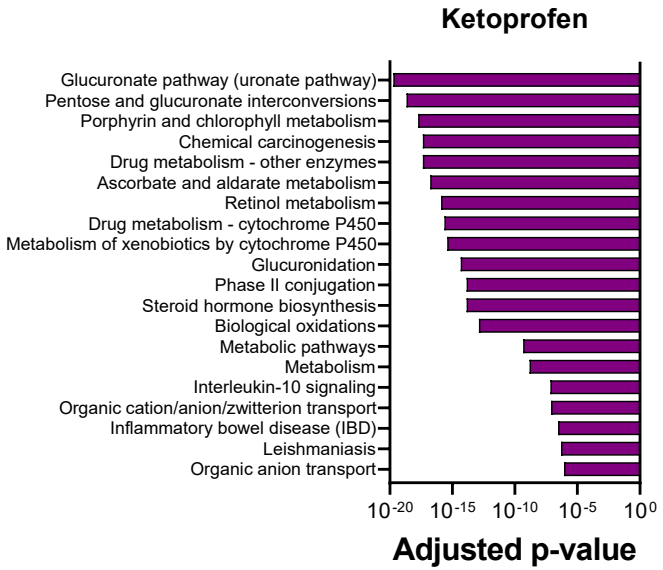

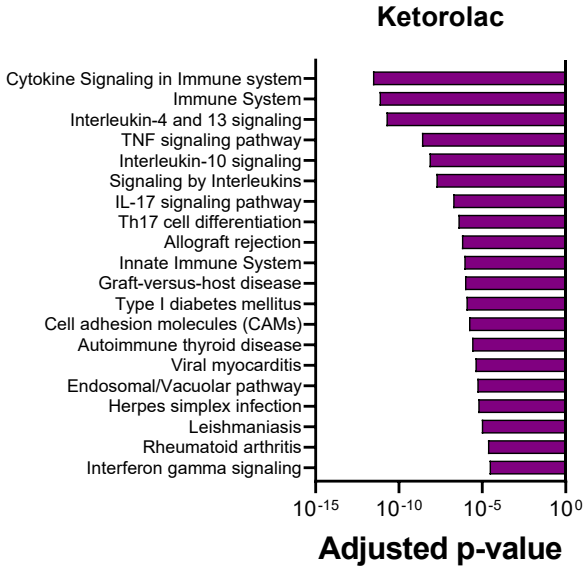

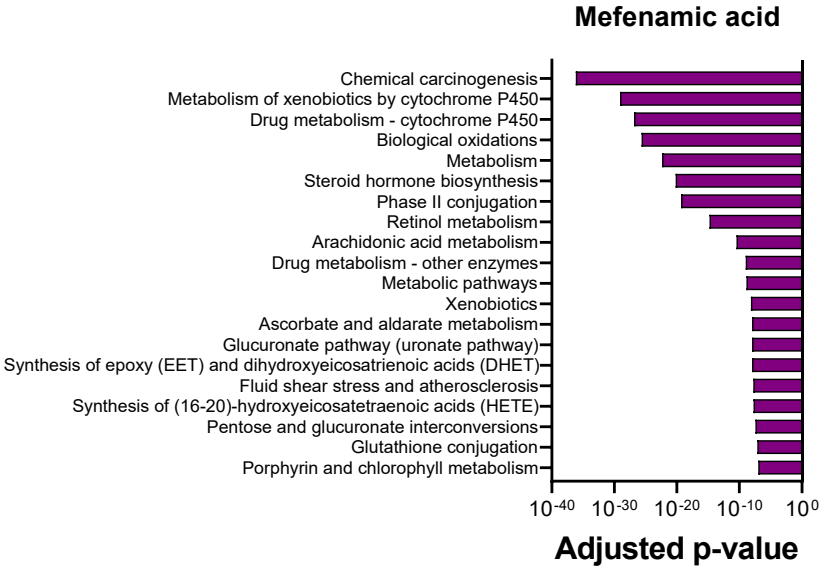

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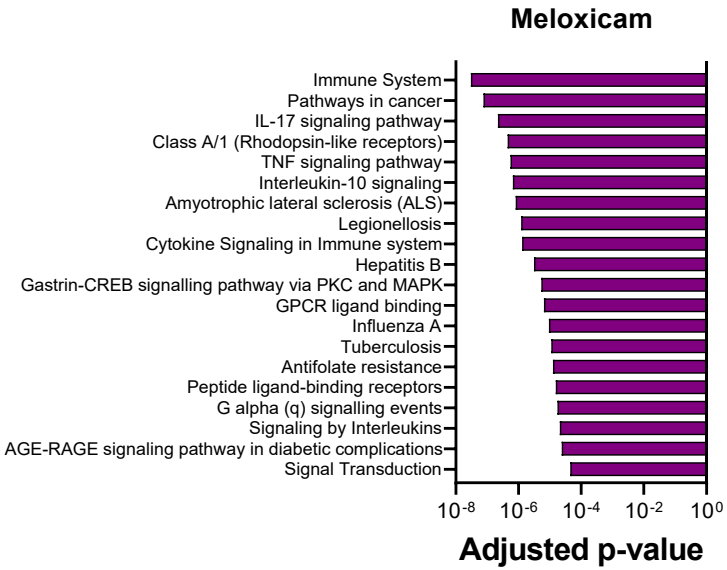

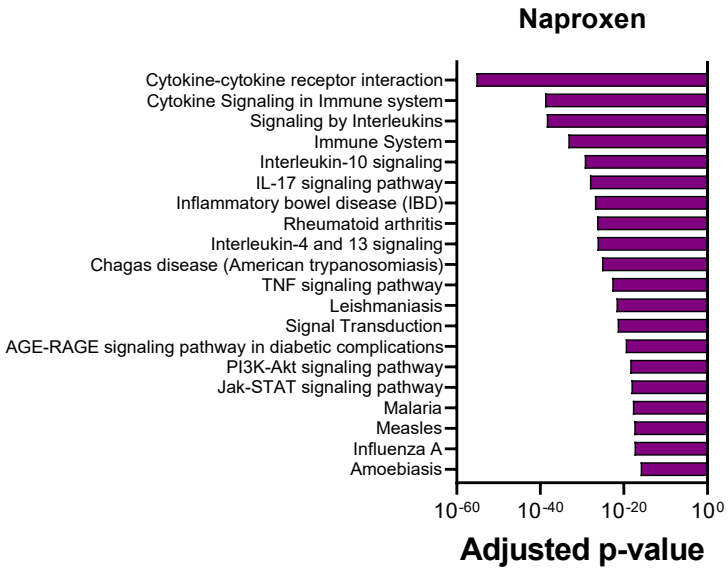

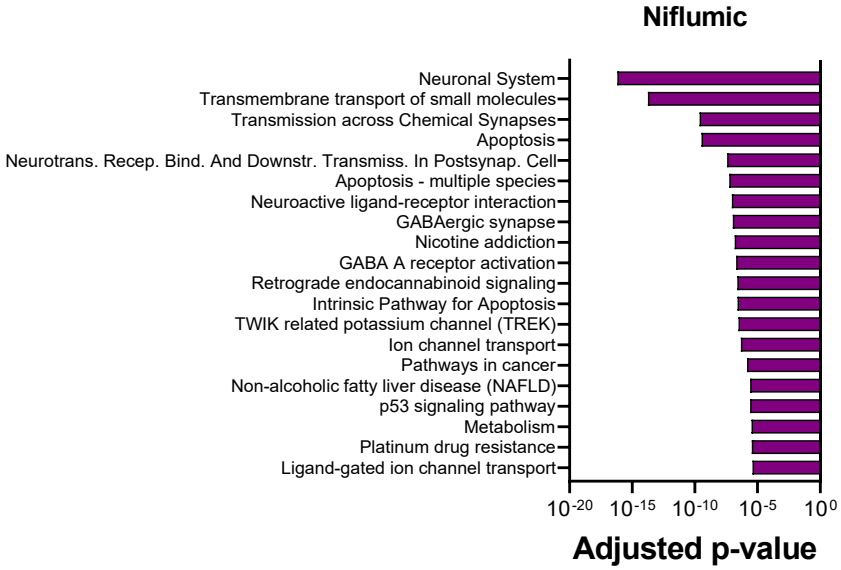

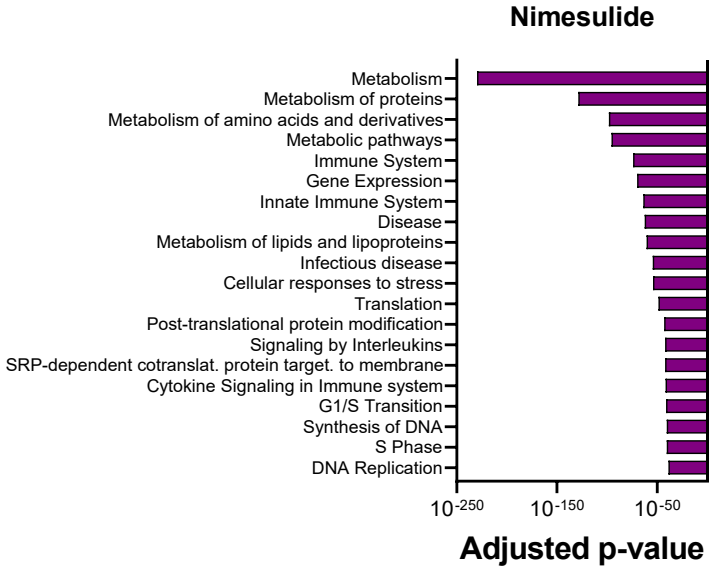

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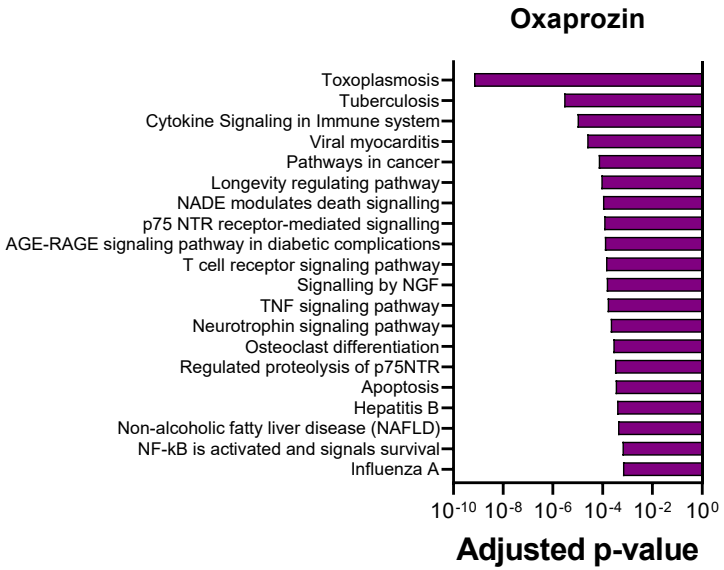

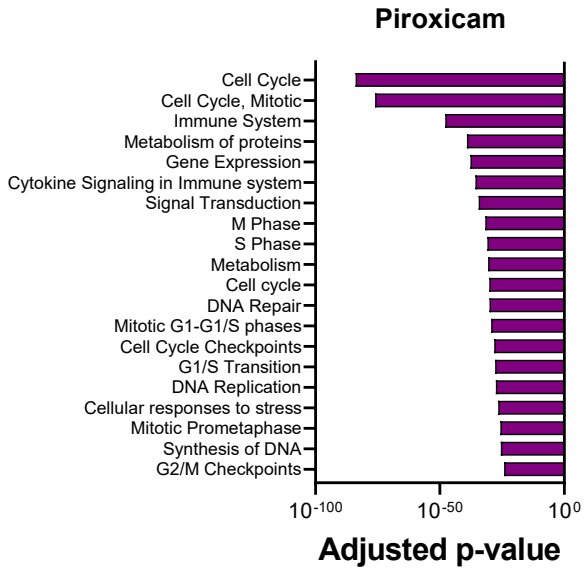

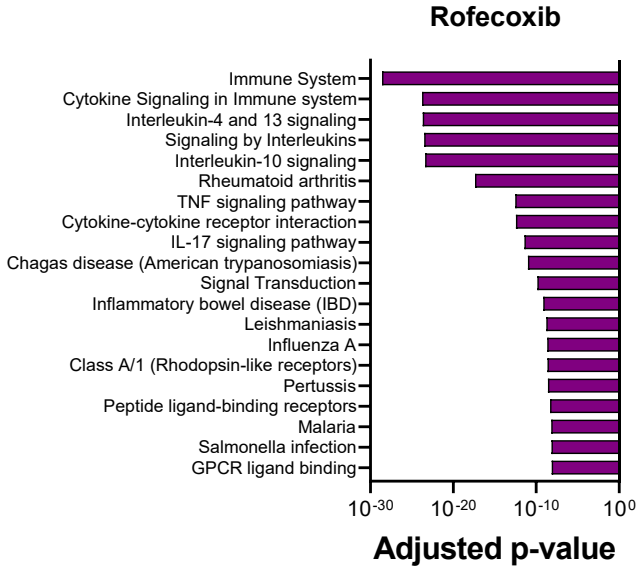

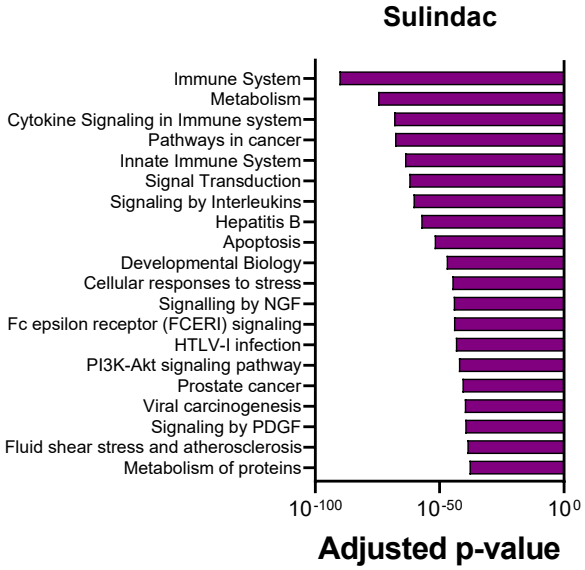

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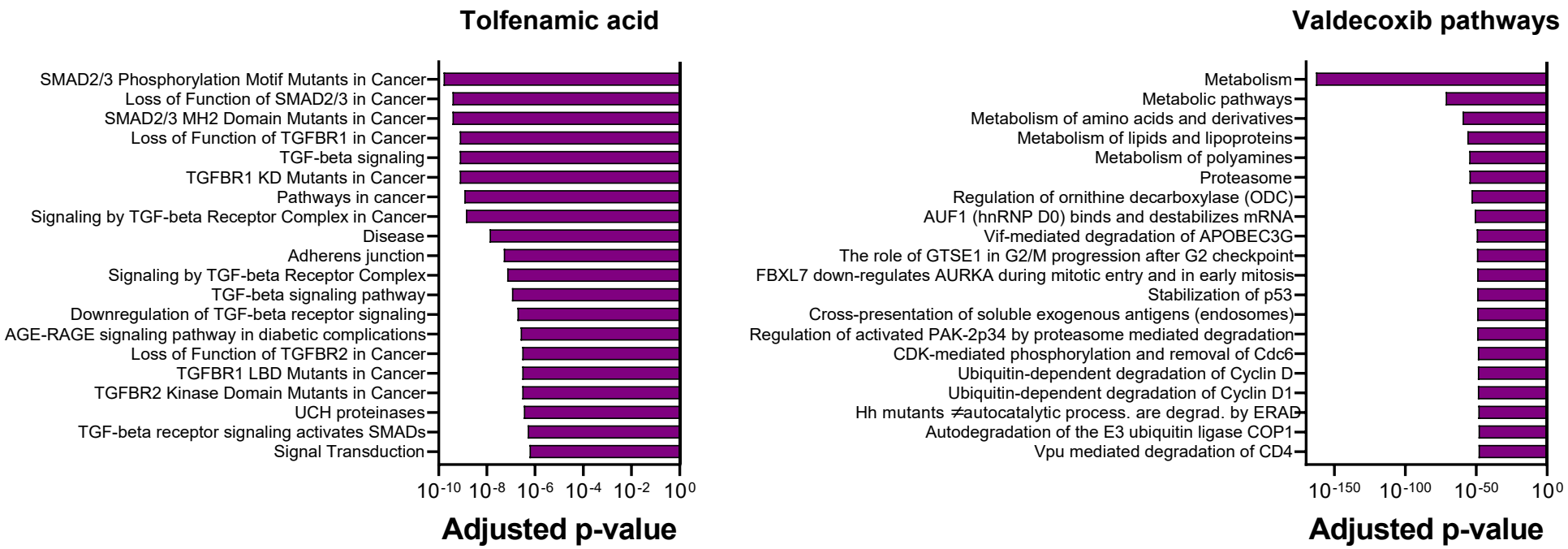
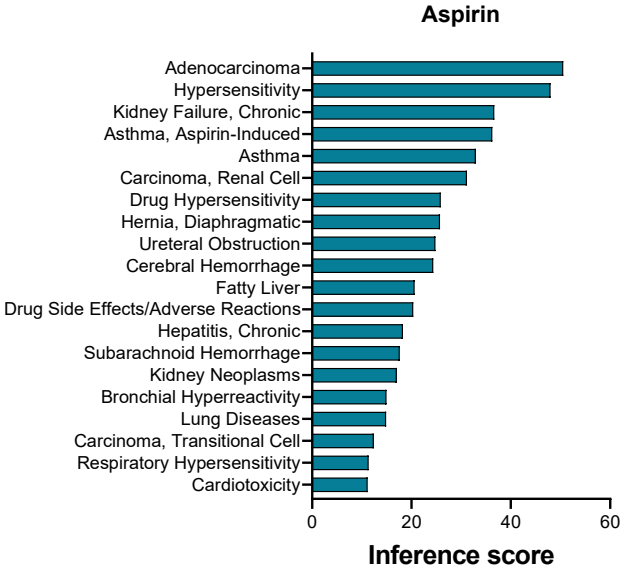
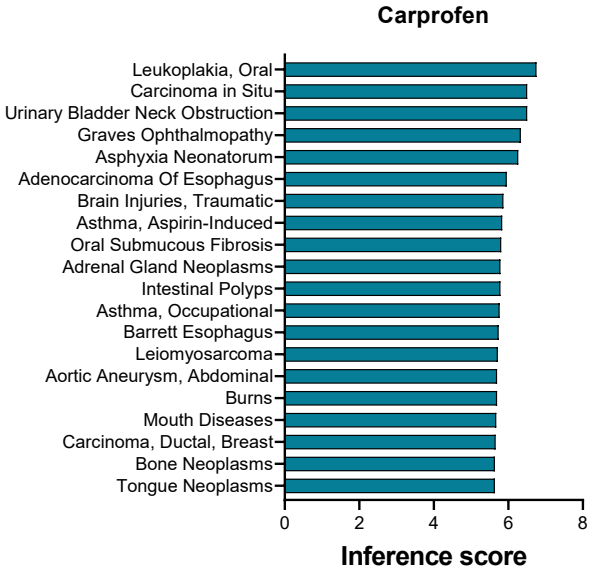
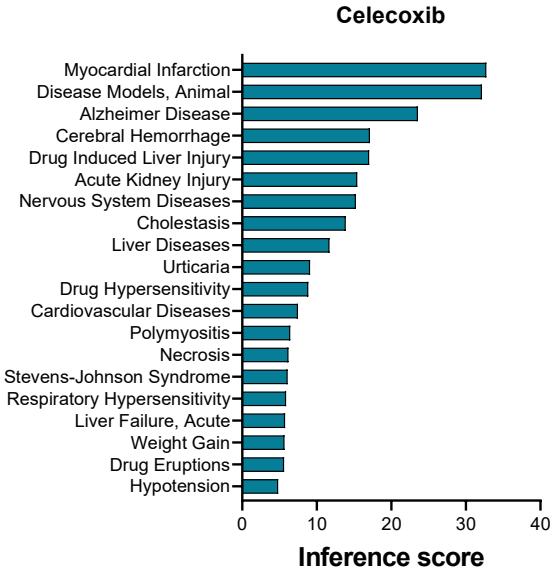
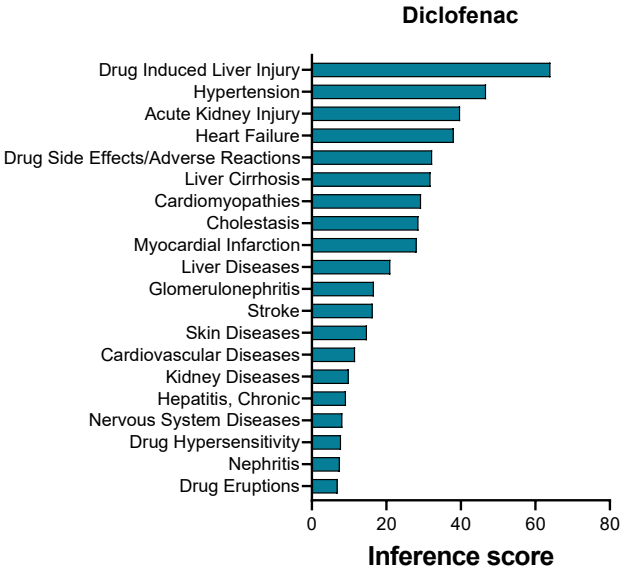


Figure 2.2. Top 20 CTD pathways enrichment for each NSAID. *These analysis are dependent upon the number of drug-gene interactions, which does not necessarily indicate each drugs' potential for biological activity, as the number of interactions is dependent upon the curation process, and the amount of available data in the literature; 22 figures in total.*









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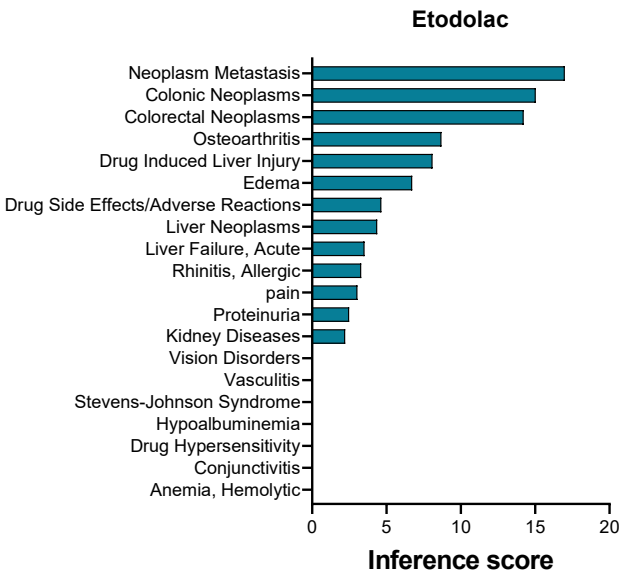

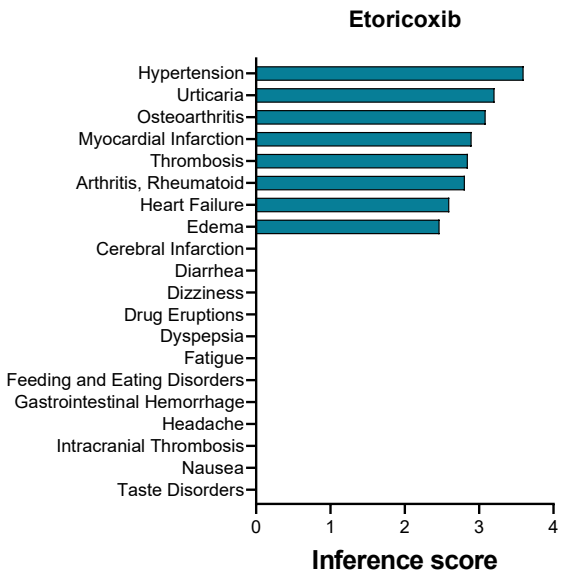

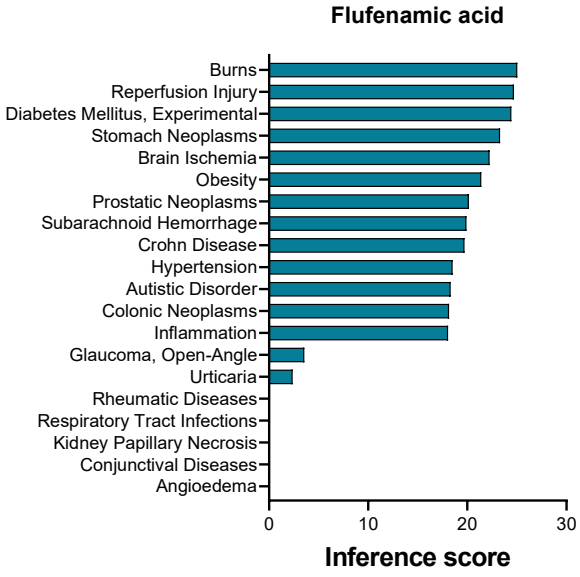

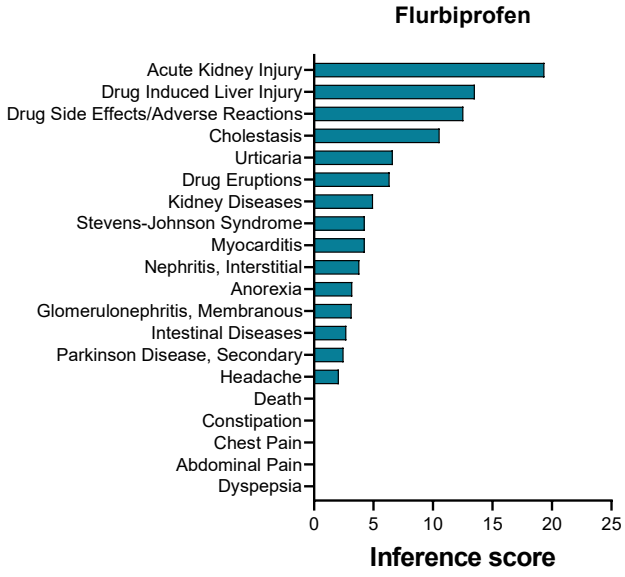

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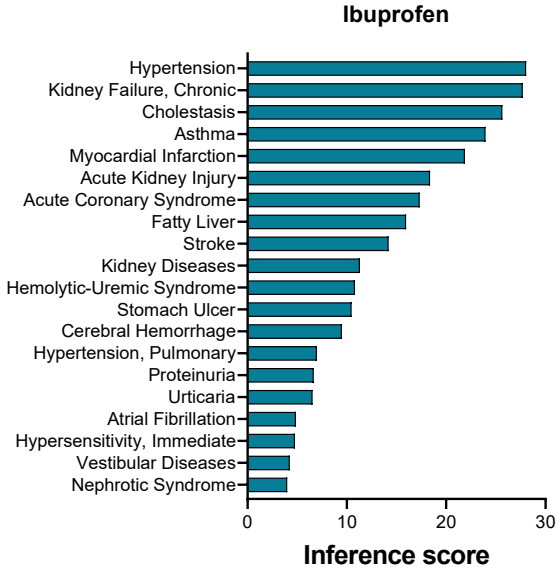

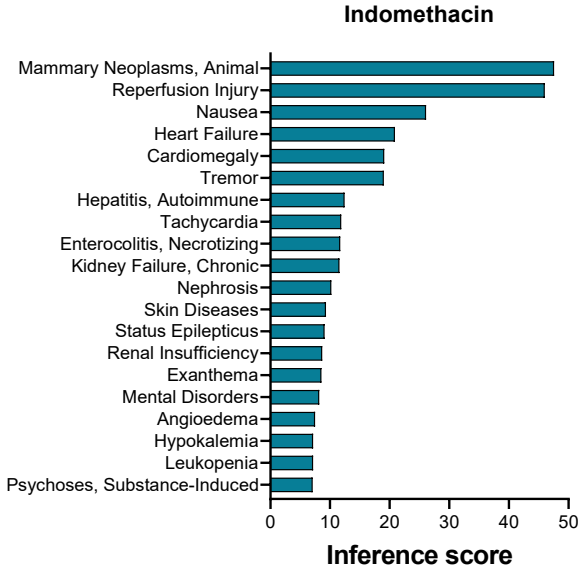

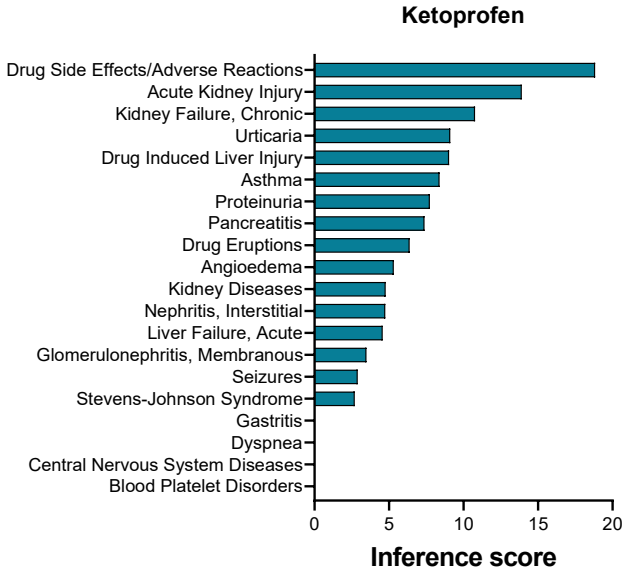

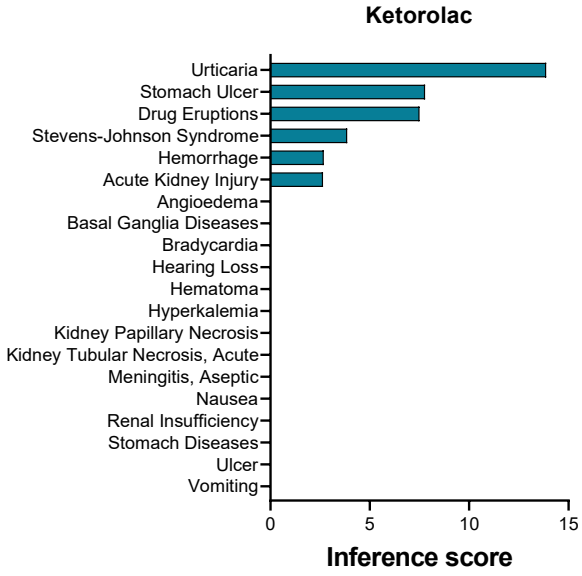

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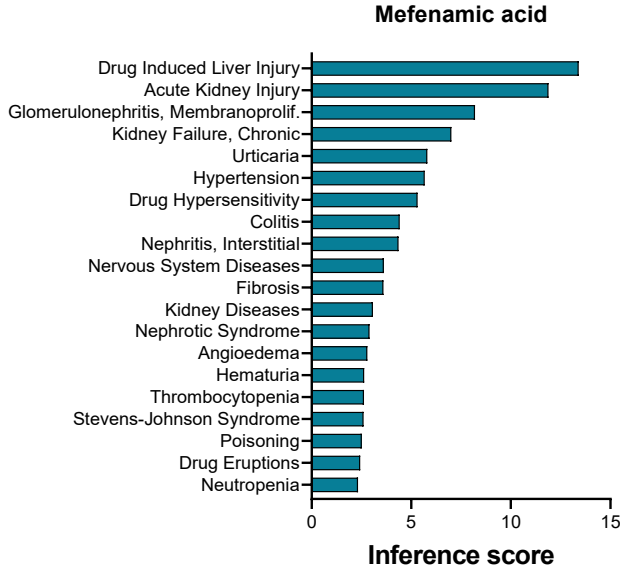

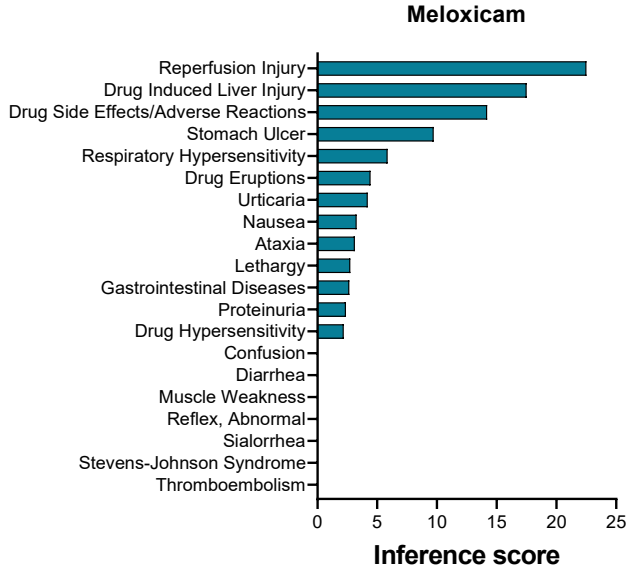

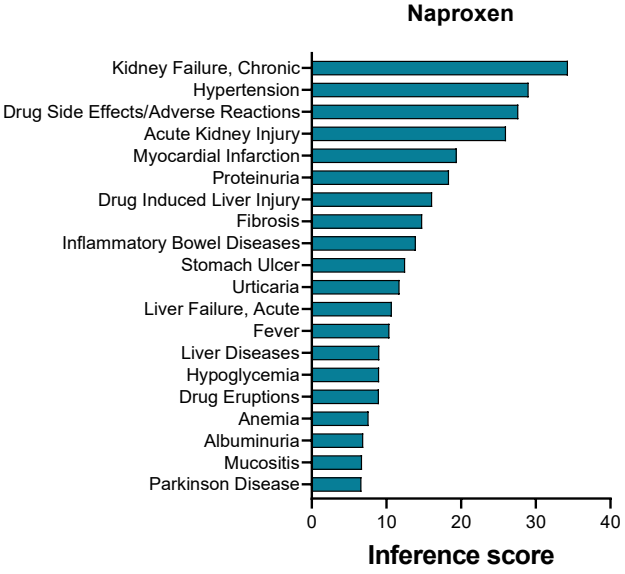

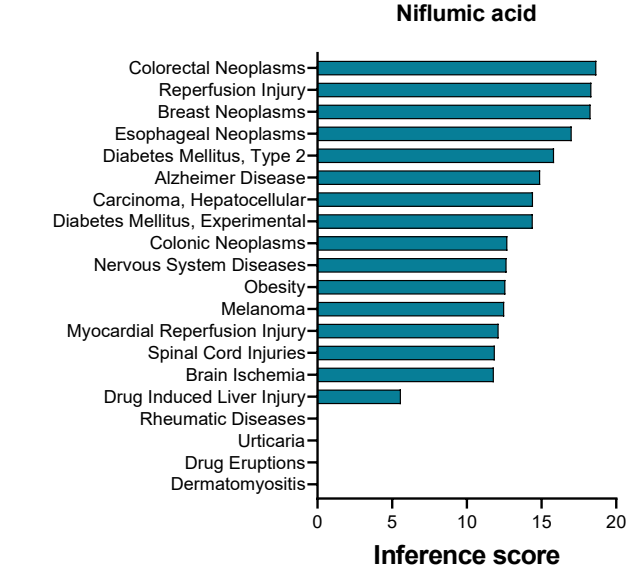

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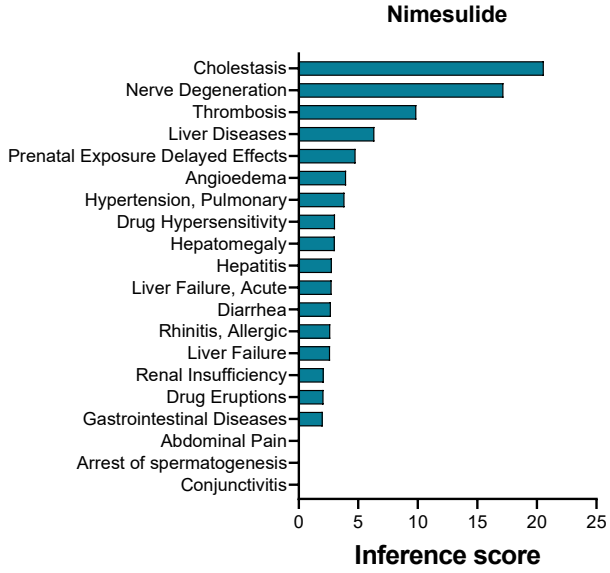

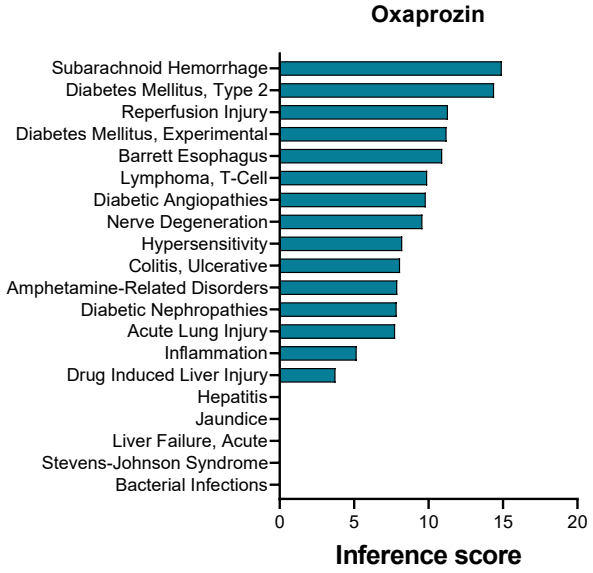

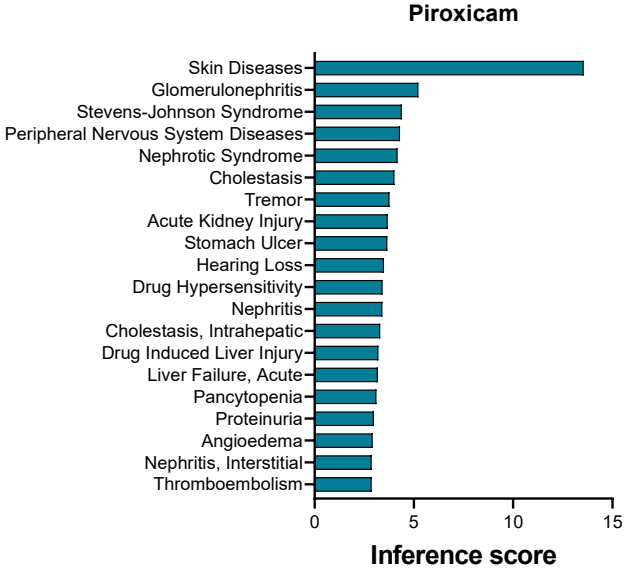

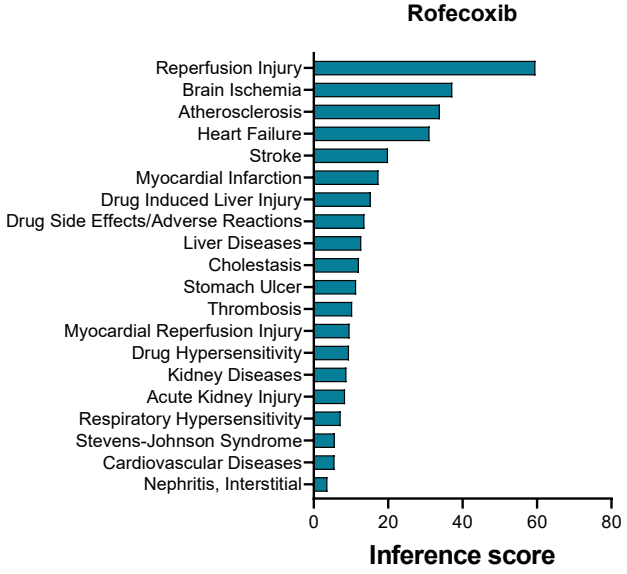

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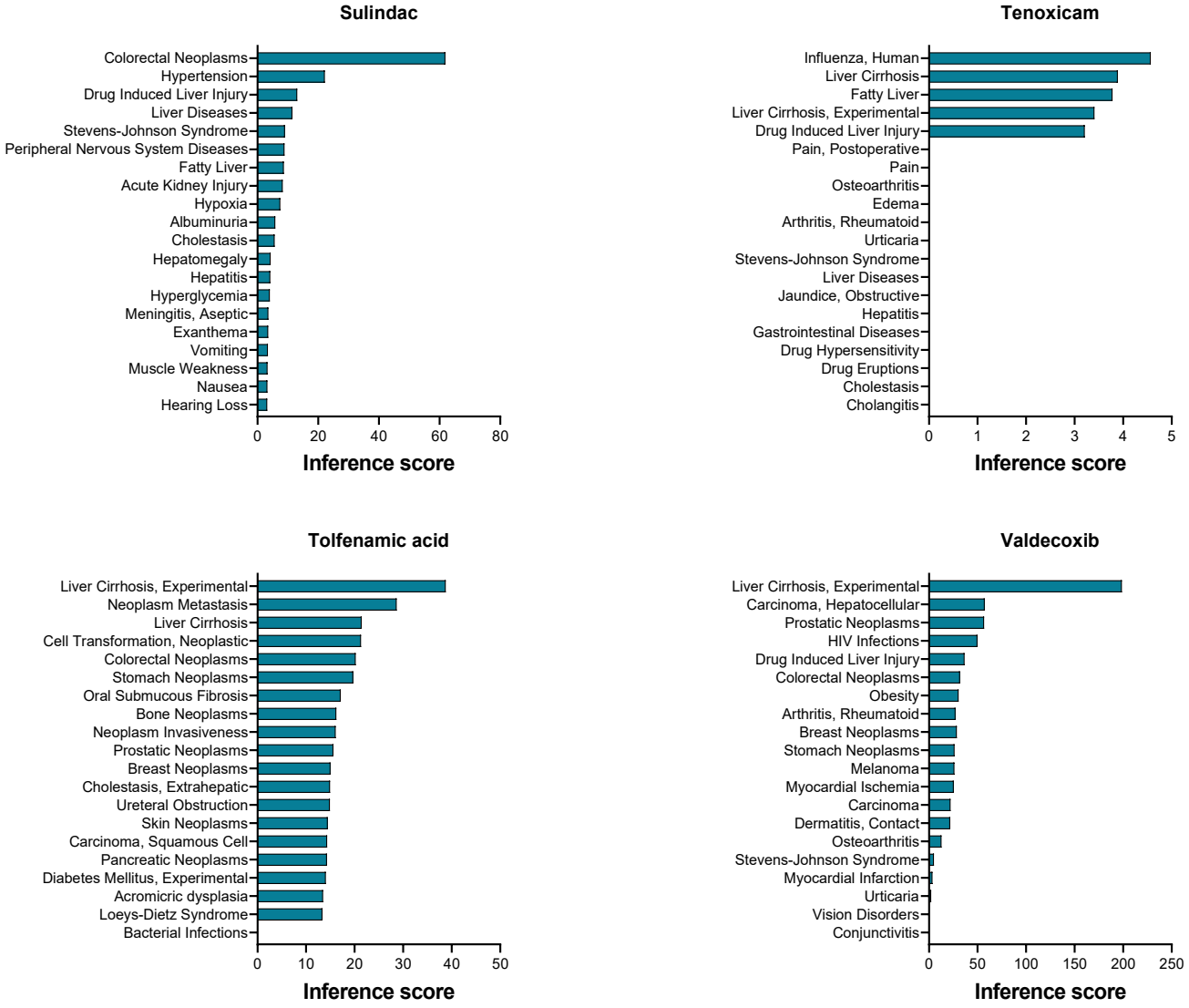
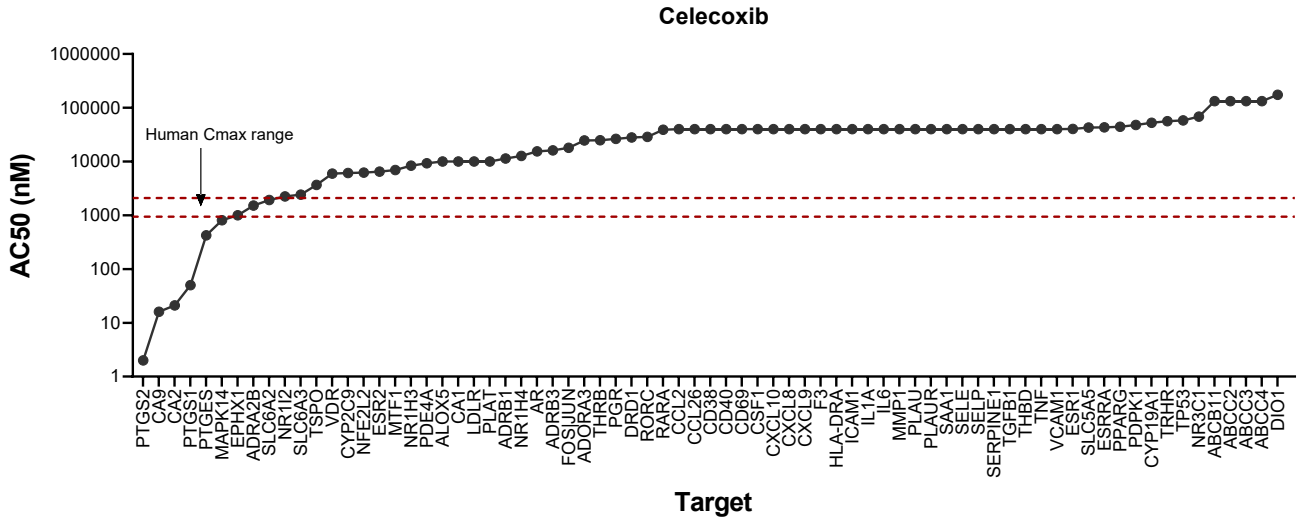
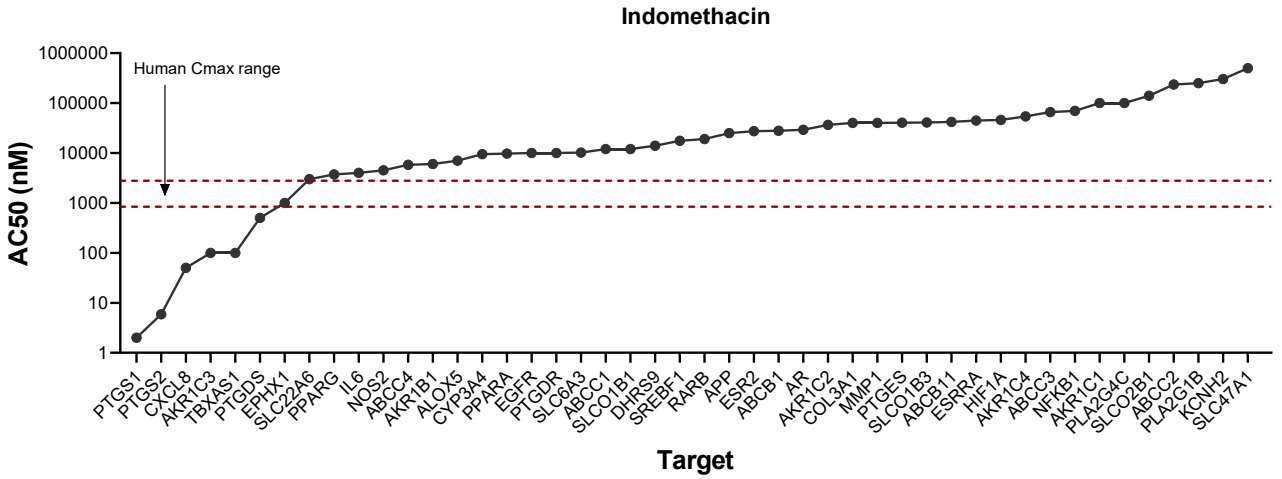


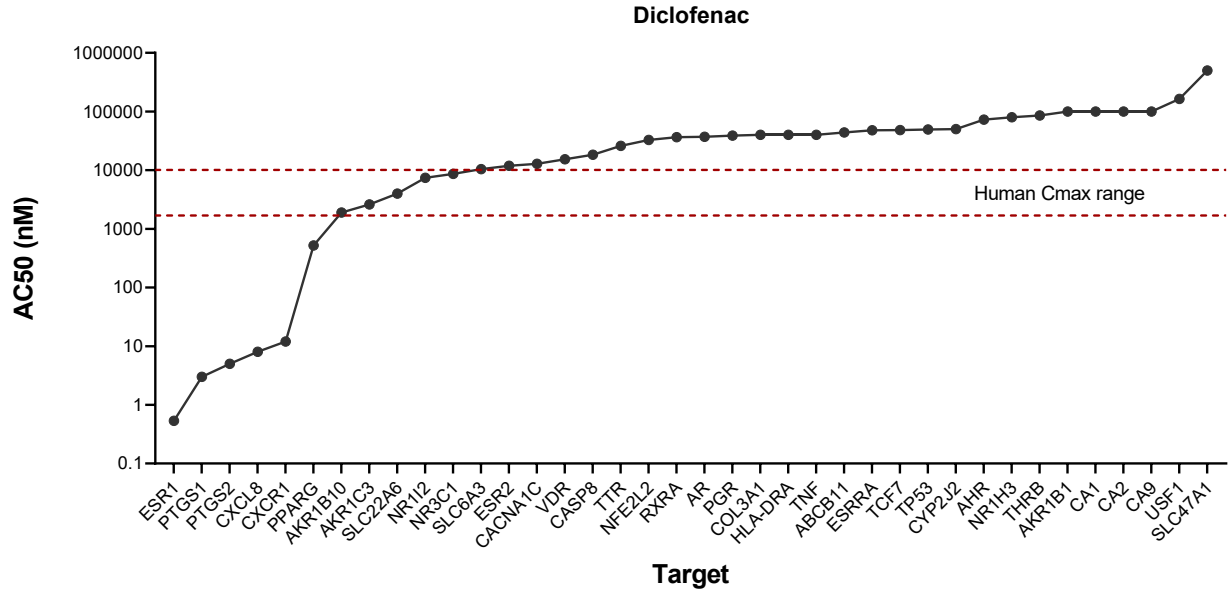
Figure 2.3. Top 20 CTD drug-disease associations. *Relationships are either inferred, or they are curated from direct evidence so have no inference score. As this analysis is based on the number of drug-gene interactions, these results do not necessarily indicate each drugs' potential for biological activity, as the number of interactions is dependent upon the curation process, and the amount of available data in the literature; 24 figures total.*

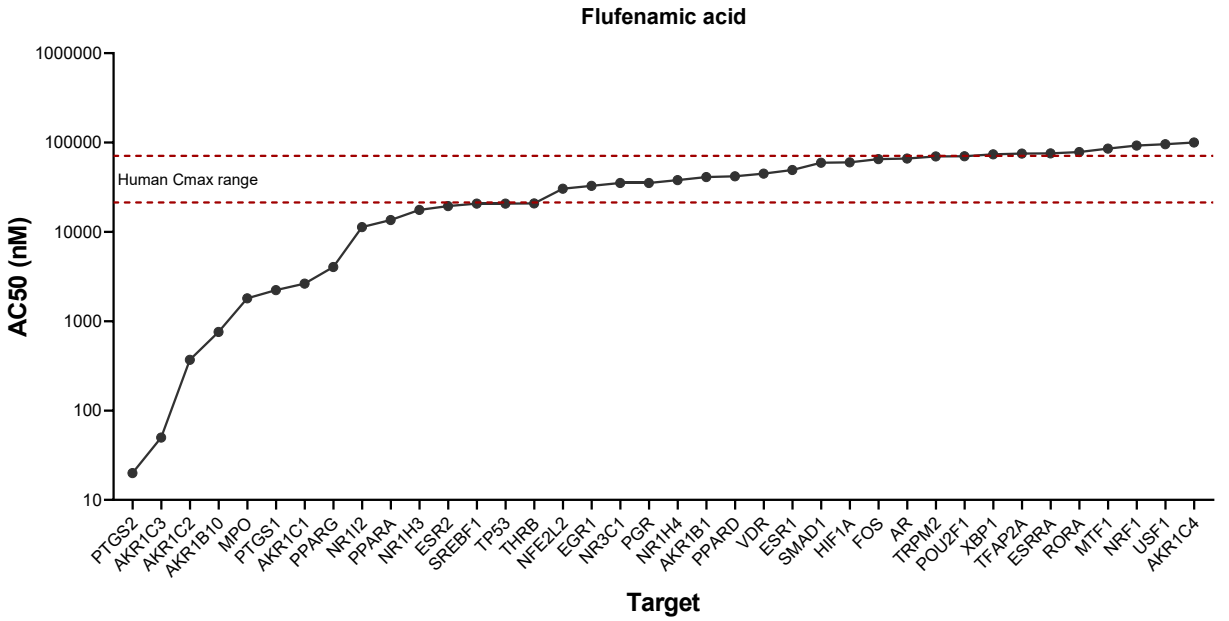
2.4.3 ToxCast/ChEMBL functional bioactivity

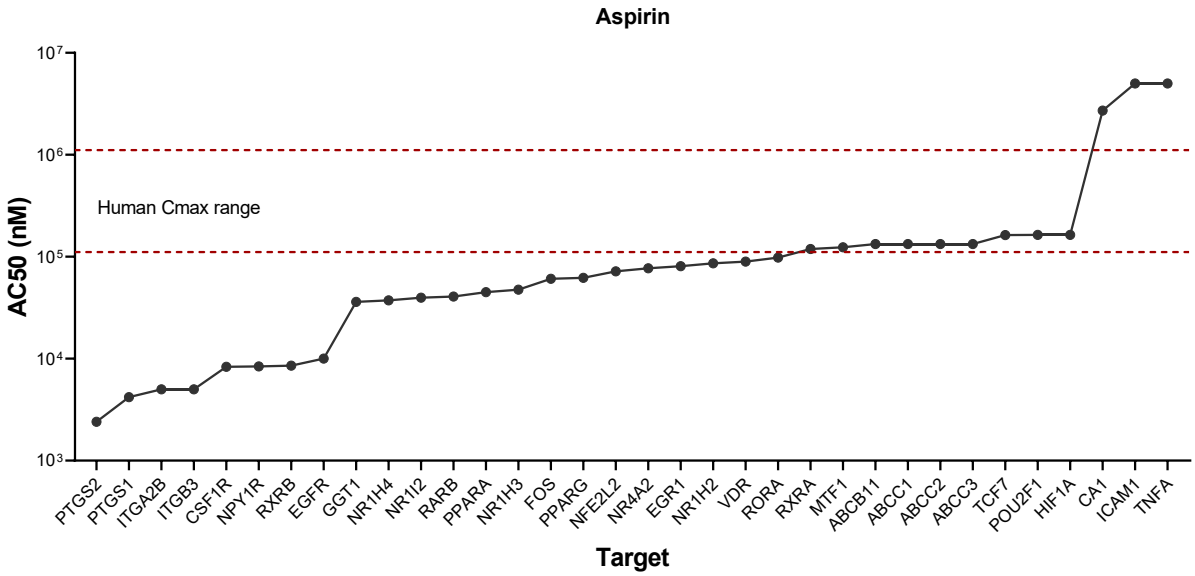
Drug-gene interaction data can provide useful information on the potential mechanism of action of a drug at the molecular level. The next step to inform mode-of-action considerations is to determine the functional interaction between drugs and proteins. To do so, we generated *in vitro* bioactivity profiles for each NSAID using the data previously extracted from the ToxCast and ChEMBL databases. Drug-target interaction data was available for all of the 25 NSAIDs identified in DrugBank, including target AC50 values. Figure 2.4 displays the bioactivity profile for each NSAID. The three drugs with the highest number of interactions were celecoxib, indomethacin, and diclofenac (73, 46, and 38, respectively), whereas etodolac, tenoxicam, and amfenac displayed the lowest number of interactions (6, 6, and 3, respectively). To support the interpretation of the clinical relevance of the observed interaction, each plot also displays the range of human therapeutic Cmax. This visualisation facilitates the interpretation of hazard and risk in a real-life scenario (i.e., the clinical setting). For example, the Cmax of amfenac ranges from 0.76 to 1.57 nM and the concentration of amfenac required to elicit a 50% response of PTGS1 and PTGS2 is 100 nM. This would suggest that at a therapeutic dose of amfenac the PTGS1 and PTGS2 proteins are not likely to be modulated by this drug despite being primary targets. The explanation for this apparent pharmacological discrepancy is that amfenac is only administered topically using eye drops (often as nepafenac, its pro-drug). Hence, the drug concentration at the target site is likely to be much higher than the circulating concentration, and high enough to inhibit PTGS1 and PTGS2. On the other hand, if we consider the bioactivity profile of aspirin all but three targets (CA1, ICAM1, TNFA) lie either within the Cmax range or below it, suggesting that the majority of these targets can be modulated by a therapeutic dose of aspirin. The data indicated that eight out of 25 NSAIDs modulate four or less targets at therapeutic doses. Meloxicam, piroxicam, and tenoxicam modulate only one or both of the cyclooxygenase enzymes, and amfenac and tolfenamic acid do not modulate any targets at therapeutically relevant plasma concentrations. Together, these 25 bioactivity profiles integrated with human plasma therapeutic concentrations provide a quantitative understanding of the risks associated with exposure to distinct NSAIDs at a functional protein level (i.e., target inhibition).

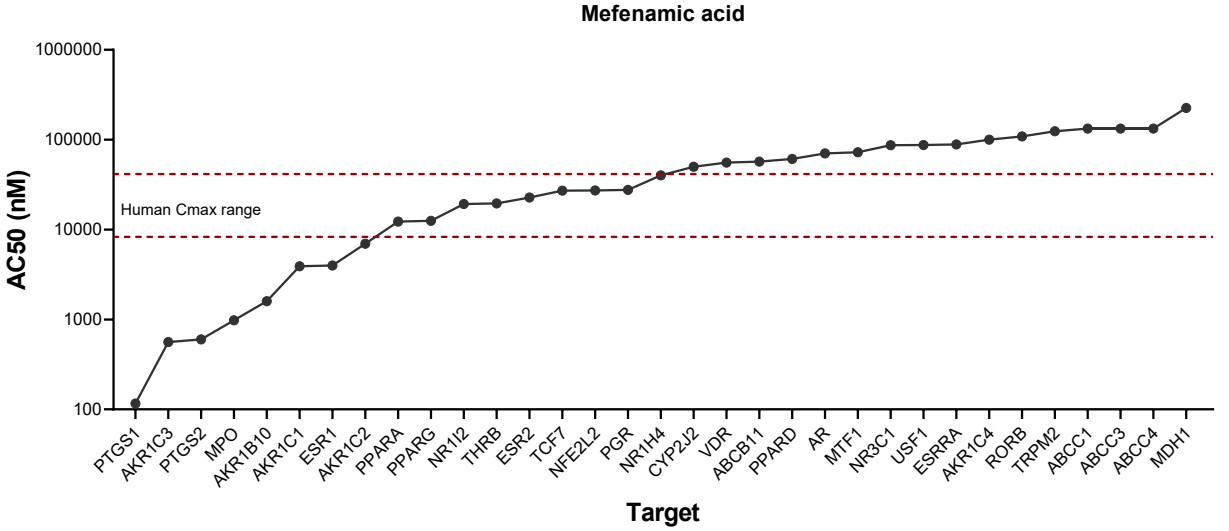

Celecoxib
AC50 (nM)
1000000
100000
10000
1000
100
10
1
Human Cmax range
PTGS2 CA9 CA2 PTGS1 PTGES MAPK14 EPHX1 ADRA2B SLC6A2 NR1I2 SLC6A3 TSPO VDR CYP2C9 NFE2L2 ESR2 MTF1 NR1H3 PDE4A ALOX5 CA1 LDLR PLAT ADRB1 NR1H4 AR ADRB3 FOSJUN ADORA3 THRB PGR DRD1 RORC RARA CCL2 CCL26 CD38 CD40 CD69 CSF1 CXCL10 CXCL8 CXCL9 F3 HLA-DRA ICAM1 IL1A IL6 MMP1 PLAU PLAUR SAA1 SELE SELP SERPINE1 TGFB1 THBD TNF VCAM1 ESR1 SLC5A5 ESRRA PPARG PDPK1 CYP19A1 TRHR TP53 NR3C1 ABCB11 ABCC2 ABCC3 ABCC4 DIO1
Target


Indomethacin
AC50 (nM)
1000000
100000
10000
1000
100
10
1
Human Cmax range
PTGS1 PTGS2 CXCL8 AKR1C3 TBXAS1 PTGDS EPHX1 SLC22A6 PPARG IL6 NOS2 ABCC4 AKR1B1 ALOX5 CYP3A4 PPARA EGFR PTGDR SLC6A3 ABCC1 SLCO1B1 DHRS9 SREBF1 RARB APP ESR2 ABCB1 AR AKR1C2 COL3A1 MMP1 PTGES SLCO1B3 ABCB11 ESRRA HIF1A AKR1C4 ABCC3 NFKB1 AKR1C1 PLA2G4C SLCO2B1 ABCC2 PLA2G1B KCNH2 SLC47A1
Target


Diclofenac
AC50 (nM)
1000000
100000
10000
1000
100
10
1
0.1
Human Cmax range
ESR1 PTGS1 PTGS2 CXCL8 CXCR1 PPARG AKR1B10 AKR1C3 SLC22A6 NR1I2 NR3C1 SLC6A3 ESR2 CACNA1C VDR CASP8 TTR NFE2L2 RXRA AR PGR COL3A1 HLA-DRA TNF ABCB11 ESRRA TCF7 TP53 CYP2J2 AHR NR1H3 THRB AKR1B1 CA1 CA2 CA9 USF1 SLC47A1
Target







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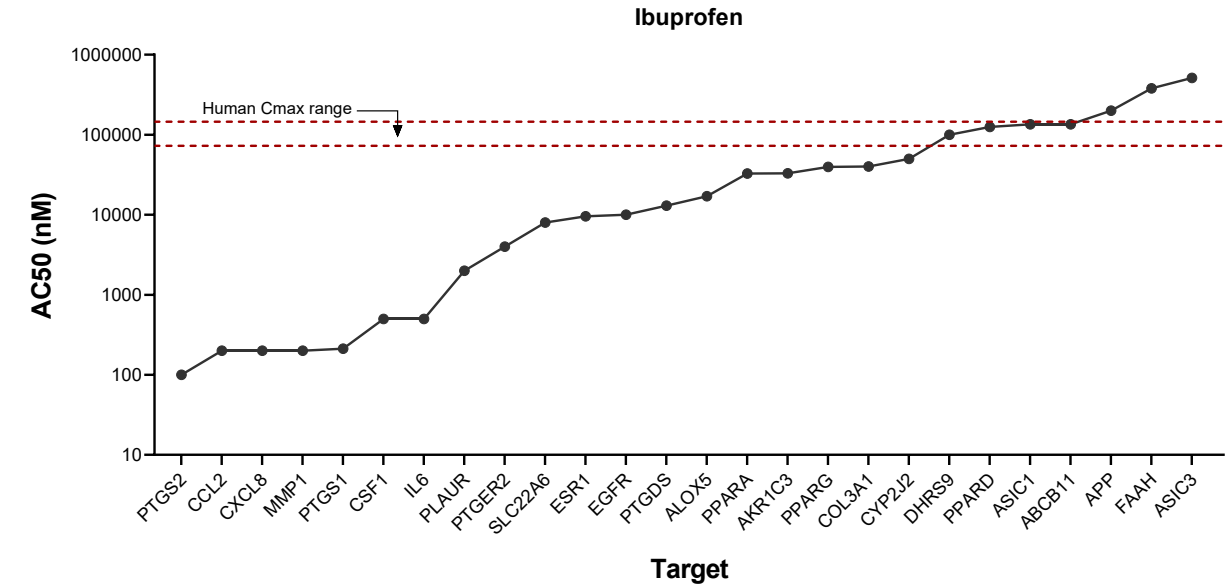

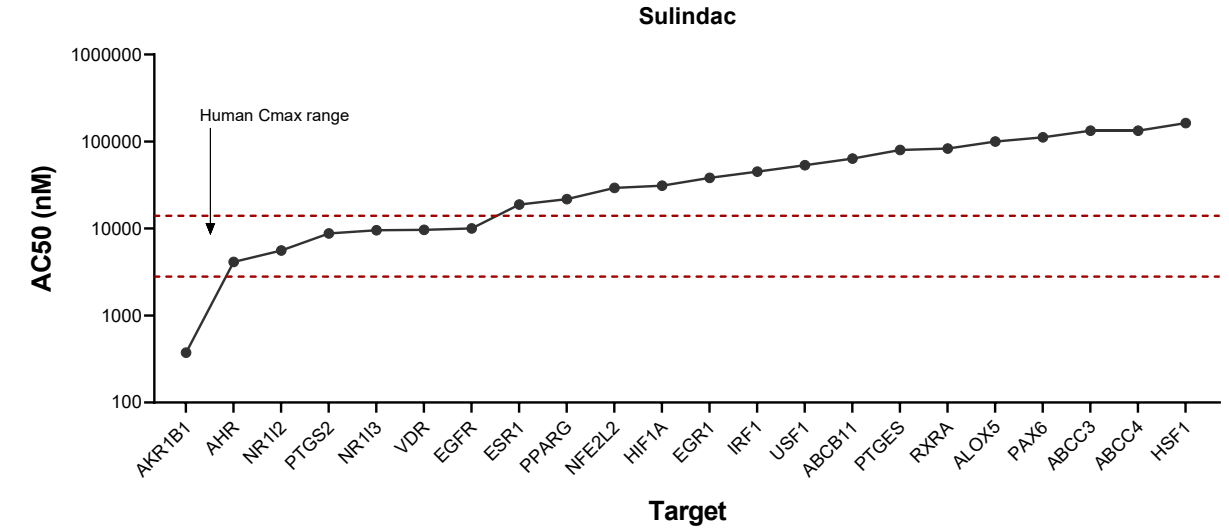

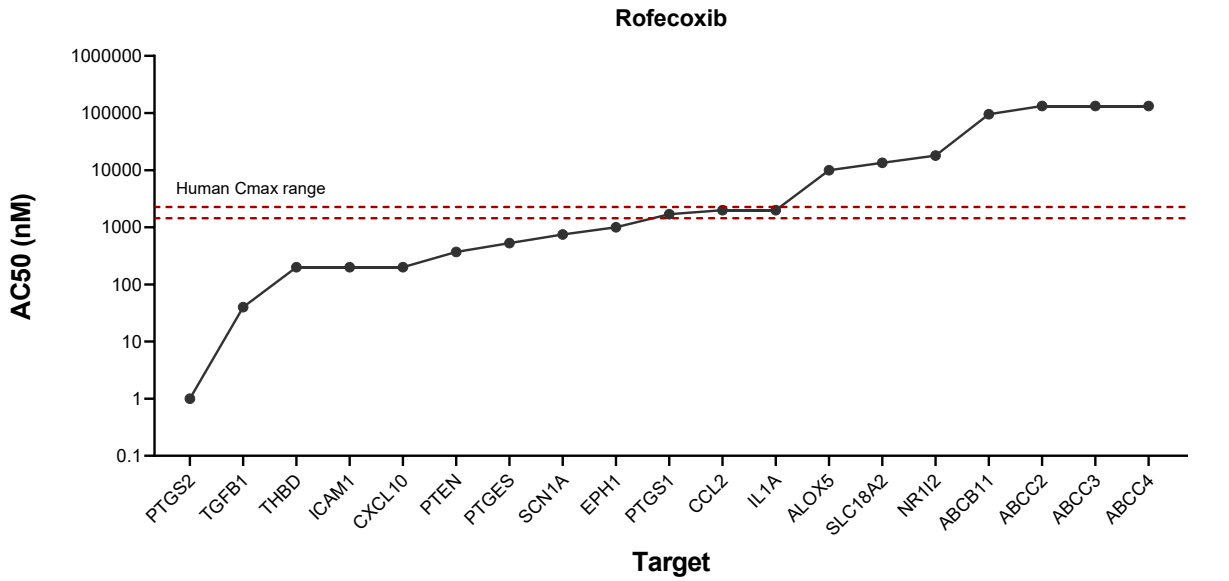

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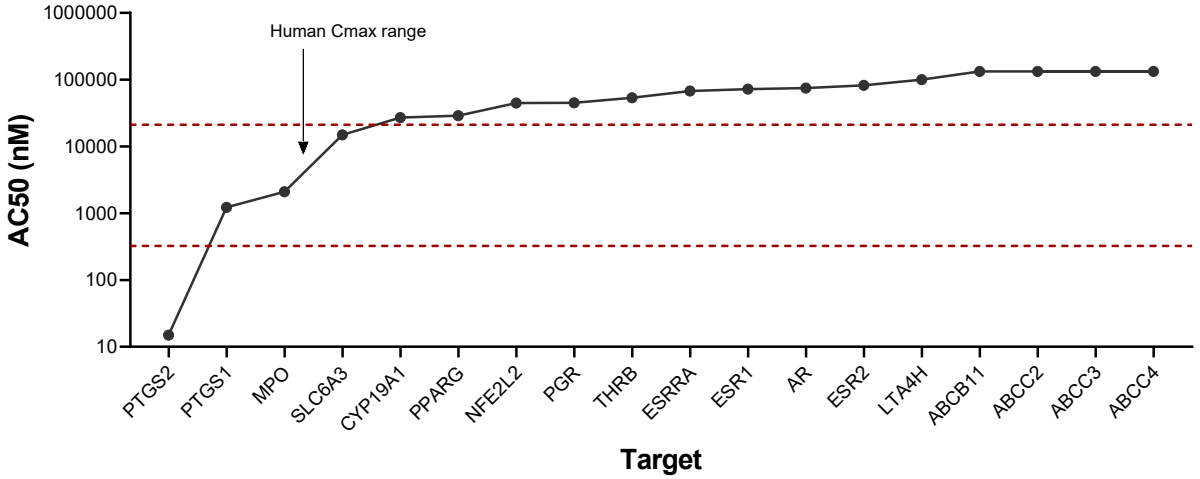
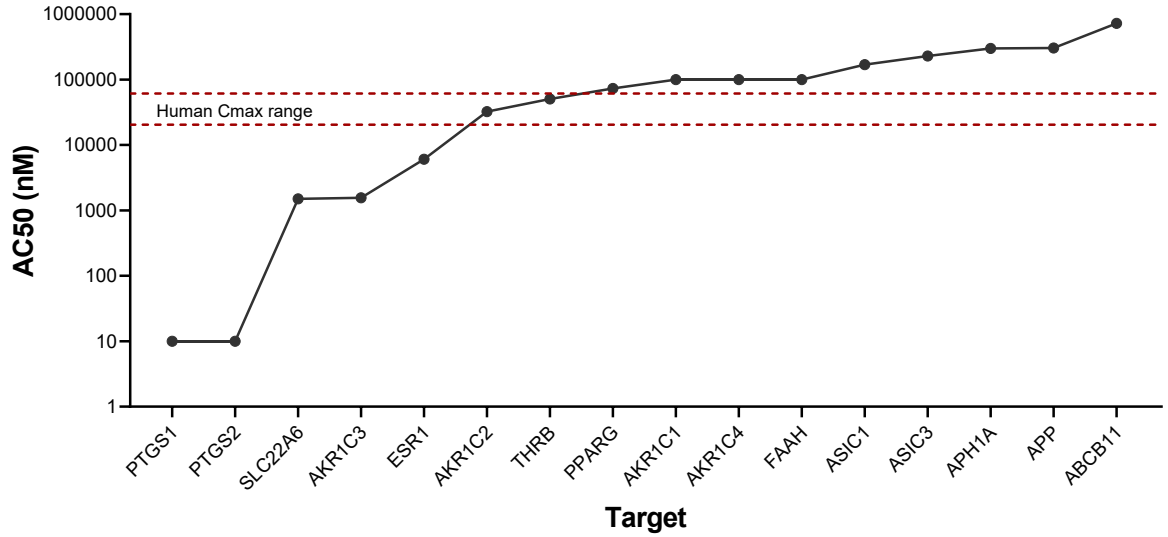
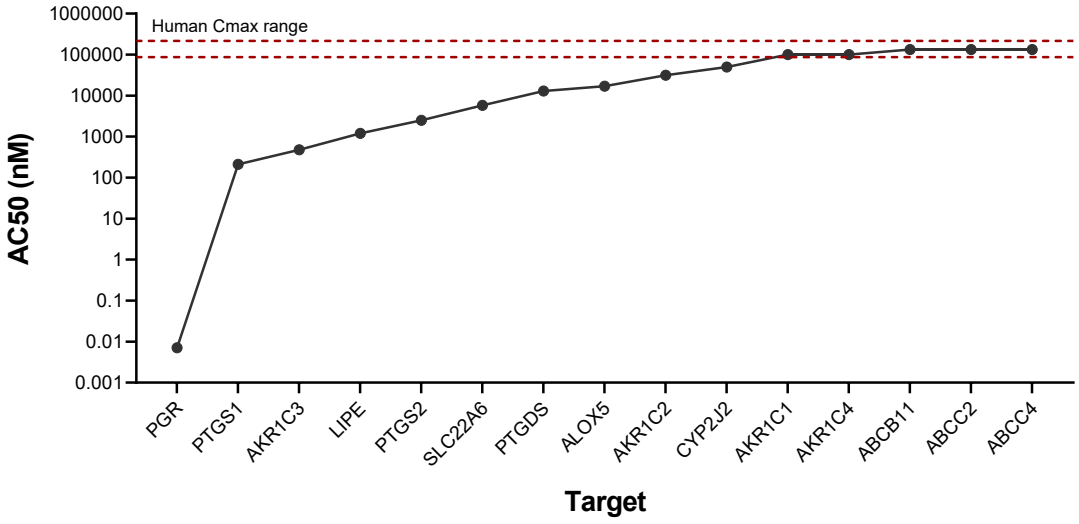
Nimesulide



Flurbiprofen



Naproxen



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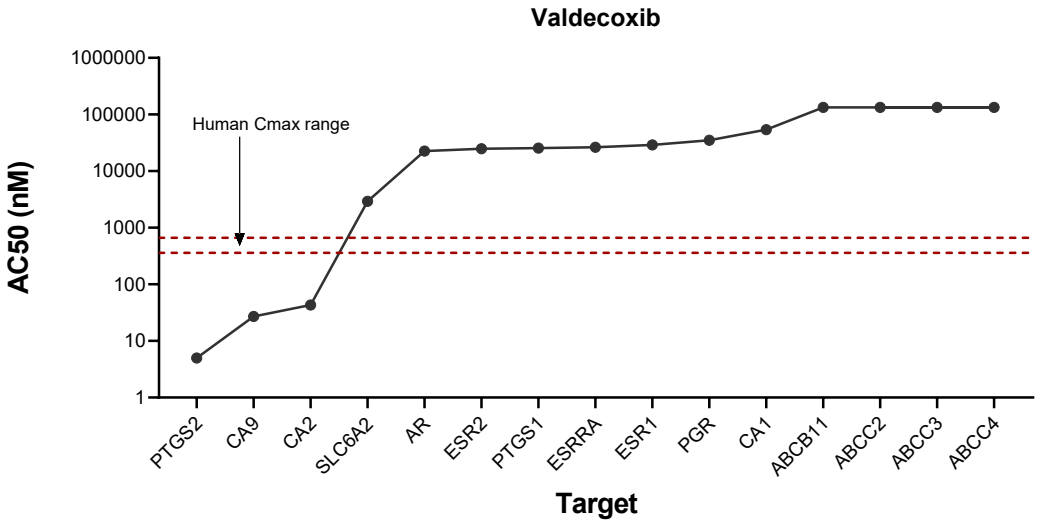

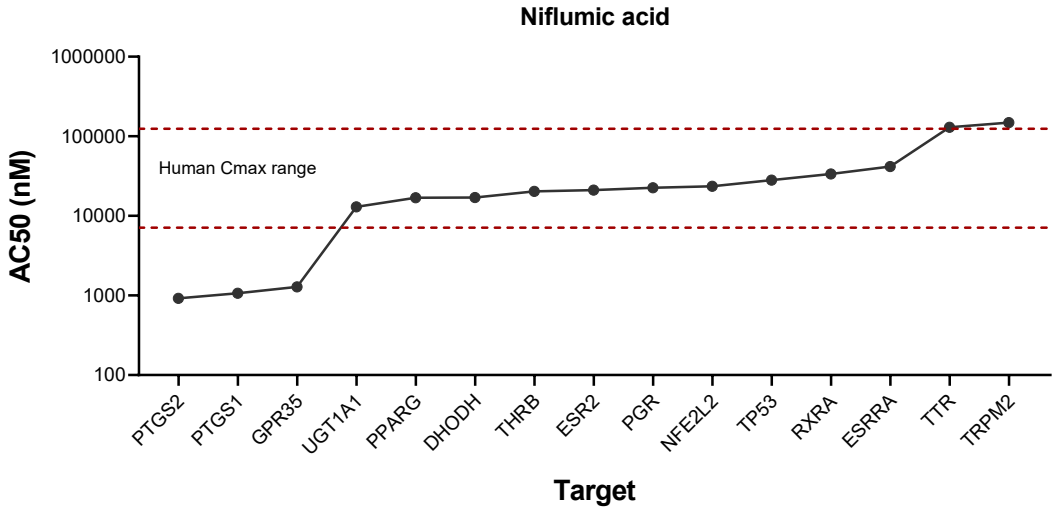

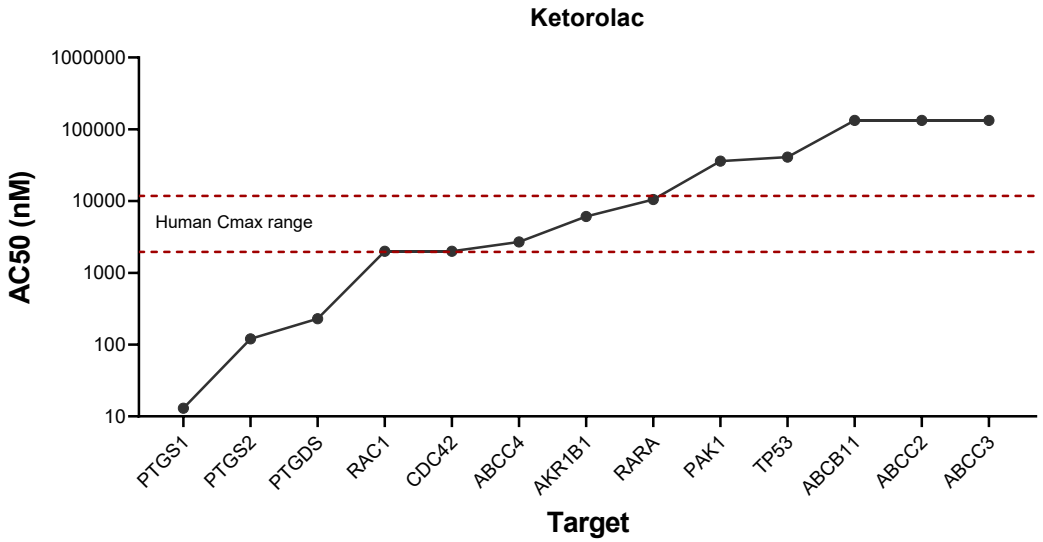

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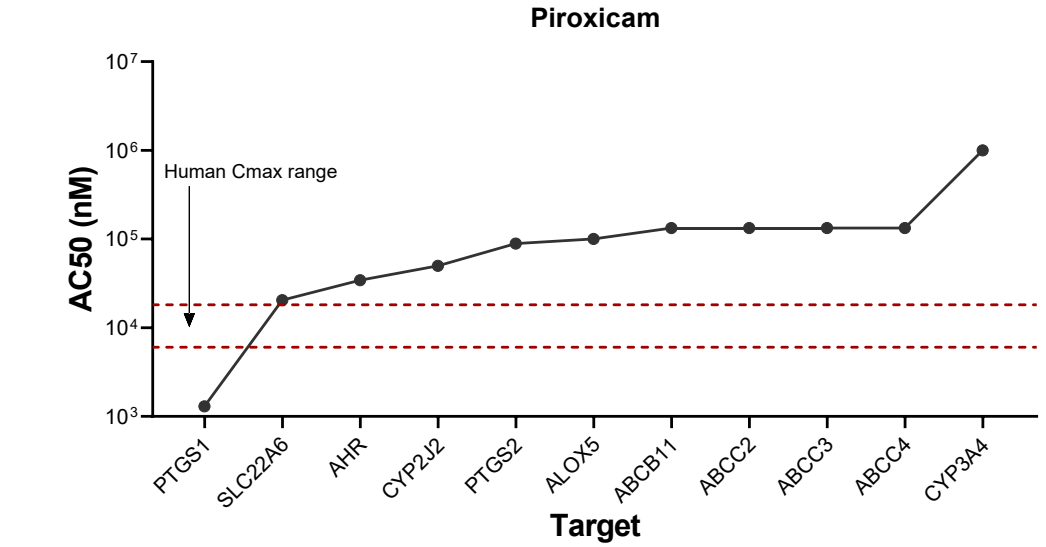

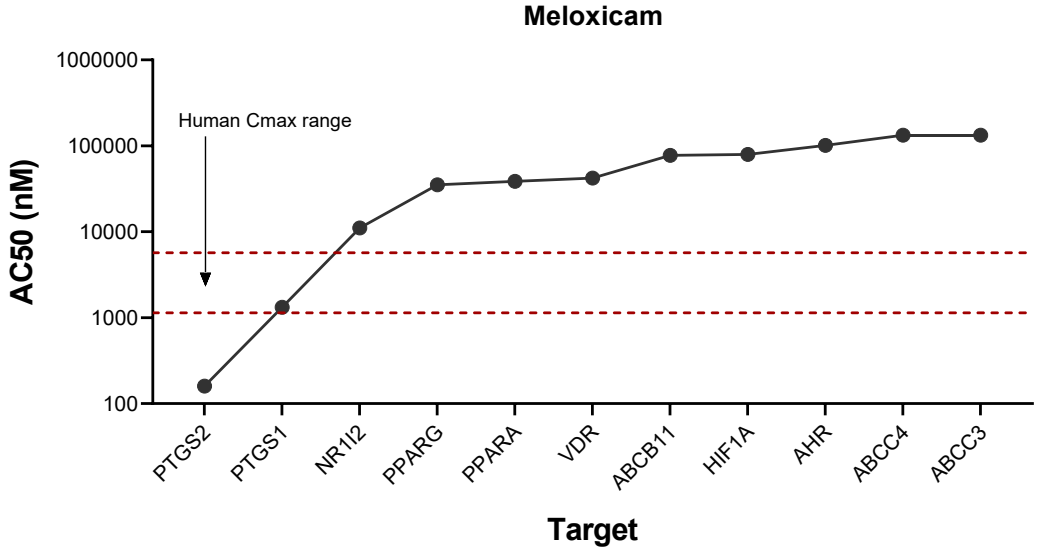

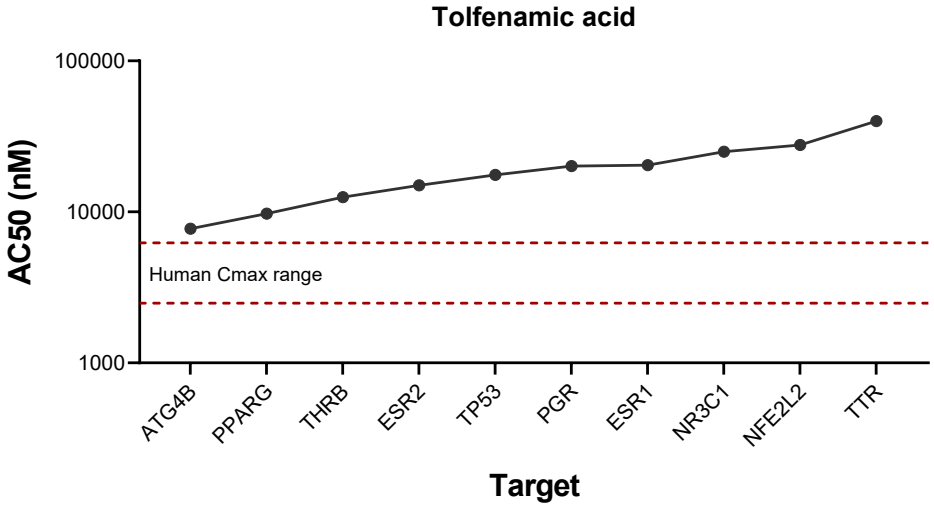

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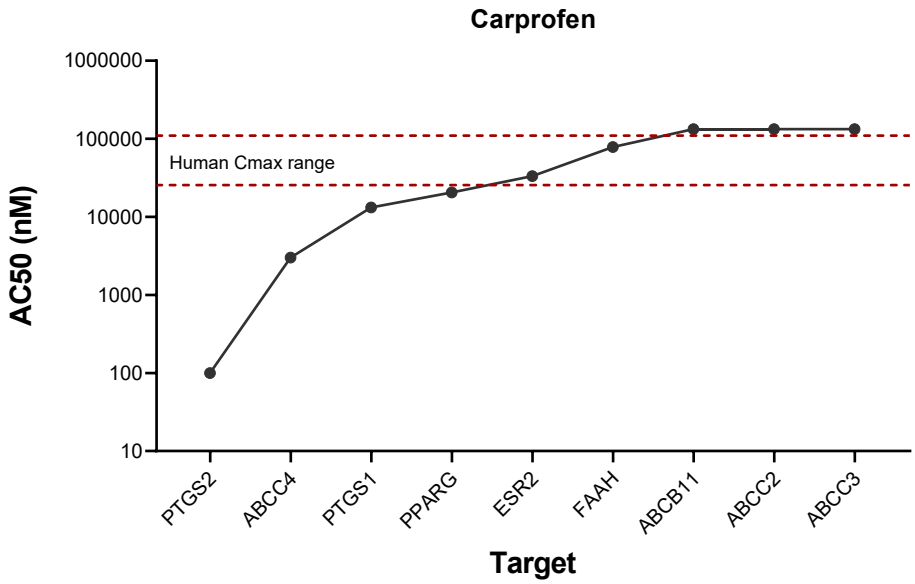

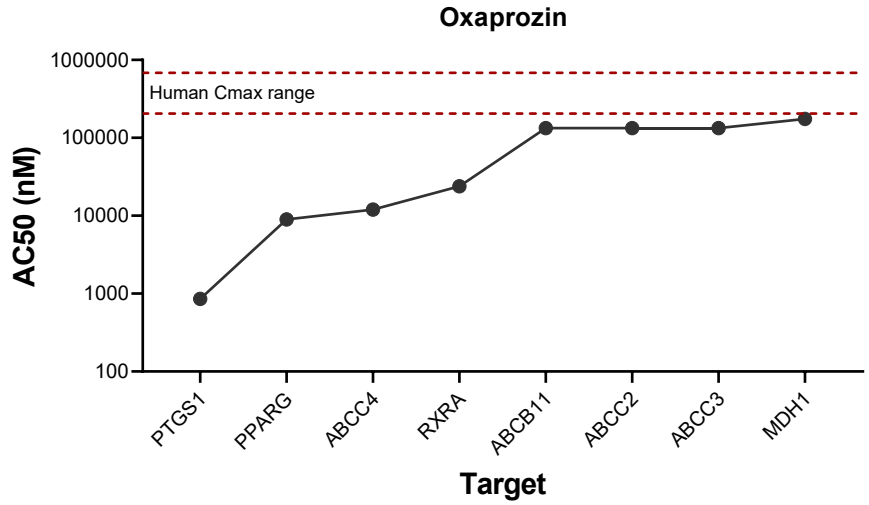

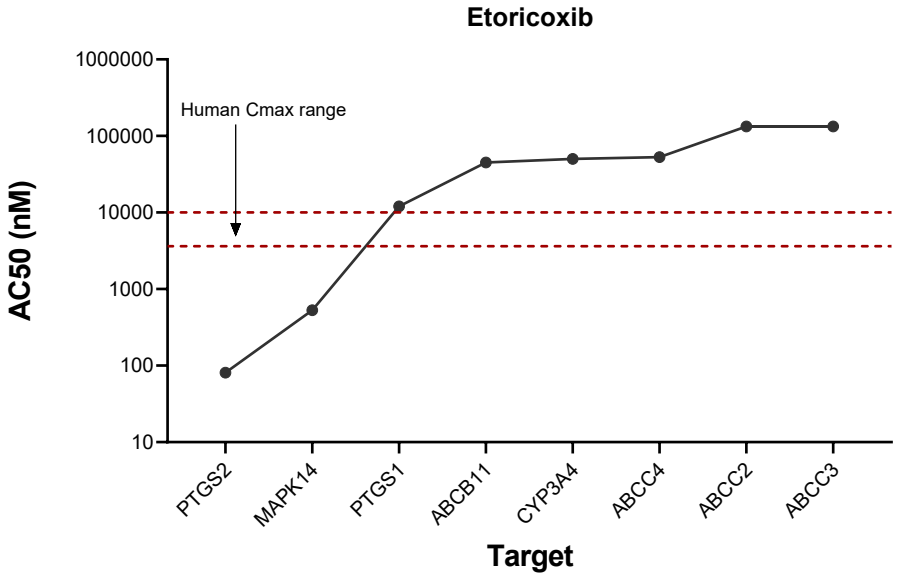

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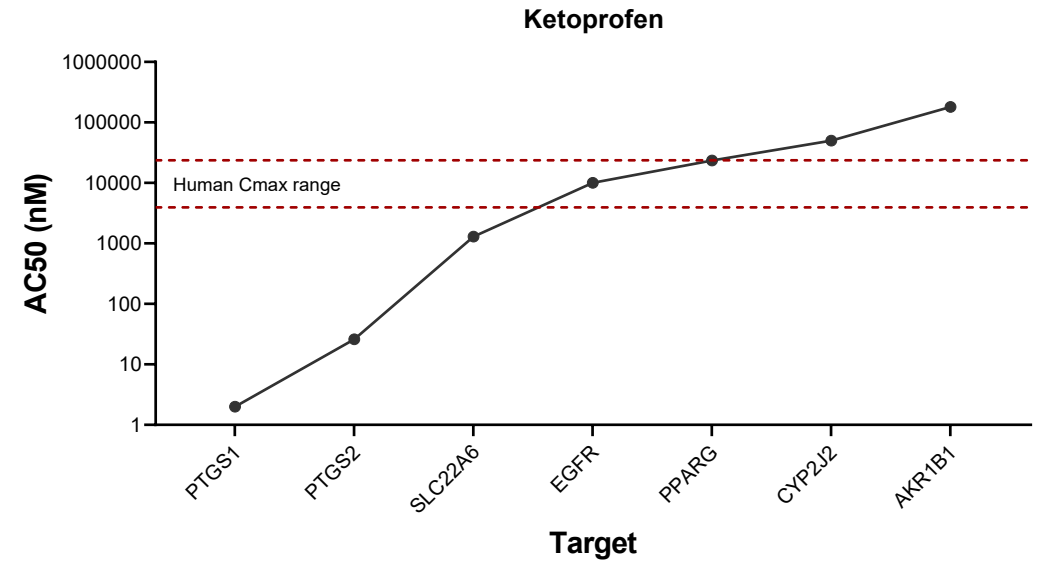

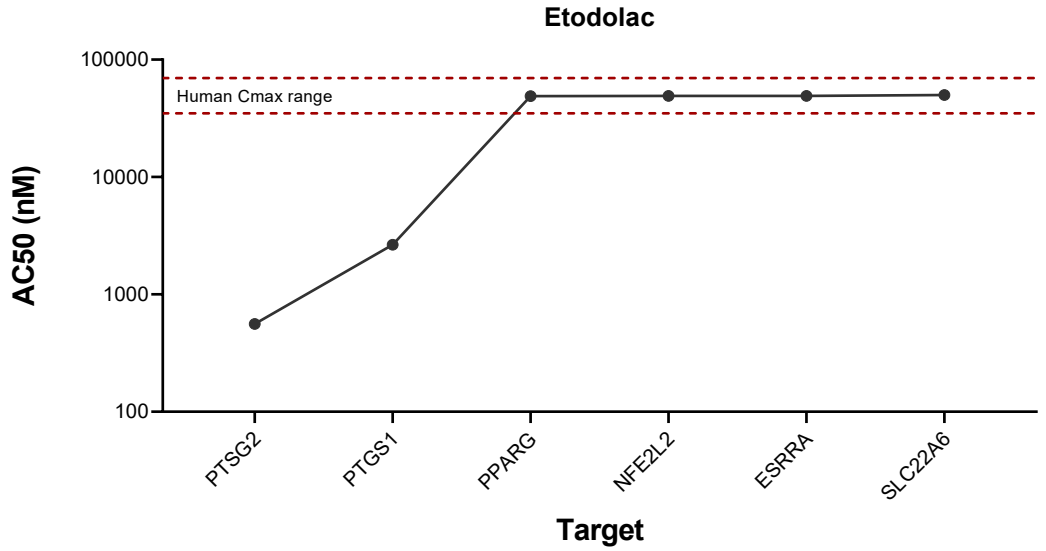

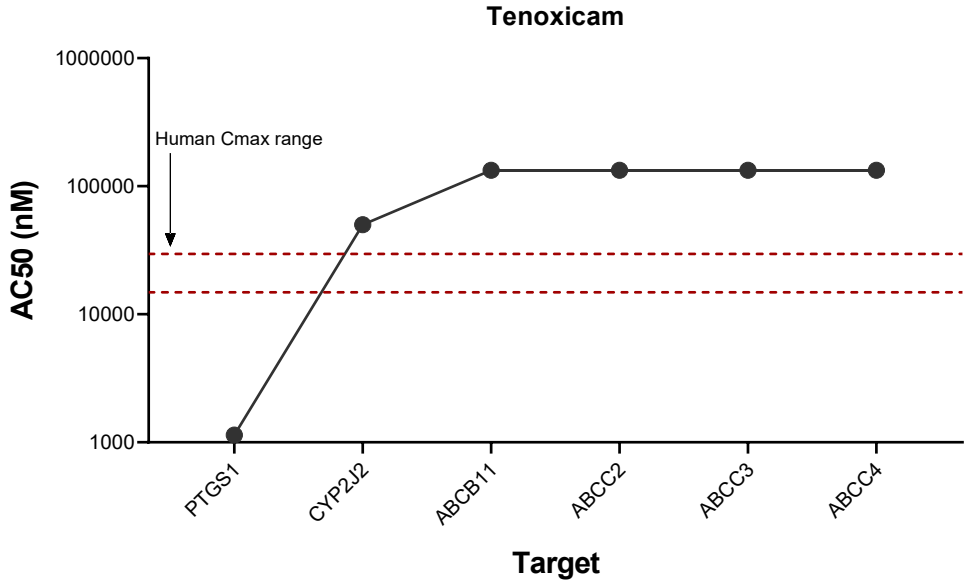

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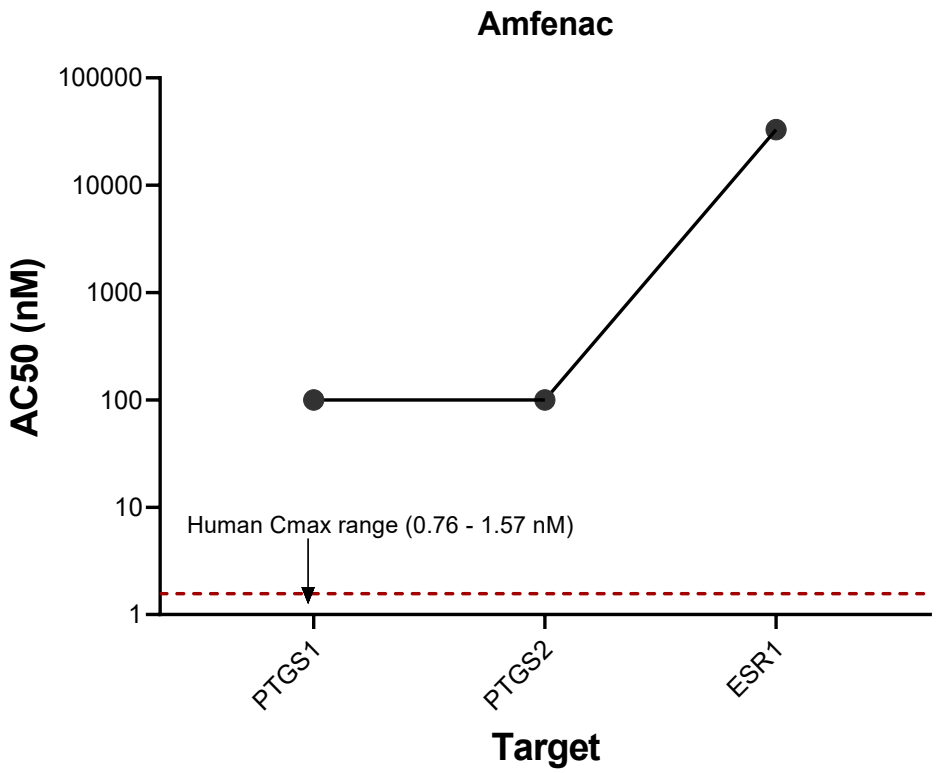

Figure 2.4. ToxCast/ChEMBL bioactivity profiles for 25 NSAIDs. *The human therapeutic Cmax range is displayed by the red dashed lines on each plot; 25 figures total.*

2.4.4 Predicted NSAIDs-mediated immunomodulation

The results obtained through the analysis of NSAID-gene interaction data indicated that NSAIDs can perturb numerous immune-related pathways. To investigate this aspect in more detail, we mined a database generated by Kidd *et al.* (2016), to perform a comparative analysis of the predicted immunomodulatory activity of NSAIDs. The data mining exercise led to the identification of 34 COX-inhibitors, of which only 14 were also present in the DrugBank selection process (used in the previous analyses). The analysis revealed that individual NSAIDs may vary in their ability to interact with the immune system (Table 2.1). The number of predicted interactions ranged from 5 to 113. The three NSAIDs predicted to display the highest number of immune interactions with immune cells were isoxicam (113 interactions), bufexamac (95 interactions), and flunixin (94 interactions). On the other hand, the three compounds with the lowest number of interactions were meclofenamic acid (6 interactions), nifenazone (6 interactions), and niflumic acid (5 interactions). The number of predicted immune interactions provides a qualitative overview of the immunomodulatory potential of each drug. However, each drug can have either a positive or negative effect on each specific immune cell state change (e.g., favouring the shift towards ‘A’ or towards ‘B’, in the state change from ‘A’ to ‘B’). This quantitative information is captured by the immunomod score, which can be either positive or negative. In order to utilise this quantitative data effectively, a hierarchical clustering analysis was carried out to identify any potential differences and similarities amongst NSAIDs effects on immune cell state transitions (Figure 2.5). The clustering exercise led to the identification of 6 main clusters with differential levels of activity. Some of the clusters contain medium-highly active drugs that induce contrasting effects on specific groups of immune cell types, in particular on T cells (e.g. isoxicam, indoprofen, acematicin, and felbinac, *versus* nimesulide, benzydamine, naproxen, acetylsalicylic acid, flurbiprofen, indomethacin, oxaprozin, and aceclofenac). Notably, isoxicam, bufexamac, and indoprofen have been withdrawn from the market due to severe adverse drug reactions. Other clusters contain low activity NSAIDs (e.g. celecoxib and NS398) with minimal predicted immunomodulatory activity that do not display any obvious pattern.

Table 2.1. Number of predicted immune interactions for 34 NSAIDs extracted from the database generated by Kidd et al. (2016).

NSAID	Number of predicted immune interactions
Isoxicam	113
Bufexamac	95
Flunixin	94
Fenoprofen	89
Etofenamate	84
Nabumetone	84
Acemetacin	79
Indoprofen	75
Acetylsalicylic acid	69
Ketoprofen	65
Felbinac	58
Nimesulide	58
Flurbiprofen	50
Oxyphenbutazone	50
Dexibuprofen	45
Diclofenac	45
Naproxen	44
Fenbufen	41
Ketorolac	40
Oxaprozin	36
Indomethacin	34
NS398	30
Epirizole	27
Etodolac	27
Azapropazone	25
Benzydamine	21
Aceclofenac	18
Triamcinolone	17
Piroxicam	16
Diflunisal	15
Celecoxib	8
Mefenamic acid	7
Meclofenamic acid	6
Nifenazone	6
Niflumic acid	5

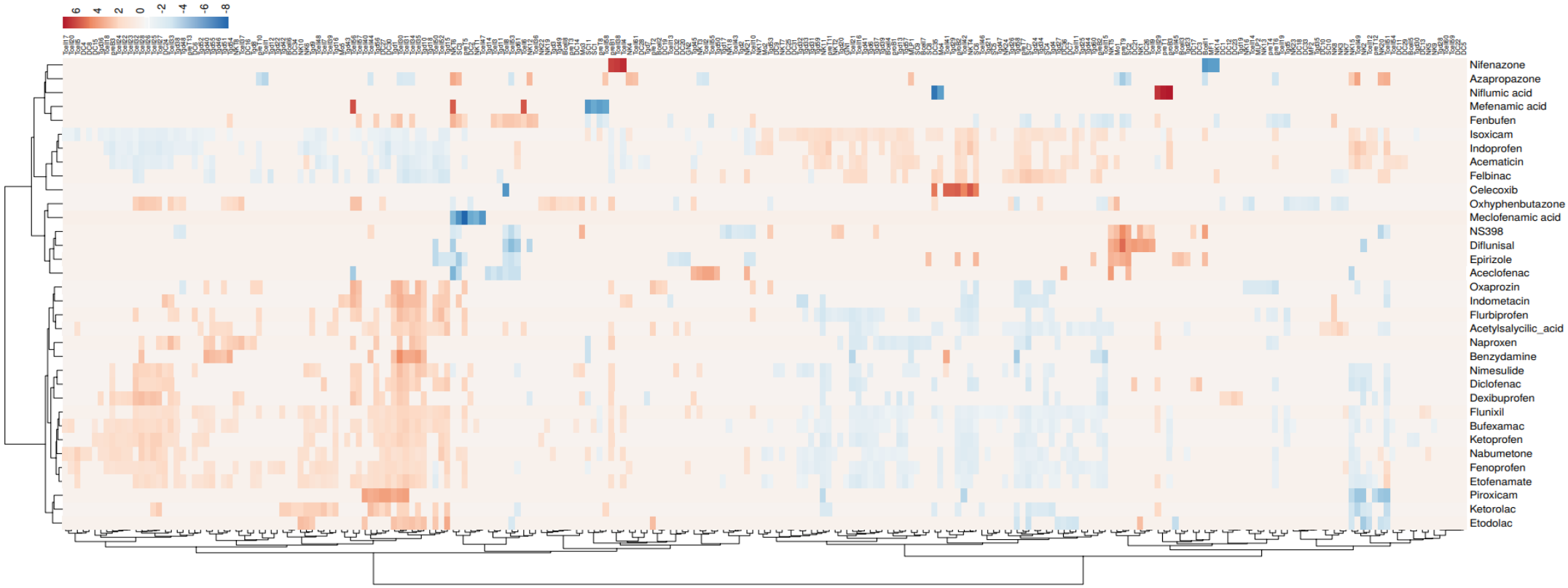

Figure 2.5. Predicted immunomodulatory activity of 34 NSAIDs. *The data was extracted from the large-scale drug immunomodulation database generated by Kidd et al. (2016) and analysed using hierarchical clustering techniques. Each column represents a distinct immune cell state labelled with a simplified code. The full name of the immune state transition corresponding to each code can be visualized in the Excel file available here: https://figshare.com/s/f5e910433971a132eac2. Each row indicates a specific NSAID. Abbreviations: Neut = neutrophil; Mono = monocyte; NKT = natural killer T cell; NK = natural killer cell; Mac = macrophage; T4 = CD4+ T cell; T8 = CD8+ T cell; Tgd = gamma-delta T cell; DC = dendritic cell.*

2.5 Discussion

The data mining of biological databases demonstrated that a substantial wealth of mechanistic data exists for over 25 distinct NSAIDs. This mechanistic profiling exercise provided a data-driven foundation able to guide hypothesis generation and experimental design of the remaining phases of the project. NSAIDs pharmacology is traditionally focused on the biological significance of COX inhibition. The analysis presented here leads to two main considerations. Firstly, we demonstrate that NSAIDs can trigger (directly and indirectly) a wide variety of biological responses that may be relevant for the assessment of drug-specific efficacy and safety. Secondly, multiple lines of evidence indicate that NSAIDs-mediated immunomodulation and the perturbation of immune and inflammatory pathways could represent a key, but underexplored, mode-of-action that warrants further investigation. Starting from these considerations, in Chapter 3 we describe the application and expansion of this data-driven approach to characterise and predict NSAIDs-mediated effects in fish species, and to assess the toxicological risk of NSAIDs mixtures in the environment. Whereas in Chapter 4 and Chapter 5, we describe an innovative experimental approach based on the use of transgenic zebrafish larvae to validate our computationally-driven hypothesis and unravel the role played by NSAIDs-mediated immunomodulation in the manifestation of gastrointestinal toxicity.

The major novelty of the present work stems from the integrated focus on multiple levels of biological and functional organisation. The CTD provided curated NSAID-gene interaction data, pathways enrichment analyses, and disease association analysis. Whereas the combined data from ToxCast and ChEMBL provided us with functional bioactivity data at the protein level. Using the available human Cmax data from the literature, and other online sources, we were able to predict the risk of each drug-target interaction occurring at therapeutic levels of exposure to each NSAID. Some of the most common targets, pathways, and diseases shared between each NSAID in the CTD and ToxCast/ChEMBL datasets appear to be immune system-related. These data analyses led us to focus on immunomodulation as a pivotal component of NSAIDs-mediated effects which directed our final data extraction process. Immunomodulatory data extracted from the publication by Kidd *et al.* (2016) provided us with data at the cellular level of interaction. Hierarchical cluster analysis enabled us to simultaneously highlight the effects of NSAIDs on a comprehensive set of immune cell types, and group together drugs with a similar interaction profile. Together, these datasets have provided us with mechanistic information at multiple levels of biological organisation and point towards the potential immunomodulatory effects of NSAIDs as an area of interest for further investigation.

One of the challenges of using biological databases that use automated or semi-automated approaches to extract data from heterogenous sources is the quantification of confidence and uncertainty. To estimate the confidence in each drug-gene interaction from the CTD we considered the 'interaction count' as a surrogate marker of reproducibility, as this value represents the number of times that each drug-gene interaction has been experimentally observed in the literature. Therefore, drugs like indomethacin which have some of the highest interaction counts per drug-gene interaction give us a high level of confidence in the likelihood of these perturbations occurring. In contrast, the NSAIDs carprofen and tenoxicam present with only one gene interaction and an interaction count of one, indicating a low level of confidence. Low numbers of interactions and low interaction counts do not give us high levels of confidence in the data. However, it is important to consider that a lack of data, or a low interaction count, does not necessarily mean that a drug is less promiscuous/less likely to cause an effect at the molecular level. Since the CTD is a database curated from the literature, it is entirely dependent upon the quality, and quantity, of research describing the effects of these drugs. Additionally, not all drugs have been fully curated in the database which could also account for a lack of interaction data. Hence, for drugs with a low number of interactions, such as carprofen, it is quite likely that an absence of evidence is not evidence of absence. Some NSAIDs are much more highly investigated than others which can have an impact on the accuracy of the CTD. For example, PubMed indicates that the popular NSAID ibuprofen has been studied in over 11,279 papers in the last 20 years. On the other hand, the less popular NSAID tenoxicam is mentioned in only 294 papers. Another important factor is that these drug-gene interactions are not associated with any quantitative data, meaning it is unclear what concentration of drug would be required to elicit an interaction with each target. The CTD also provides pathways enrichment analysis for each of the drug-gene interaction sets, from which we extracted the top 20 most significantly enriched pathways (when available). This gives us an idea of the types of pathways most likely to be affected by modulation of the genes in each respective NSAIDs gene interaction set. Similarly, we extracted disease association data from the CTD to give us an idea about the types of diseases that are likely to be implicated by exposure to each NSAID. However, it is important to note that these associations are largely made through the use of an 'inference score' statistic calculated by the CTD software, which may not necessarily be accurate. The inference score is based upon the curated drug-gene interactions from the literature, and so the quality of the inference score is also wholly reliant upon the quality of the underlying data. As this underlying data is curated from the literature there is the danger that publication bias, due to under-reporting of negative data, may skew the results of downstream analyses, such as the generation of the inference score. Evidence demonstrating the existence of publication bias supports this notion (Kicinski, 2014), and so it is important to consider these limitations when

interpreting the inference score in this case. Nevertheless, using existing data from the CTD alone allowed us to predict the perturbation of molecular level targets, pathway level interactions, and disease level implications for each drug, to the best of our ability. Other than the aforementioned limitations, a lack of quantitative data associated with the drug-gene interactions provides us with only an estimation of the hazard, since it is unclear if these interactions are likely to occur at realistic exposure concentrations. Nonetheless, the CTD provides a good initial indication of the potential mechanistic effects that exposure to 24 distinct NSAIDs may have. Some of the most common pathways and disease associations shared between our drugs involved some level of immunomodulation.

Out of the 24 NSAIDs, pathways enrichment was only available for 22 of them, and 18 of these drugs included immune-related pathways within their top 20 significantly enriched pathways (aspirin, celecoxib, diclofenac, etodolac, etoricoxib, flurbiprofen, ibuprofen, indomethacin, ketoprofen, ketorolac, mefenamic acid, meloxicam, naproxen, nimesulide, oxaprozin, piroxicam, rofecoxib, and sulindac). In terms of disease association, 22 out of the 24 NSAIDs included immune-related diseases within their top 20 associations (aspirin, carprofen, celecoxib, diclofenac, etodolac, etoricoxib, flurbiprofen, ibuprofen, indomethacin, ketoprofen, ketorolac, mefenamic acid, meloxicam, naproxen, niflumic acid, nimesulide, oxaprozin, piroxicam, rofecoxib, sulindac, tenoxicam, valdecoxib). From a mechanistic perspective, the immunomodulatory potential of NSAIDs may be mediated both directly (i.e., via the inhibition of the primary targets PTGS1 and PTGS2, and the perturbation of their immunomodulatory functions) and indirectly (i.e., via the indirect/secondary perturbation of genes and pathways involved in immunomodulatory functions). We know that inhibition of PTGS1 (COX-1) and PTGS2 (COX-2) can lead to a variety of diverse effects, as discussed in Chapter 1, many of which are related to the immune system. For example, COX-2 has been identified as both a key pro-inflammatory mediator, and a crucial component for the resolution of inflammation (Wallace and Devchand, 2005). Moreover, COXs' downstream products such as PGE_2 and PGI_2 have been shown to significantly increase oedema, vascular permeability, and leukocyte infiltration (Smyth *et al.*, 2009). Hence, modulation of PTGS1 and/or PTGS2 is incredibly likely to significantly impact immune-related homeostatic mechanisms. Many of the secondary targets modulated by NSAIDs, either directly or indirectly, in the CTD are also important immune system components; TNF, CXCL8, and IL1B represent just a few of these key immunomodulatory genes which are modulated by the majority of NSAIDs, according to the CTD. TNF-α is one of the most important pro-inflammatory cytokines, inducing oedema and vasodilation, as well as leukocyte adherence to the vascular endothelium (Zelova and Hosek, 2013). In fact, Page *et al.* (2010) found that NSAIDs are able to indirectly upregulate TNF-α synthesis in rheumatoid arthritis tissues through the inhibition of PGE_2, paradoxically

exacerbating inflammation. CXCL8 (IL8; interleukin-8) is a potent chemokine which regulates many functions including cell trafficking and activation of polymorphonuclear (PMN) leukocytes, during inflammatory and homeostatic conditions (Russo *et al.*, 2014). Clearly, significant modulation of any of the aforementioned genes by NSAIDs has the capacity to disrupt normal immune system function.

The transition from hazard to risk assessment requires the consideration of quantitative information, such as potency and effect concentration data. Compared to the CTD data, which is largely focused on gene expression, the bioactivity profiles generated using ToxCast/ChEMBL data provide an overview of the functional interaction between NSAIDs and protein targets. This profiling exercise also indicates the NSAID concentrations required to elicit a half maximal response for each drug-protein interaction. Furthermore, by overlaying the range of human therapeutic Cmax values for each NSAID, we are able to assess the risk of each target being modulated at therapeutically relevant concentrations. Out of the 25 NSAIDs assessed, eight of these drugs appear able to modulate four or less targets at therapeutically relevant plasma concentrations (amfenac, etoricoxib, meloxicam, nimesulide, piroxicam, tenoxicam, tolfenamic acid, and valdecoxib). Out of those eight NSAIDs, only three can modulate PTGS1 and/or PTGS2 (meloxicam, piroxicam, and tenoxicam). In contrast, the remaining 17 NSAIDs can modulate a higher number of targets at therapeutically relevant plasma concentrations, suggesting that these drugs may carry an increased level of risk of effects beyond COX inhibition. Many of the targets modulated by the 25 NSAIDs appear to be immune-related, some of which are potent regulators of the immune response. For example, celecoxib modulates 73 targets of which 17 are important immune system components (PTGS1, PTGS2, PTGES, MAPK14, ALOX5, CCL2, CCL26 CXCL8, CXCL9, CXCL10, VCAM1, ICAM1, IL1A, IL6, TGFB1, TNF, PPARG, SELE, and SELP). Some important factors to again consider include the quality and quantity of data being produced by each database. Similar to the limitations surrounding the CTD data, if the research has not been done for a particular drug, then the data will not be available in the database. So in terms of ToxCast, one NSAID may not show any activity data for a particular target, but this does not necessarily mean that this drug lacks the capacity to modulate that target. It may simply mean that this particular NSAID has not been tested in the specific *in vitro* assay required to assess this interaction. For example, the data from ToxCast suggests that naproxen modulates PGR (progesterone receptor), and that meloxicam does not. However it is unclear whether meloxicam was ‘inactive’ in this assay or simply was not tested. Aside from issues surrounding the quantity of data, and amount of testing, the quality of these *in vitro* assays with regards to their reliability has certainly come under scrutiny. For example, one such paper highlighted that a large percentage of the chemicals classified as PPARγ agonists in ToxCast were in fact

false positives (Janesick *et al.*, 2016). Other issues surrounding reliability of the data from these sources comes from inter-assay variability. For example, diclofenac was tested in 17 *in vitro* assays designed to detect ESR1 activity and was only active in three of those assays (Marmon, Owen, and Margiotta-Casaluci, 2021).

The immunomodulatory potential of NSAIDs was highlighted by both the CTD and the ToxCast/ChEMBL data analyses, at multiple levels of biological organisation. To further investigate this emerging pharmacological feature, we data-mined and analysed predicted drug immunomodulation data generated by Kidd *et al.* (2016). This analysis confirmed that NSAIDs have the potential to modulate several immune cell types and cell state transitions. This prediction is based on complex bioinformatics evaluations of NSAIDs transcriptomic signatures. However, several studies in the literature suggest that such immunomodulation may also occur *in vivo* via both COX-dependent and COX-independent mechanisms (Bancos *et al.*, 2009; Chen *et al.*, 2021; Villalonga *et al.*, 2010).

The interaction between drugs and the immune system may have implications for both drug efficacy and safety assessment. For example, unintended immunomodulation may play a role in the manifestation of adverse drug reactions. Our analysis revealed that isoxicam is the NSAID with the highest number of predicted immune interactions (n=113). This compound was withdrawn from the market in the 1980s due to its ability to trigger toxic epidermal necrolysis (Roujeau *et al.*, 1990; Garcia-Doval *et al.*, 2000), a severe cutaneous adverse drug reaction involving significant inflammatory and immune responses (Abe, 2015). This side effect and other types of skin toxicity also represented the driving factor that led to the withdrawal of oxyphenbutazone (Biron, 1986), and bufexamac (EMA, 2010), which are predicted to induce 50 and 95 distinct interactions with a variety of immune cell state changes, respectively. Other NSAIDs included in our analysis were also withdrawn from the market due to other major adverse effects, such as indoprofen (gastrointestinal bleeding) (Aronson, 2016), nimesulide (hepatotoxicity) (Donati *et al.*, 2016), rofecoxib and valdecoxib (cardiotoxicity) (Dieppe *et al.*, 2004; Nussmeier *et al.*, 2005). It is important to consider that these types of toxicity also represent the major drivers that led to regulatory action. However, this does not imply that these are the only types of toxicities detected, as immune-related adverse effects are also apparent. This consideration is supported by analysis of the data stored in the World Health Organisation (WHO) database for the global monitoring of Adverse Drug Reactions (ADRs) – VigiAccess™. For example, 2,935 and 682 Individual Case Safety Reports (ICSRs) of gastrointestinal disorders and hepatobiliary disorders, respectively, have been submitted for nimesulide in 110 countries. This data is not surprising as increased risk of liver toxicity was the trigger for its withdrawal from the market. However, the data indicate that 254 immune system disorders and 3,393 skin and subcutaneous tissue disorder ICSRs were also

submitted for this same compound. Moreover, cardiac disorder ICSRs represent 15% of all ICSRs submitted for rofecoxib (withdrawn due to increased risk of cardiotoxicity). Whereas the combination of immune-related ADRs (blood and lymphatic system disorders, immune system disorders, infections, and skin and subcutaneous tissue disorders) represent 8.4% of all submitted ICSRs for this same compound.

These examples highlight the possibility that the ability of NSAIDs to modulate the immune system may not be negligible and may explain the risk of developing certain types of side effects. Understanding and predicting the intrinsic immunomodulatory potential of drugs can therefore enhance drug safety assessment and, for approved drugs, inform clinical decision making and personalised medicine approaches. In recent years, several *in vitro* immunotoxicity methods have been developed and applied to preclinical safety testing, and some of them have been endorsed and proposed in the guidance document ICH S8 (Immunotoxicity Studies for Human Pharmaceuticals). Nonetheless, the complexity of the immune network is such that our ability to predict system-wide drug-induced effects using *in vitro* approaches remains limited. On the other hand, the implementation of tailored immunotoxicity endpoints during standard animal toxicity testing, when possible, is limited by the low-throughput nature of *in vivo* testing. These limitations suggest that the integration of *in silico* predictive approaches into the testing workflow may allow the characterisation of drug immunomodulatory potential prior to *in vivo* testing, leading to an overall improvement of the process. The methodology used by Kidd *et al.* (2016) is an excellent example of the huge potential for this type of approach.

2.6 Conclusions

Here we present multiple lines of evidence highlighting the potential diversity of biological targets and processes which may be perturbated by NSAIDs. Some of these key processes include, but are not limited to, steroid hormone signalling and several immunomodulatory pathways. Immunomodulation may well represent a key mode-of-action that underlies both safety and efficacy considerations for NSAIDs. Our data demonstrates the utility of existing mechanistic data for specific hypotheses generation and lays the foundations for the development of more sophisticated *in silico* analyses. Considering the fundamental importance of the 3R's concept throughout all areas of research, this work highlights the value of using predictive *in silico* analyses for the refinement of future *in vivo* study design.

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Chapter 3

Pharmacology-informed prediction of the risk posed to fish by mixtures of non-steroidal anti-inflammatory drugs (NSAIDs) in the environment

3.1 Abstract

The presence of non-steroidal anti-inflammatory drugs (NSAIDs) in the aquatic environment has raised concern that chronic exposure to these compounds may cause adverse effects in wild fish populations. This potential scenario has led some stakeholders to advocate a stricter regulation of NSAIDs, especially diclofenac. Considering their global clinical importance for the management of pain and inflammation, any regulation that may affect patient access to NSAIDs will have considerable implications for public health. The current environmental risk assessment of NSAIDs is driven by the results of a limited number of standard toxicity tests and does not take into account mechanistic and pharmacological considerations. Here we present a pharmacology-informed framework that enables the prediction of the risk posed to fish by 25 different NSAIDs and their dynamic mixtures. Using network pharmacology approaches, we demonstrated that these 25 NSAIDs display a significant mechanistic promiscuity that could enhance the risk of target-mediated mixture effects near environmentally relevant concentrations. Integrating NSAIDs pharmacokinetic and pharmacodynamic features, we provide highly specific predictions of the adverse phenotypes associated with exposure to NSAIDs, and we developed a visual multi-scale model to guide the interpretation of the toxicological relevance of any given set of NSAIDs exposure data. Our analysis demonstrated a non-negligible risk posed to fish by NSAID mixtures in situations of high drug use and low dilution of waste-water treatment plant effluents. We anticipate that this predictive framework will support the future regulatory environmental risk assessment of NSAIDs and increase the effectiveness of ecopharmacovigilance strategies. Moreover, it can facilitate the prediction of the toxicological risk posed by mixtures via the implementation of mechanistic considerations and could be readily extended to other classes of chemicals.

3.2 Introduction

Millions of people worldwide use non-steroidal anti-inflammatory drugs (NSAIDs) to treat a wide variety of health conditions involving inflammation and pain (Gunaydin and Bilge, 2018). One of the consequences of such widespread therapeutic use is that subsequent to excretion from the human body, NSAIDs and their metabolites enter the domestic waste-waters and can reach the aquatic environment where they are detected at low concentrations (Aus der Beek *et al.,* 2016; Lonappan *et al.,* 2016). Administration of NSAIDs to humans, especially when long-term, is associated with an increased risk of adverse events in multiple organs/systems, including gastrointestinal and cardiovascular systems (Conaghan, 2012; Fanelli *et al.,* 2017). These safety concerns led to various regulatory actions during the last twenty years in both North America and Europe, which required drug manufacturers to update product labels with explicit warnings that NSAIDs may increase the risk of serious adverse events (e.g. UK Medicines and Healthcare products Regulatory Agency, 2007; UK Medicines and Healthcare products Regulatory Agency, 2012; US Food and Drug Administration, 2015). In parallel with the clinical safety considerations, the presence of low, but sustained, concentrations of NSAIDs in the aquatic environment has raised the concern that chronic exposure to these compounds may also cause adverse effects in wild fish populations. In 2015, this concern triggered regulatory action and one specific NSAID, diclofenac, was included in the European Union (EU) Watch List of emerging pollutants under the European Water Framework Directive (European Commission, 2015). Diclofenac was subsequently removed from the Watch List in 2018 (European Commission, 2018) once a larger volume of high-quality monitoring data was gathered to allow a refined risk assessment. However, the regulatory and academic discussions concerning the environmental risk assessment (ERA) of NSAIDs continued and have reached the point that some stakeholders are advocating a stricter regulation of over-the-counter NSAIDs, such as diclofenac, and even the substitution with compounds associated with a lower environmental risk (OECD, 2019).

Considering the global clinical importance of NSAIDs for the management of pain and inflammation, any regulation that may affect patient access to NSAIDs will have considerable implications for public health. Thus, it is of paramount importance that all relevant scientific evidence, beyond the boundaries of ecotoxicology, is used to inform regulatory decision-making. The inclusion of diclofenac in the EU Watch List highlighted three potential limitations of the current risk assessment of NSAIDs. Firstly, from a toxicological perspective, the original decision to include diclofenac in the list was driven by a relatively small set of experimental data (e.g. Hoeger *et al.,* 2005; Mehinto *et al.,* 2010; Schwaiger *et al.,* 2004; Triebskorn *et al.,* 2004) concerning chronic effects in fish species, which were subsequently the object of scientific debate (Memmert *et al.,* 2013). The reasons underlying the debate were not related

to the widely accepted notion that diclofenac may trigger adverse effects in fish (hazard assessment), but rather to the degree of reproducibility of the experiments that characterised those effects, and to the range of environmental concentrations that may trigger them (risk assessment). Secondly, the current ERA of diclofenac (and any other pharmaceutical) does not incorporate mechanistic and mode-of-action considerations, limiting the potential to implement predictive toxicology approaches to support decision-making. Finally, more than 20 different NSAIDs are currently available on the market, and all of them exert their pharmacological effects by inhibiting one or both isoforms of the cyclooxygenase enzyme (COX-1 and COX-2). This pharmacological aspect implies that diclofenac may not be the only NSAID of concern, and that mixture effects might occur.

To overcome these challenges, we developed a novel pharmacology-informed framework that enables the prediction of the risk posed to fish by NSAIDs and their mixtures under realistic exposure scenarios. Our framework is centred on the integration of two mechanistic perspectives, network-centred and target-centred, and on the consideration of drug concentrations inside the organism (rather than in the surrounding water) as an essential parameter for the generation of accurate and realistic risk predictions. This research aims at providing a valuable tool that can facilitate the implementation of mechanistic considerations into the future regulatory environmental risk assessment of NSAIDs and ecopharmacovigilance strategies.

3.3 Methods

3.3.1 Compound identification

To identify the NSAIDs currently present on the market, we screened the database DrugBank (www.drugbank.ca; Wishart *et al.,* 2018) and selected all pharmaceuticals labelled as ”COX-inhibitor” or “NSAID”. The physico-chemical properties of each compound – including LogKow and LogD7.4 - were retrieved from the database ChemSpider (www.chemspider.com).

3.3.2 Prediction of blood concentrations of NSAIDs in wild fish

Measured surface water concentrations for each compound were retrieved from a database curated by the German Environment Agency (Umweltbundesamt – UBA) (https://www.umweltbundesamt.de/en/database-pharmaceuticals-in-the-environment-0). At the date of access (November 2019), the database contained environmental concentrations of human and veterinary pharmaceutical residues in 53 environmental matrices from 75 countries, extracted from 1519 publications, and 240 review articles (Eike *et al.,* 2019).

Measured concentrations in UK freshwaters were used as an example of environmentally realistic exposure scenario. Specifically, we used the highest average measured concentrations in treated wastewater treatment plant (WWTP) effluents and surface waters (i.e. freshwaters). These water concentrations were subsequently used to predict the concentration of each compound in the blood of wild fish by applying the Fish Plasma Model, as described by Margiotta-Casaluci *et al.,* 2014, Margiotta-Casaluci *et al.,* 2016 (Table 3.1).

3.3.3 Network-centred approach

The network-centred approach was driven by the hypothesis that NSAID-mediated adverse effects are induced through the perturbation of a network of drug targets (i.e. drug polypharmacology and bioactivity profile).

3.3.4 Extraction of drug-target interaction and *in vitro* bioactivity profiling data

In vitro bioactivity profiling data for 25 different NSAIDs was extracted from two sources: 1) the 'US Environmental Protection Agency (US EPA) Toxicity Forecaster (ToxCast) database (U.S. EPA. 2015. ToxCast and Tox21 Summary Files from invitrodb_v3.2. Retrieved from https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data between May 2019 and October 2019. Data released May 2018) (Williams *et al.,* 2017), and 2) the European Bioinformatics institute (EBI) ChEMBL database (http://www.ebi.ac.uk/chembl; Gaulton *et al.,* 2017). The data extracted from ToxCast included drug target identifier and drug concentration at 50% maximum activity (AC50). Data extraction was limited to the interactions labelled as 'active', hence, those labelled as 'inactive' were excluded from the analysis. On the other hand, the data extracted from ChEMBL included drug target identifier and half-maximum inhibitory concentrations (IC50). Similarly, in this case, data extraction was limited to the interactions labelled as 'active', whereas those labelled as 'not active' or 'not determined' were excluded from the analysis. The bioactivity profiling data used in this study was uploaded onto the Brunel Data Repository System (FigShare), and can be retrieved from the following address: https://figshare.com/s/acb737422927d3416c70.

3.3.5 Data harmonisation and processing

The data extracted from ToxCast and ChEMBL were manually harmonised to ensure inter-database comparability and maximise data usability. AC50 and IC50 values were converted to, and uniformly expressed as, ng/mL. When data from multiple species were available, human data was used as the first choice; if unavailable, rodent data was used instead. When

multiple datapoints were available for the same target, the lowest AC50 (or IC50) value was selected for the final analysis. ToxCast and ChEMBL use different target annotation strategies, hence all drug target identifiers were converted into human gene symbols to ensure target specificity and allow dataset merging. The gene symbol nomenclature was harmonised using GeneCards as the reference source (GeneCards.org; Stelzer *et al.,* 2016). The harmonised datasets from ToxCast and ChEMBL were finally combined to assess the database-specific bioactivity coverage (i.e. degree of overlap between ToxCast and ChEMBL interactions), after which duplicate interactions were removed. As before, the lowest AC50 (or IC50) value for each target was retained for the final analysis. This process led to the generation of combined ToxCast/ChEMBL drug-target interaction profiles, which were used in the subsequent network analyses (https://figshare.com/s/acb737422927d3416c70).

3.3.6 Generation of hazard-based and risk-based drug-target interaction networks

Drug-target interaction networks were generated using the Cytoscape software (Shannon *et al.,* 2003). The initial network included all the drug-target interactions present in our database, irrespective of any effect concentration data. For this reason, this network represented a hazard bioactivity network, which was used as the point of departure for the subsequent analyses. To determine the meaningfulness of the network under realistic exposure scenarios, each drug-target interaction node was filtered using the drug concentrations predicted to be present in the blood of wild fish in the UK (i.e. using the highest average measured concentrations in treated WWTP effluents). Using this approach, the refined network contained only those interactions that occur at concentrations equal to, or lower than, the exposure levels of interest. To evaluate the impact of integrating exposure data within the network, we simulated a realistic exposure scenario: the highest average measured concentrations in UK treated WWTP effluents.

3.3.7 Target-to-phenotype analysis

To predict the phenotypic meaning of the risk-based drug-target interaction network (i.e. the one occurring at a realistic exposure scenario only), we first identified the gene involved in each interaction (e.g. cyclooxygenase 1 inhibition → PTGS1 gene), and subsequently we performed a gene-phenotype anchoring analysis using the Monarch Initiative platform (www.monarchinitiative.org). The latter is a “collaborative, open science effort that aims to semantically integrate genotype–phenotype data from many species and sources in order to support precision medicine, disease modelling, and mechanistic exploration” (Mungall *et al.,* 2017). Using Monarch, we extracted all the available zebrafish-specific phenotypic data

associated with alterations of the target genes (e.g. mutations, variants, and artificial alterations such as knock out or knock down). This analysis generated an array of phenotypes that might potentially occur in wild fish under the considered exposure scenario (i.e. highest average measured concentrations in treated WWTP effluents in the UK).

3.3.8 Target-centred approach

The target-centred approach was driven by the hypothesis that NSAID-mediated adverse effects are induced by the inhibition of COX-1 and COX-2, which are the primary targets of NSAIDs.

3.3.9 Literature review and data extraction

To identify all the relevant effects caused by NSAIDs in fish, we performed a literature review to identify relevant medium-to-long term *in vivo* freshwater ecotoxicity studies (four days or longer). The literature search was conducted via PubMed and Google Scholar using a combination of keywords (e.g. drug name, endpoint name, species, and toxicity) and was restricted to English language publications only. Statistically significant effect data was extracted from each paper. Whenever available, we also extracted the average value for each parameter and the relative uncertainty measure (e.g. standard deviation) to calculate the effect size reported in each study. For the studies that reported multiple concentration and/or time responses, each dose and/or time point was considered as an independent data point in the database. Other extracted information included exposure concentrations, duration of exposure, fish species, life stage, and effect direction (increase or decrease). A quality assessment of all extracted data and relative database was performed by two different operators to evaluate the consistency between extracted data and original values. Considering the highly variable vocabulary used in different papers (e.g. same endpoint defined using different terms), we carried out a harmonisation process to ensure data comparability.

3.3.10 Prediction of internal effect concentrations and equivalence calculation

To account for the different uptake profile of each drug, we transformed water exposure concentrations for all the identified drug-effect combinations into predicted effect plasma concentrations using the Fish Plasma Model (FPM) as described by Margiotta-Casaluci *et al.* (2014), and Margiotta-Casaluci *et al.* (2016). Considering the hypothesis that NSAID-induced effects are mediated by COX-1 and COX-2 inhibition, and that all NSAIDs act via inhibiting COX-1 and/or COX-2, we expressed each drug plasma concentration as equivalent to a

reference NSAID (diclofenac). To do so, we considered the COX-1 inhibition IC50 of diclofenac as the reference value (=1); successively, we calculated a "diclofenac-equivalence conversion factor" for every other NSAID using the formula "COX-1-inhibition IC50(diclofenac) / COX-1-inhibiton IC50(other NSAID)". The resulting conversion factor was used to express all NSAIDs plasma concentrations as "diclofenac-equivalent plasma concentrations". The focus on COX-1 rather than COX-2 was justified by two observations: a) all NSAIDs tested *in vivo* were dual COX inhibitors; b) in human pharmacology, COX-1 inhibition is generally considered to be the main driver of NSAIDs-mediated side effects, as COX-2 is generally expressed at low levels and is induced when the organism is experiencing an inflammation (Rouzer and Marnett, 2009).

3.3.11 Generation of a multi-scale COX-1-centred model to predict the risk of *in vivo* chronic effects

The data described above was integrated to generate a multi-scale model displaying the range of NSAID plasma concentrations (expressed as diclofenac-equivalents calculated using "diclofenac human COX-1 IC50" as the reference value), associated with mode-of-action-relevant adverse effects, under medium/long-term exposure scenarios. To facilitate the interpretation of the model and its relevance for the ERA process, we incorporated three threshold levels. Two of these thresholds represent the concentration of the NSAIDs mixture predicted to occur in the plasma of wild fish in a) UK WWTP effluents, and b) UK surface waters. The third threshold level represents the range of predicted NSAIDs plasma concentrations that are likely to induce mortality.

3.4 Results

3.4.1 NSAIDs selection and environmental occurrence

25 NSAIDs were identified in the DrugBank database: amfenac, aspirin, carprofen, celecoxib, diclofenac, etodolac, etoricoxib, flufenamic acid, flurbiprofen, ibuprofen, indomethacin, ketoprofen, ketorolac, mefenamic acid, meloxicam, naproxen, niflumic acid, nimesulide, oxaprozin, piroxicam, rofecoxib, sulindac, tenoxicam, tolfenamic acid, and valdecoxib. Four of these compounds were classified as COX-2 selective inhibitors (celecoxib, etoricoxib, rofecoxib, and valdecoxib), whereas 21 compounds were classified as non-selective COX inhibitors. According to the UBA database of pharmaceuticals in the environment, 18 out of 25 NSAIDs were detected in the aquatic environment, in 66 different countries around the world. This supports our hypothesis that the overall environmental risk of NSAIDs should be

addressed from a mixture perspective. This data mining exercise revealed a wide range of concentrations detected worldwide in surface waters and wastewater treatment plant (WWTP) effluents (Figure 3.1); however, when multiple measurements for the same compounds were available, the observed median value was generally below 1 µg/L. Carrying out a detailed analysis of the environmental levels of NSAIDs is beyond the scope of the present work. Hence, for the next steps of our analysis we only considered the concentrations of NSAIDs measured in UK surface waters and WWTP effluents as the default exposure scenario for our toxicity predictions (Table 3.1). Specifically, seven out of 25 NSAIDs were detected in UK WWTP effluents, and six in surface waters. These numbers are in line with the number of NSAIDs detected in other countries characterised by intensive environmental monitoring activity (e.g. Canada, USA, Germany, Sweden, Japan). It is important to note that the exposure concentrations were selected to represent a worst-case scenario in the UK. For example, based on the data generated from two large UK-wide wastewater treatment plant monitoring programmes, Comber *et al.* (2018) estimated a diclofenac median effluent concentration equal to 0.33 µg/L, whereas the 95th percentile is 0.5 µg/L. As a term of comparison, the effluent concentration of diclofenac used in our simulation was 0.42 µg/L, indicating a good degree of agreement with other worst-case scenarios estimated in other studies.

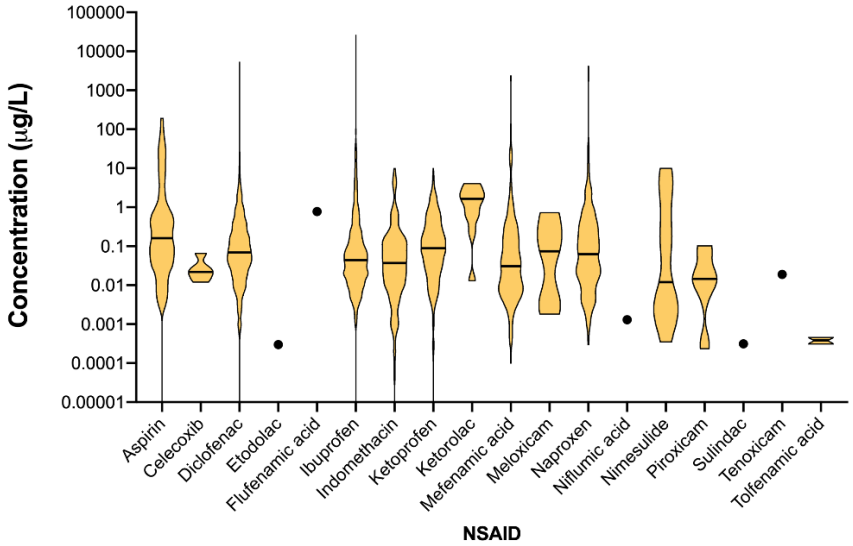

Figure 3.1. Range of measured concentrations (µg/L) in surface waters and wastewater treatment plant (WWTP) effluents from 66 countries. *Data was retrieved from the German Environmental Agency (UBA) database "Pharmaceuticals in the Environment" (aus der Beek et al., 2015). The line within each violin plot represents the median value. Only one concentration value was available for etodolac, flufenamic acid, niflumic acid, sulindac, tenoxicam.*

Table 3.1. *Average measured environmental concentrations of NSAIDs in the UK and corresponding predicted plasma concentrations in wild fish. This specific exposure scenario was used to generate the toxicological predictions described in the present study. The data was retrieved from the German Environmental Agency (UBA) database "Pharmaceuticals in the Environment" (aus der Beek et al., 2015).*

Drug	Environmental concentration 1 (UK, µg/L)	Environmental matrix 1	Predicted fish plasma concentration 1 (UK, ng/mL)*,**	Environmental concentration 2 (UK, µg/L)	Environmental matrix 2	Predicted fish plasma concentration 2 (UK, ng/mL)*,**
Aspirin	0.0064	Surface waters	0.01	0.0235	WWTP (treated) effluent	0.02
Carprofen	Not detected	/	0	Not detected	/	0
Celecoxib	Not detected	/	0	Not detected	/	0
Diclofenac	0.04	Surface waters	4.71	0.42	WWTP (treated) effluent	50.71
Etodolac	Not detected	/	0	Not detected	/	0
Etoricoxib	Not detected	/	0	Not detected	/	0
Flufenamic acid	Not detected	/	0	Not detected	/	0
Flurbiprofen	Not detected	/	0	Not detected	/	0
Ibuprofen	0.03	Surface waters	1.76	0.94	WWTP (treated) effluent	64.36
Indomethacin	0.009	Surface waters	0.22	0.02	WWTP (treated) effluent	0.47
Ketoprofen	0.006	Surface waters	0.09	0.017	WWTP (treated) effluent	0.25
Ketorolac	Not detected	/	0	Not detected	/	0
Mefenamic acid	0.007	Surface waters	7.18	0.05	WWTP (treated) effluent	51.27
Meloxicam	Not detected	/	0	Not detected	/	0
Naproxen	0.047	Surface waters	0.95	1.23	WWTP (treated) effluent	25.07
Niflumic acid	Not detected	/	0	Not detected	/	0
Nimesulide	Not detected	/	0	Not detected	/	0
Oxaprozin	Not detected	/	0	Not detected	/	0
Piroxicam	Not detected	/	0	Not detected	/	0
Rofecoxib	Not detected	/	0	Not detected	/	0
Sulindac	Not detected	/	0	Not detected	/	0
Tenoxicam	Not detected	/	0	Not detected	/	0
Tolfenamic acid	Not detected	/	0	Not detected	/	0
Valdecoxib	Not detected	/	0	Not detected	/	0

3.4.2 Analysis of the primary pharmacological activity of NSAIDs

The inhibition of COXs is the primary mechanism of action of NSAIDs. The analysis of COXs-inhibitory activity of the 25 compounds revealed a wide range of pharmacological potencies (Figure 3.2). IC50 values for COX-1 inhibition ranged from 2 to 3 nM (indomethacin, ketoprofen, and diclofenac) to over 25,000 nM (valdecoxib) (Figure 3.2.a). Similarly, IC50 values for COX-2 inhibition ranged from 1 to 2 nM (rofecoxib, and celecoxib) to over 89,000 nM (piroxicam) (Figure 3.2.b). The analysis of the ratio between COX-1 and COX-2 inhibition IC50s revealed the selectivity of each compound for the two isoforms of the enzyme (Figure 3.2.c). Unsurprisingly, COX-2 selective inhibitors such as rofecoxib, valdecoxib, and etoricoxib displayed the highest selectivity for COX-2. These compounds have been specifically developed to display such a pharmacological feature. However, non-selective NSAIDs - such as carprofen, flufenamic acid, nimesulide, and meloxicam – also showed considerable COX-2 selectivity. Piroxicam was the NSAID with the highest COX-1 selectivity, followed by naproxen and ketoprofen (Figure 3.2.c).

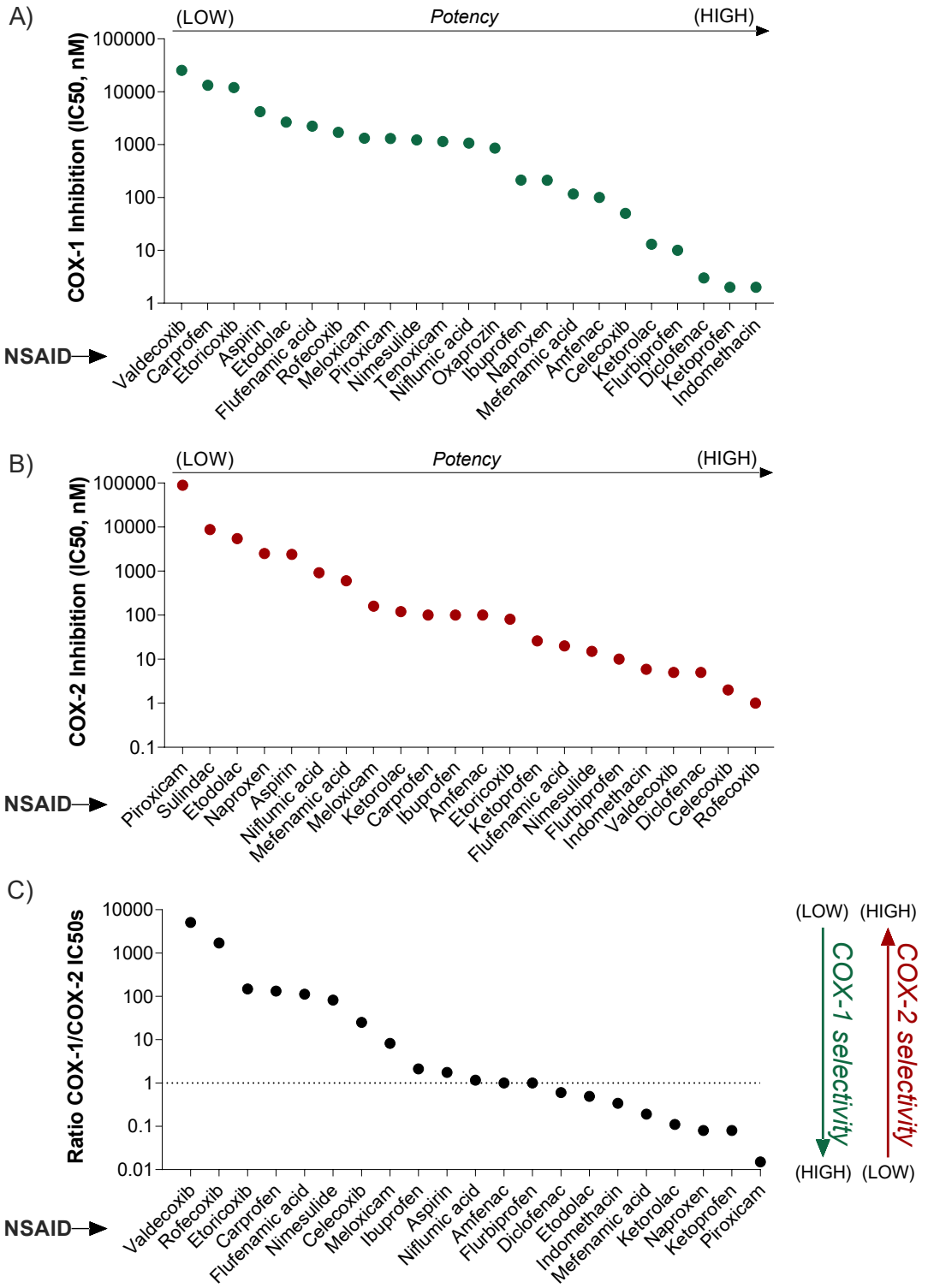

Figure 3.2. Pharmacological activity of NSAIDs on the primary targets COX-1 and COX-2*. A) Lowest COX-1 inhibition IC50 values retrieved from ToxCast/ChEMBL. B) Lowest COX-2 inhibition IC50 values retrieved from ToxCast/ChEMBL. C) Ratio of COX-1/COX-2 IC50 values, indicating the selectivity of each compound towards either COX-1 or COX-2.*

The AC50 and IC50 values used in this study were retrieved with the explicit intention to simulate a worst-case scenario (i.e. when multiple values were available, the lowest value was selected for the final analysis). However, it is important to consider that the inter-experiment variability in IC50 values can be considerable. To assess such variability, we compared the ToxCast/ChEMBL data used in our model with two additional IC50 values retrieved from the literature (Figure 3.3). The comparative analysis of COX-1 IC50s confirmed that our data were at the bottom of the variability range, except for flurbiprofen. The analysis of COX-2 IC50 values revealed a less consistent scenario, where ToxCast/ChEMBL values were at the bottom of the variability range in only seven out of 25 cases. In some cases, the gap between the ToxCast/ChEMBL values and the literature values was considerable (e.g. rofecoxib COX-2 IC50s: 1, 340, 510 nM). Notably, we also observed a surprising variability between the two alternative IC50 values retrieved from Rainsford (2004). It is important to note that those values were generated using different test systems. A similar degree of variability was also observed in the COX-1/COX-2 IC50 ratios, influencing the interpretation of the selectivity of the compound for either COX-1 or COX-2.

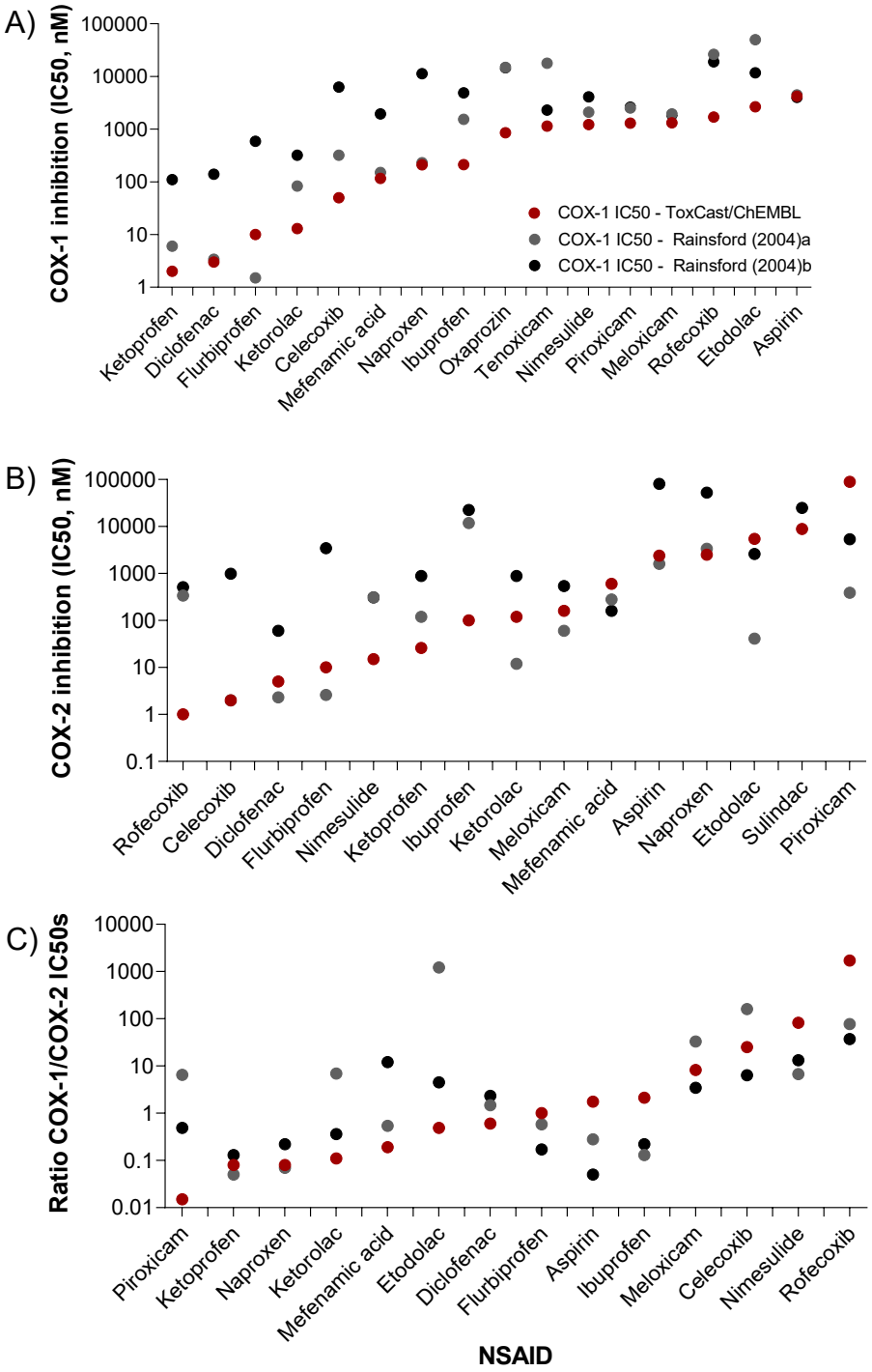

Figure 3.3. Analysis of the variability of COX IC50 values retrieved from multiple sources (ToxCast/ChEMBL and Rainsford 2004). *Note that the AC50 or IC50 values used in the present analysis were selected to be the lowest available in the database, in accordance with the precautionary principle. The three panels display the variability in A) COX-1 inhibition IC50s; B) COX-2 inhibition IC50s; C) COX-1/COX-2 inhibition ratio. The IC50 values labelled as "Rainsford (2004)a" were generated using human recombinant enzymes, whereas those labelled as "Rainsford (2004)b" were generated using whole human blood.*

3.4.3 Hazard-based bioactivity networks of NSAIDs mixtures

Understanding the secondary mechanisms of action of drugs can significantly enhance the prediction of their toxicity profile. To explore the mechanisms of action of NSAIDs beyond COX inhibition, we leveraged the ToxCast and ChEMBL platforms to generate a bioactivity network for the mixture of 25 NSAIDs (Figure 3.4). The combination of the ToxCast and ChEMBL databases was aimed at expanding the biological space covered in our analysis. To evaluate this aspect, we analysed the gain in biological space due to the merging exercise (Figure 3.5). The analysis revealed that the degree of overlap between ToxCast and ChEMBL data was minimal (i.e. zero shared interactions for 21 NSAIDs out of 25). The combination of the two data sources allowed us to expand the biological space, while increasing the relevance of the pharmacological network.

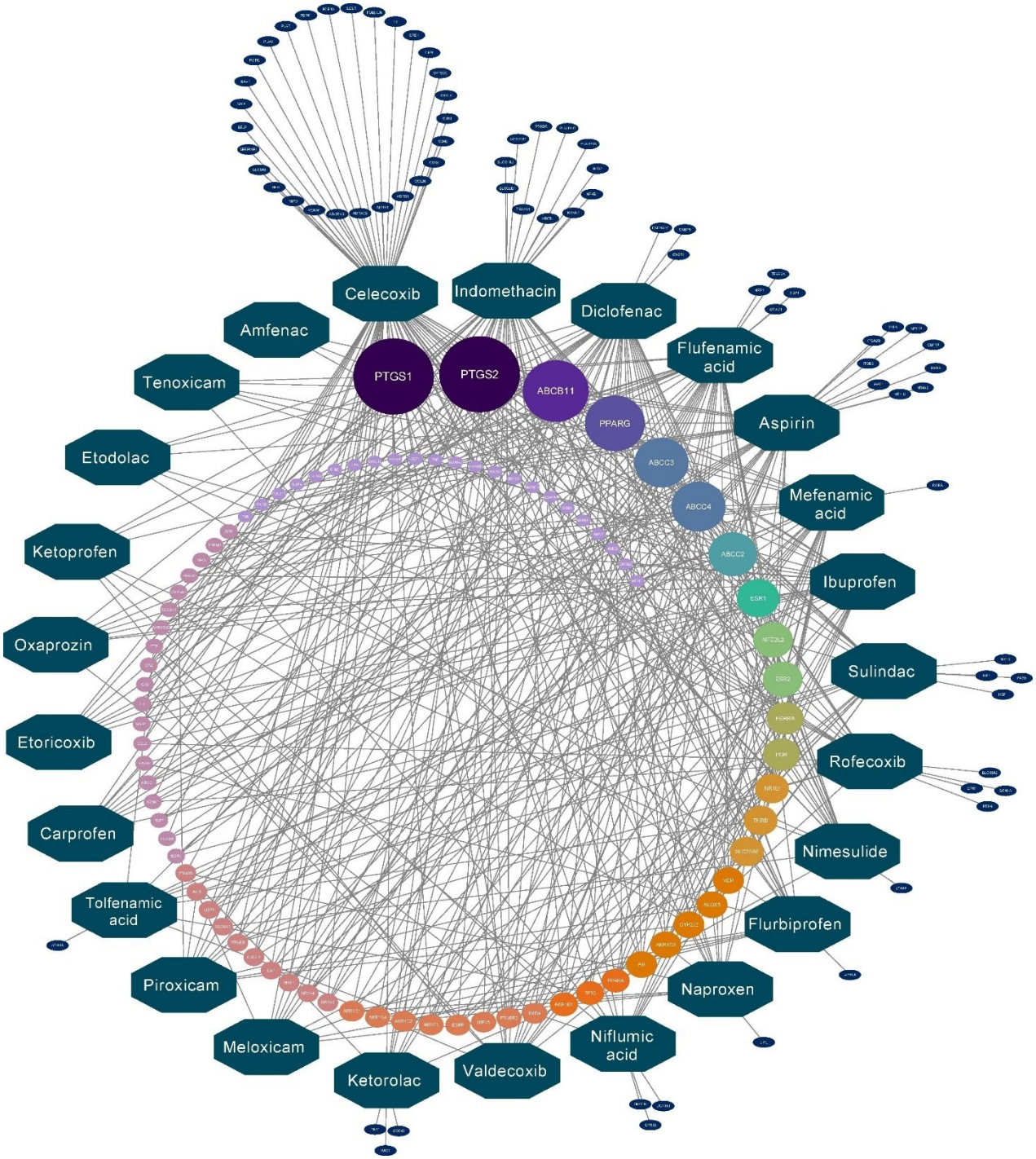

Figure 3.4. Drug-target interaction network for a mixture of 25 NSAIDs. *The green octagons indicate the single drugs. The nodes on the external layer indicate the drug-target interactions that are unique for each compound. The nodes in the inner area indicate the drug-target interactions shared by at least two different NSAIDs. The larger the size of the inner nodes, the higher the number of NSAIDs that interact with that target. The different colours of the inner nodes indicate a different number of NSAIDs sharing the target. Each drug-target relationship is associated to a specific AC50 (or IC50) value, retrieved from a combined ToxCast/ChEMBL database. No exposure data is incorporated into this model (e.g. some AC50 or IC50 values represent unrealistic exposure levels); hence, this network can be considered as a hazard-based bioactivity network. (PTGS1 = COX-1, and PTGS2 = COX-2).*

The mechanistic analysis revealed that the 25 NSAIDs have a wide range of mechanisms of action beyond COXs inhibition. The number of recorded interactions ranged from 3 to 74 (Figure 3.4). The compound with the highest number of interactions was celecoxib (n = 74), followed by indomethacin (n = 47), and diclofenac (n = 40). On the other hand, the compounds with the lowest number of recorded interactions were etodolac (n = 6), tenoxicam (n = 6), and amfenac (n = 3). In total, the mixture of 25 NSAIDs was associated with 507 interactions, involving 157 distinct targets; 83 of these targets were shared by at least two NSAIDs, whereas 74 targets were modulated only by individual drugs. PTGS1 and PTGS2 (corresponding to COX-1 and COX-2) were the targets with the highest levels of promiscuity, and were shared by 23 and 22 NSAIDs, respectively. Notably, both ToxCast and ChEMBL did not contain any information concerning the COXs inhibitory activity for two NSAIDs, sulindac and tolfenamic acid, despite the known COX-inhibitory activity of these compounds. After PTGS1 and PTGS2, the targets with the highest levels of promiscuity were the bile salt export pump (ABCB11; shared by 19 NSAIDs) and the peroxisome proliferator-activated receptor gamma (PPARγ; shared by 17 NSAIDs). Other targets shared by 10 or more NSAIDs were the transporters ABCC4 (n = 15), ABCC3 (n = 15) and ABCC2 (n = 13), the estrogen receptors ESR1 (n = 11) and ESR2 (n = 10), and the nuclear factor erythroid 2-related factor 2 (NFE2L2, n = 10). A detailed list of interactions for each target and for each drug is available in the data file located at: https://figshare.com/s/acb737422927d3416c70, whilst the full drug-target interaction network is represented in Figure 3.4. Of the 25 NSAIDs, 14 interacted with unique targets that were not shared by any other compound. The drug with the highest number of unique interactions was celecoxib (n = 28), followed by indomethacin (n = 11), and aspirin (n = 9) (Figure 3.4). It is important to note that the bioactivity network described above does not include any information about the concentration of the drug needed to modulate each target, hence it should be considered as a hazard network.

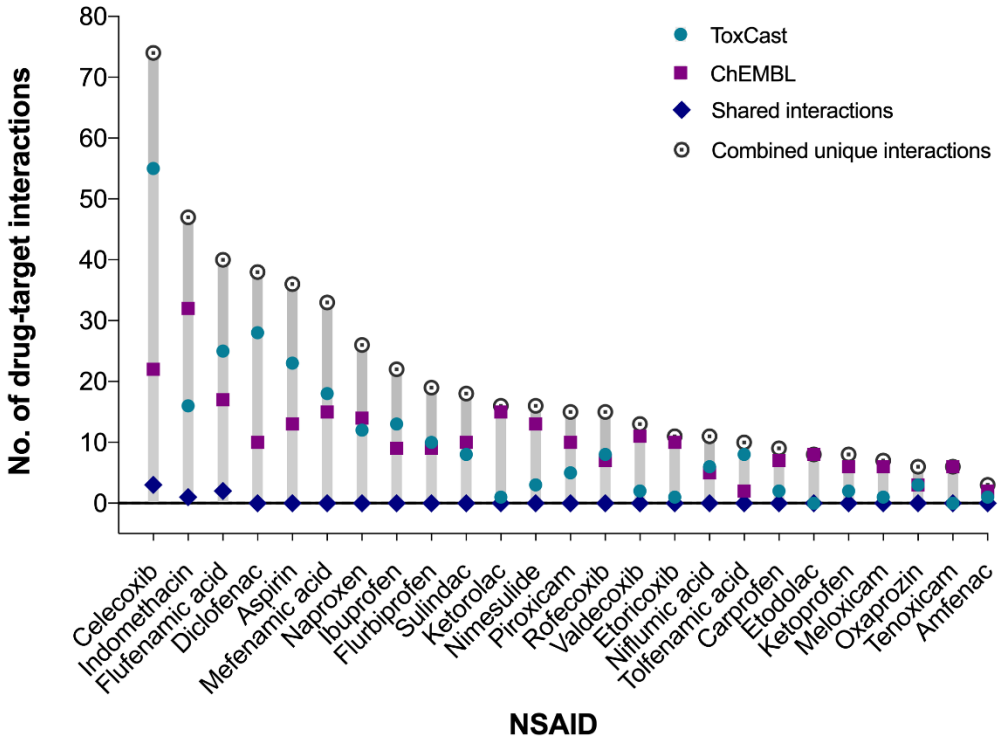

Figure 3.5. Effect of ToxCast and ChEMBL data integration on the biological space covered by the bioactivity network. *Note the minimal overlap between ToxCast and ChEMBL datasets (i.e. blue diamonds close, or equal to, zero).*

3.4.4 Risk-based bioactivity networks of NSAIDs mixtures

To interpret the environmental relevance of the hazard bioactivity network, we filtered each drug-target interaction using the concentrations of NSAIDs predicted to be present in the blood of wild fish in the UK. The targets remaining in the network are only those with drug-specific AC50 values below these predicted blood concentrations of NSAIDs. Hence, the resulting network displays only the drug-target interactions predicted to occur at the defined exposure scenario (i.e. highest average measured concentrations in UK WWTP treated effluents) (Figure 3.6) and can be considered as a risk-based network. In the specific example used here, the refined network suggests that only eight targets are likely to be modulated in wild fish exposed to those effluent concentrations: C-C motif chemokine 2 (CCL2), interleukin-8 (CXCL8), C-X-C chemokine receptor type 1 (CXCR1), estrogen receptor 1 (ESR1), progesterone receptor (PGR), interstitial collagenase (MMP1), prostaglandin G/H synthase 1 (PTGS1), and prostaglandin G/H synthase 2 (PTGS2) (note that the latter two targets correspond to COX-1 and COX-2). Three out of eight targets are shared by multiple NSAIDs, whereas the other five targets are only modulated by single drugs. To identify the drivers of the risk within the interaction network, we calculated the ratio between predicted blood

concentrations and the AC50 (or IC50) values associated with each drug-target interaction. The analysis showed that the targets with the highest risk are the two steroid receptors PGR and ESR1, as blood concentrations of naproxen and diclofenac were predicted to be 15,375-fold and 321-fold higher than the drug-specific AC50 values. These high values were driven by the low ToxCast AC50s reported for naproxen-induced PGR modulation and diclofenac-induced ESR1 modulation, which were 0.007 nM and 0.5 nM, respectively. The data for PGR was generated employing a GAL4 β-lactamase reporter gene technology using PR-UAS-bla HEK 293 T cells, whereas the data for ESR1 was generated with a luciferase-coupled ATP quantitation technology using human breast tissue cells. Diclofenac was also the driver of the risk for modulation of PTGS1 (ratio = 57), PTGS2 (ratio = 34), CXCL8 (ratio = 21), and CXCR1 (ratio = 14).

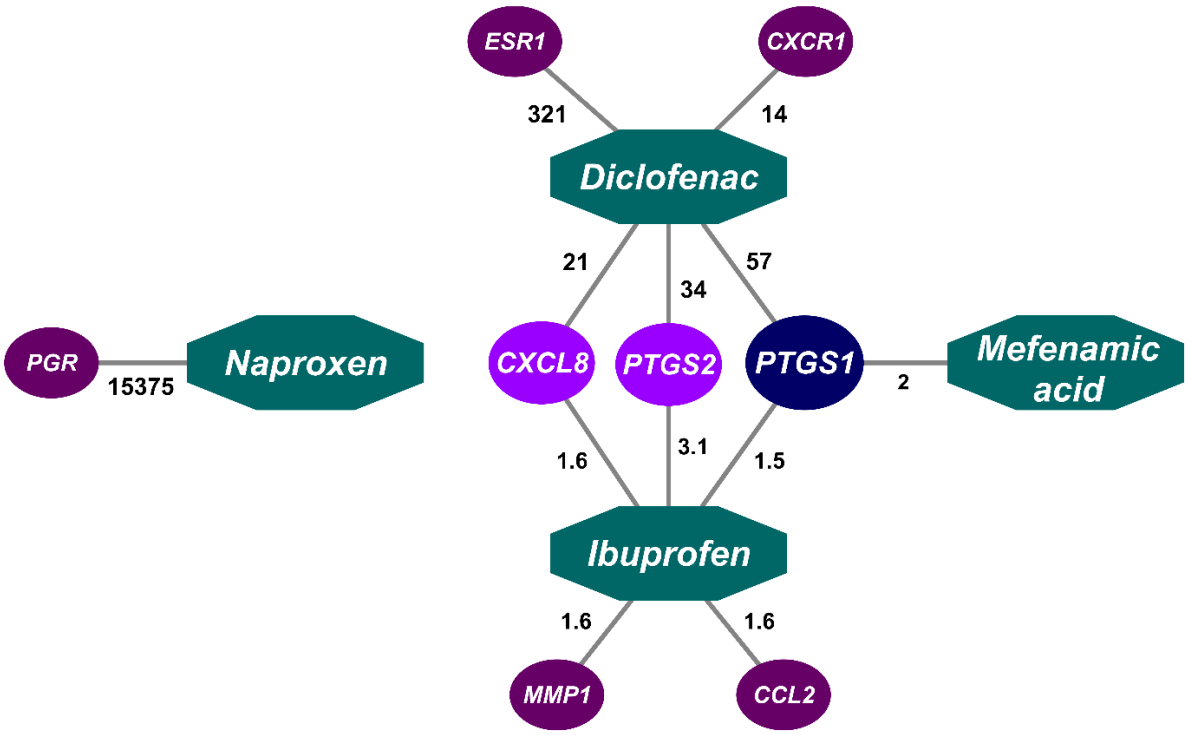

***Figure 3.6. Drug-target interaction network, for a mixture of 25 NSAIDs, predicted to occur at a worst-case exposure scenario (UK highest average measured concentration in wastewater treatment plant effluents)**. The green octagons indicate the single drugs. Drug targets are represented by color-coded nodes. Each colour indicates the different number of drugs that act on the associated target (PTGS1: 3 drugs, PTGS2: 2 drugs, CXCL8: 2 drugs, CCL2: 1 drug, CXCR1: 1 drug, ESR1: 1 drug, MMP1: 1 drug, PGR: 1 drug). The numbers indicated next to each drug-target connection represent the ratio between the ToxCast/ChEMBL AC50 (or IC50) value and the drug concentration predicted to be present in the blood of wild fish in the UK, under the considered exposure scenario. For example, the concentration of diclofenac present in the blood of wild fish is predicted to be 57-times above the considered IC50 for PTGS1. Abbreviations: CCL2: C-C Motif Chemokine Ligand 2; CXCL8:*

C-X-C Motif Chemokine Ligand 8; CXCR1: C-X-C Motif Chemokine Receptor 1; ESR1: Estrogen Receptor 1; MMP1: Matrix Metallopeptidase 1; PGR: Progesterone Receptor; PTGS1: Prostaglandin-Endoperoxide Synthase 1; PTGS2: Prostaglandin-Endoperoxide Synthase 2.

3.4.5 Phenotypic anchoring of the risk-based bioactivity network

To elucidate the phenotypic relevance of the targets displayed in the risk-based network (Figure 3.6), we performed a gene-phenotype association analysis by data-mining available databases. The analysis generated a list of highly specific zebrafish phenotypes that may be observed following perturbation of the eight targets of interest (Table 3.2). These phenotypes indicate that the risk-based NSAIDs bioactivity network may lead to profound effects on the cardiovascular and immune systems, the liver, pancreas, kidneys, growth, reproduction, and general development. It is important to note that the effects on development, growth, and reproduction have high regulatory relevance as they are considered as apical endpoints that, in turn, may perturbate population dynamics. From a risk-assessment perspective, this analysis cannot provide quantitative indications on the likelihood that each phenotype may occur. However, it provides a highly granular prediction of the endpoints that could be used for a potential experimental assessment of the case-specific risk.

***Table 3.2**. Zebrafish-specific phenotypes associated with the perturbation of the NSAIDs-targets predicted to be modulated at environmentally relevant exposure scenarios (i.e. UK). (Targets: CXCL8, CXCR1, ESR1, MMP1, PGR, PTGS1, PTGS2 (no data available for CCL2)).*

Target	Function/system/organ	Phenotype	Phenotype ID
PTGS1	Development	Abnormal otolith in otic vesicle	ZP_0003813
ESR1	Development	Altered sex ratio	ZP_0103077
MMP1	Development	Curled notochord	ZP_0005644
PTGS1, PTGS2	Development	Disrupted cilium development	ZP_0018462
PTGS1	Development	Disrupted gastrulation	ZP_0000567
ESR1	Development	Disrupted neuromast development	ZP_0001566
PTGS1	Development	Disrupted skeletal muscle plasticity	ZP_0100172
PTGS1, PTGS2	Development	Hydrocephalus	ZP_0018285
MMP1	Development	Hyperplastic epithelium	ZP_0005645
CXCL8	Development	Increased progenitor cells	ZP_0022176
MMP1	Development	Kinked post-vent region	ZP_0001145
MMP1	Development	Malformed caudal fin actinotrichia	ZP_0005646
MMP1, PTGS1	Development	Ventrally curved trunk	ZP_0000636
MMP1	Development	Yolk sac oedema	ZP_0002060
PTGS1	Cardiovascular system	Abnormal heart symmetry	ZP_0002925
MMP1	Cardiovascular system	Decreased blood flow	ZP_0003573
PTGS1, PTGS2	Cardiovascular system	Decreased hematopoietic stem cells	ZP_0000022
PTGS1	Cardiovascular system	Disrupted heart looping	ZP_0002506
CXCL8	Cardiovascular system	Disrupted vasculogenesis	GO_0001570
CXCL8	Cardiovascular system	Increased hematopoietic stem cells	ZP_0021393
MMP1	Cardiovascular system	Pericardial oedema	ZP_0000038
PTGS1	Reproduction	Decreased egg viability	ZP_0000212
ESR1	Reproduction	Decreased testis size	ZP_0019448
PGR	Reproduction	Disrupted ovulation	ZP_0017606
PGR	Reproduction	Disrupted reproduction	ZP_0017607
PGR	Reproduction	Increased ovary size	ZP_0019913
PGR	Reproduction	Sterile female	ZP_0004113
CXCL8, CXCLR1	Immune system	Abnormal leukocyte migration	GO_0002523
MMP1	Immune system	Abnormal macrophage chemotaxis	GO_0048246
CXCL8, CXCLR1	Immune system	Abnormal response to bacteria	GO_0009617
CXCL8, CXCLR1	Immune system	Abnormal response to wounding	GO_0009611
CXCR1	Immune system	Decreased neutrophil number	ZP_0011617
MMP1	Growth	Decreased trunk size	ZP_0000027
PGR	Growth	Increased trunk size	ZP_0014050
PGR	Growth	Increased weight	ZP_0015745
PTGS1	Liver	Abnormal liver	ZP_0018785
PTGS1, PTGS2	Liver	Decreased liver size	ZP_0000720
PTGS1, PTGS2	Pancreas	Decreased exocrine pancreas size	ZP_0002701
PTGS1	Kidney	Abnormal pronephric distal late tubule	ZP_0019006

3.4.6 Multi-scale COX-1-centred model to predict the risk of *in vivo* chronic effects

The gene-phenotype association analysis, described above, provides a solely qualitative result. To overcome this challenge and provide a quantitative estimation of the toxicological risk, we generated a multi-scale model portraying the range of blood concentrations of NSAIDs (expressed as diclofenac-equivalents, ng/mL - calculated using "diclofenac human COX-1 IC50" as the reference value) associated with statistically significant adverse phenotypes; under conditions of medium-to-long term exposure (longer than four days) (Figure 3.7). The model was based on 151 data points generated in 26 *in vivo* studies, carried out using 10 different fish species (Bhandari and Venables, 2011; Bickley *et al.,* 2017; Collard *et al.,* 2013; Flippin *et al.,* 2007; Ghelfi *et al.,* 2016; Gröner *et al.,* 2017; Han *et al.,* 2010; Hoeger *et al.,* 2005; Ji *et al.,* 2013; Lister and Van Der Kraak, 2008; Mathias *et al.,* 2018; Mehinto *et al.,* 2010; Memmert *et al.,* 2013; Morthorst *et al.,* 2013; Morthorst *et al.,* 2018; Näslund *et al.,* 2017; Pandey *et al.,* 2017; Patel *et al.,* 2016; Praskova *et al.,* 2014; Ribas *et al.,* 2017; Saravanan *et al.,* 2012; Schwaiger *et al.,* 2004; Stancova *et al.,* 2015; Triebskorn *et al.,* 2004; Yokota *et al.,* 2016; and Yokota *et al.,* 2017). The data included nine different types of *in vivo* effect, at various level of biological organisation, such as: prostaglandin levels, male and female testosterone, immunomodulation, liver damage, gill damage, kidney damage, reproduction, and growth. To facilitate the interpretation of the data, we incorporated three different reference concentrations (threshold levels) into the model: 1) The lethal range of blood concentrations starting at 388,105 ng/mL diclofenac-equivalents; 2) the predicted plasma levels of the mixture of seven NSAIDs, detected in UK WWTP effluents, corresponding to 54 ng/mL diclofenac-equivalents; 3) the plasma levels of the mixture of seven NSAIDs, detected in UK surface waters (i.e. freshwaters), corresponding to 5.2 ng/mL diclofenac-equivalents. A total of 46 out of 152 effect data points corresponded to plasma concentrations lower than 54 ng/mL diclofenac-equivalents (exposure scenario considering UK WWTP effluents); whereas only 14 out of 152 data points corresponded to plasma concentrations lower than 5.2 ng/mL diclofenac-equivalents (exposure scenario considering UK surface waters). The conversion factors used to convert all relevant NSAIDs into diclofenac-equivalents are provided in the file located at the following address: https://figshare.com/s/acb737422927d3416c70.

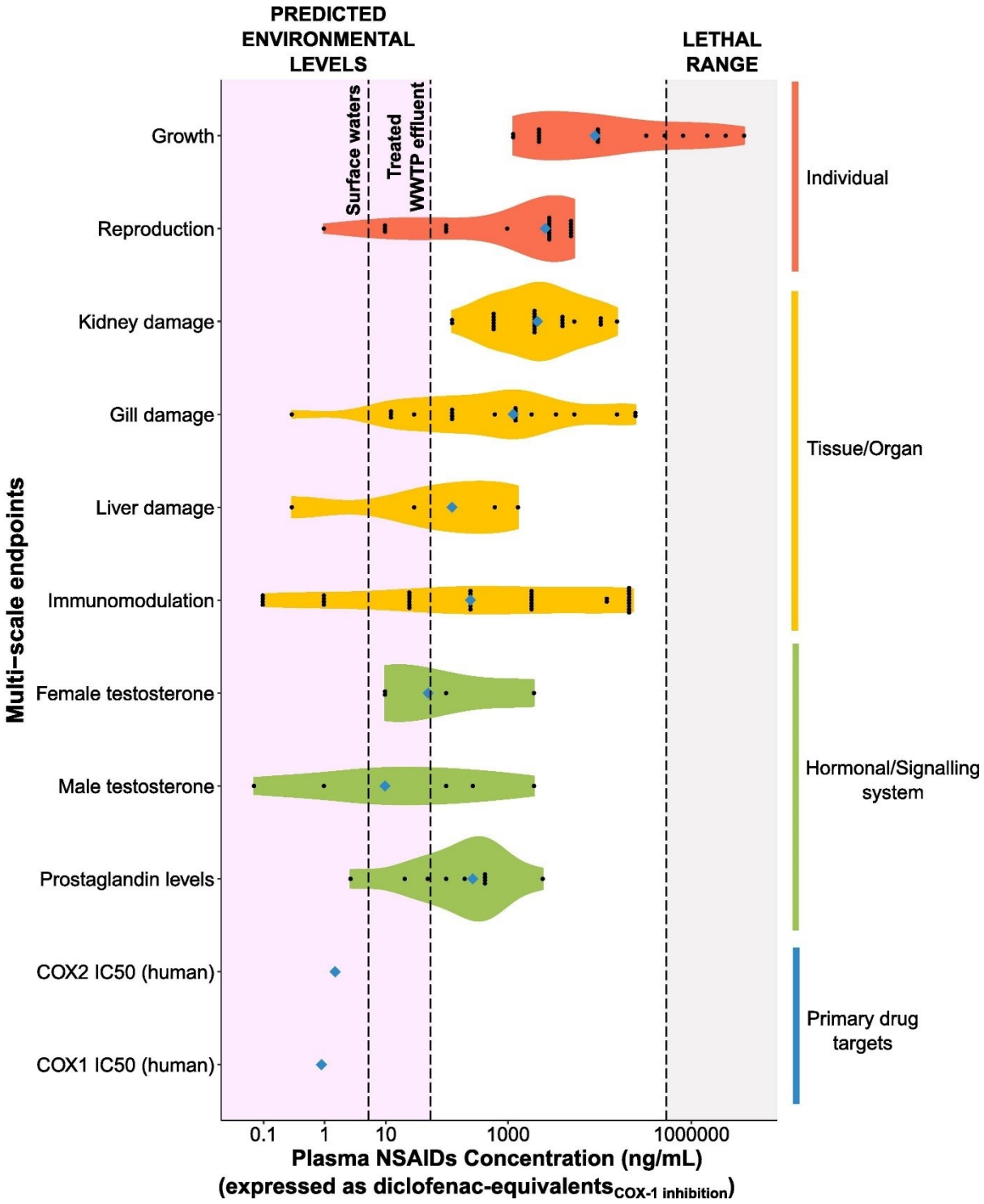

Figure 3.7. Predicted NSAID plasma concentrations associated with the manifestation of adverse phenotypes at multiple levels of biological organisation. *NSAID plasma concentrations are expressed as 'diclofenac-equivalents(COX-1inhibition) (ng/mL)'. Each violin displays the distribution of plasma concentrations that caused a statistically significant effect in in vivo studies that involved the exposure of fish species to NSAIDs, for a minimum of 4 days and a maximum of 132 days. The 151 experimental data points portrayed in the graph were retrieved from 26 studies published between 2004 and 2018. The dotted line on the right represents the plasma concentration of 'diclofenac-equivalents' associated with mortality. The dotted vertical lines on the left indicate the environmental levels of the*

mixture of NSAIDs detected in the UK (i.e. the highest measured average concentrations in surface waters, and waste-water treatment plant effluents). These lines can be used to interpret the environmental relevance of the effect data and the related risk. Each black dot represents a statistically significant experimental value, and the blue diamond represents the median value of each endpoint.

The analyses provided here were based on the assumption that the Fish Plasma Model represents a reliable tool to predict the plasma concentration of drugs in adult fish. To validate this assumption, we screened the literature to identify a set of experimentally determined plasma bioaccumulation factors (plasma BCF) for diclofenac and ibuprofen (Bickley *et al.,* 2017; Brown *et al.,* 2007; Cucklev *et al.,* 2011; Lahti *et al.,* 2011). The comparison of this data with the plasma BCFs predicted by the Fish Plasma Model revealed that the experimental values were always within the range of concentrations predicted by the model (Figure 3.8). The use of LogKow as the input parameter of the Fish Plasma Model tended to overestimate the plasma BCF of the two compounds; whereas the use of LogD7.4 tended to underestimate it. The predictions generated in this work were based on the use of LogKow; hence, it is plausible that our analysis overestimated the plasma concentrations of NSAIDs in fish. Nonetheless, this overestimation is in agreement with the precautionary principle that was applied throughout the workflow. The predictive model described here does not currently consider drug metabolism in fish, mainly due to the existing knowledge gaps in this field. Some studies have demonstrated that NSAIDs reactive metabolites may play a role in the manifestation of organ toxicity in mammalian models (Oda *et al.,* 2017). However, the ecotoxicological relevance of those findings is currently unknown.

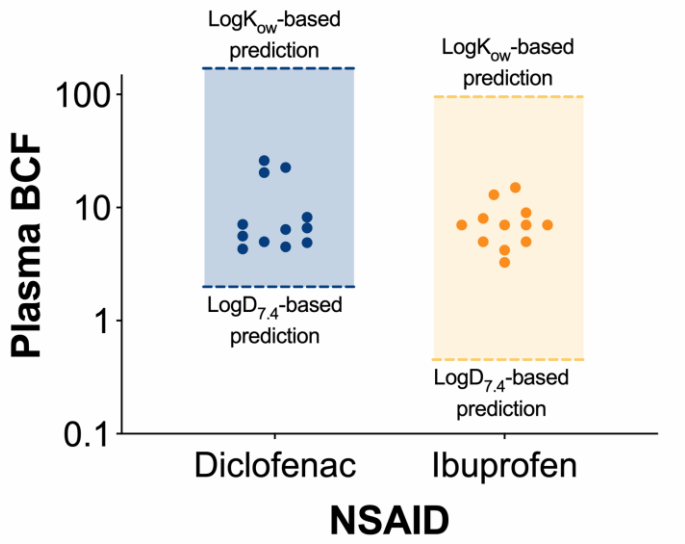

***Figure 3.8. Predicted versus measured plasma BCF**. The range of predicted values were generated by considering both LogKow and Log D7.4 as the input parameters of the fish plasma model. Measured BCF values were retrieved from four in vivo studies published in the literature (Bickley et al., 2017; Brown et al., 2007; Cuklev et al., 2011; Lahti et al., 2011).*

The interpretation of mode-of-action driven effects can be strengthened by the analysis of effect direction and magnitude. To assess this aspect, we retrospectively analysed those parameters for one of the endpoints with the highest regulatory importance, egg production (Figure 3.9). Out of 13 experimental cases (retrieved from Flippin *et al.,* 2007; Han *et al.,* 2010; Ji *et al.,* 2013; Lister and Van Der Kraak, 2008; Yokota *et al.,* 2017; and Yokota *et al.,* 2015), NSAIDs (i.e. diclofenac, ibuprofen, indomethacin) induced a decrease in egg production in 10 cases, and an increase in three cases. Notably, the observed discrepancy was related to ibuprofen, with three cases of decrease and three cases of increase. The effect magnitude was 60% or lower in the cases of decreased egg production, and up to 200% in the cases of increased egg production. It is currently unknown if the observed discrepancy across the literature has a genuine biological explanation, or if it may be due to methodological artefacts.

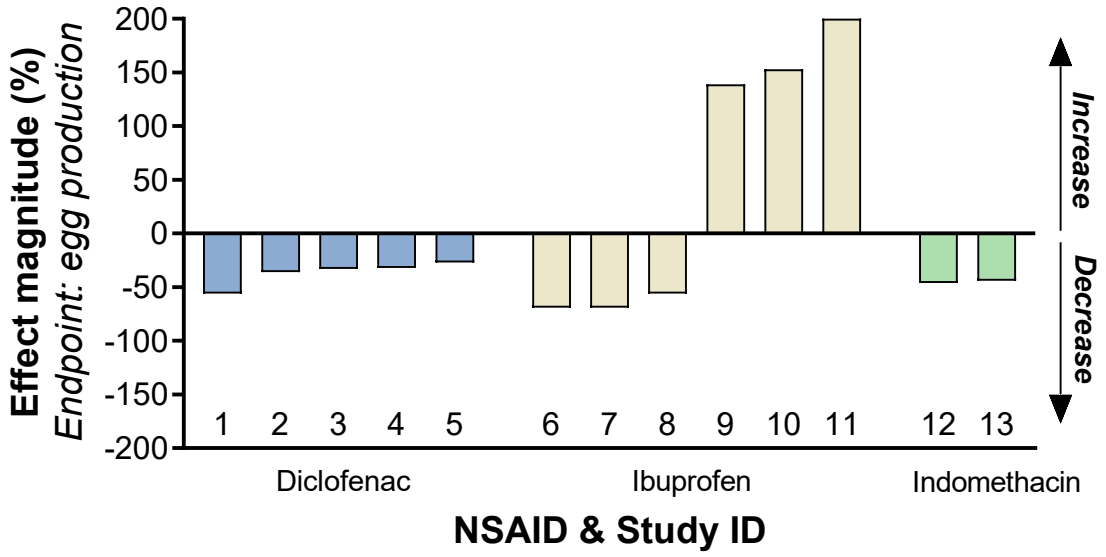

Figure 3.9. Effect of NSAIDs on fish reproduction. *The figure displays effect magnitude and direction for the endpoint "egg production". The data were retrieved from 6 studies published in the literature (1,2,4,5: Yokota et al., 2017; 3: Yokota et al., 2016; 6,8: Ji et al., 2013; 7,9,10: Han et al., 2010; 11: Flippin, Huggett and Foran, 2007; 12, 13: Lister and Van Der Kraak, 2008).*

3.5 Discussion

Implementing pharmacological and mechanistic considerations into the environmental risk assessment of pharmaceuticals can facilitate the interpretation of the risk and enable the application of modern predictive toxicology approaches. In the last decade, a number of experts have called for such implementation (e.g. Ågerstrand *et al.,* 2015; Caldwell *et al.,* 2014; Gunnarsson *et al.,* 2019; Rand-Weaver *et al.,* 2013; Winter *et al.,* 2010). Several studies have experimentally demonstrated the positive impact of this approach (e.g. Margiotta-Casaluci *et al.,* 2014; Margiotta-Casaluci *et al.,* 2016; Valenti *et al.,* 2012), and dedicated comparative pharmacology tools have been developed to facilitate the process (e.g. ECOdrug, Verbruggen *et al.,* 2018; SeqPASS, LaLone *et al.,* 2016). Despite these efforts, the current ERA process remains mechanistically agnostic and solidly centred on traditional fate/exposure predictions, with toxicity levels of individual compounds experimentally determined using simple tests focused on apical endpoints (Lee and Choi, 2019). This limitation acquires even more significance when mixtures of drugs are considered. In this case, the lack of mechanistic rationale behind the ERA of the individual components prevent the application of predictive approaches for the assessment of potential mixture effects. Moreover, the virtually endless number of exposure scenarios that may occur globally implies that the experimental determination of the risk is impossible to achieve, hence predictive approaches are vital to

reach the desired future protection goals. The present work - focused on NSAIDs - paves the way for the development of an innovative pharmacology-informed ERA of drug mixtures by proposing a predictive framework that integrates both drug pharmacokinetic and pharmacodynamic features.

In Europe, the regulatory concern about NSAIDs has until now been focused on diclofenac and its effects on fish (European Commission, 2015; European Commission, 2018; Loos *et al.,* 2018). A few academic studies have started to explore the potential effects of mixtures of NSAIDs, using a limited set of compounds - such as diclofenac, ibuprofen, naproxen, and aspirin - mostly using invertebrates (Cleuvers, 2004; Parolini and Binelli, 2012) or, when using fish, in combination with other chemicals (Parrott and Bennie, 2009; Stancova *et al.,* 2014; Schmitz *et al.,* 2018). Our analyses suggest that the problem of NSAIDs mixtures may be more significant than initially thought, as 19 out of 25 NSAIDs considered in the present study were detected in the aquatic environment worldwide. What is currently unknown is the level of co-occurrence of all these compounds in the same water body, as only a few of them are targeted in water monitoring programmes (Comber *et al.,* 2018). Considering the impossibility of determining an average exposure scenario, in the present work we considered the mechanistic hazard of all 25 NSAIDs. The risk assessment, however, was performed using measured NSAIDs concentrations in the UK as the reference exposure scenario. This choice was justified by several factors. Firstly, the UK is characterised by a high market penetration of NSAIDs (McGettigan and Henry, 2013); secondly, the UK has one of the lowest average WWTP effluent dilution factors in the world (Keller *et al.,* 2014); and thirdly, the UK carries out intensive environmental monitoring programmes that target pharmaceuticals (Comber *et al.,* 2018). Nonetheless, the model presented here can be adapted to interpret the risk of any exposure scenario once the concentrations of each component of the mixture are provided.

From a mechanistic standpoint, in a clinical context, the primary target of pharmaceuticals is generally involved in the disease pathophysiology, thus its modulation is aimed at achieving the desired therapeutic effect. Sometimes the interaction with the primary target is also the cause of adverse drug reactions. This is the case with NSAID-mediated COX-inhibition, which is considered the driving mechanism underlying many important side effects associated with NSAIDs treatment in patients (Grosser *et al.,* 2017; Ricciotti and FitzGerald, 2011). However, in many other cases adverse drug reactions are driven by the unintended interaction of pharmaceuticals with secondary targets (Lounkine *et al.,* 2012). From an ERA perspective, the exposure to pharmaceuticals is always unintended, thus the distinction between primary and secondary targets does not apply, and all drug-target interactions should be considered relevant for the mechanistic hazard assessment. This consideration led us to generate a bioactivity hazard network that captures the mechanistic promiscuity for a mixture of 25

NSAIDs, which was indeed significant in demonstrating that NSAIDs can act on many different targets beyond COXs (Figure 3.4).

As expected, the complexity of the mechanistic network was drastically reduced when realistic internal exposure scenarios were considered (Figure 3.6). Whereas some of the risk-based drug-target interactions were highly predictable (i.e. effects on COXs and interleukins) others were, to some extent, surprising. Specifically, the perturbation of estrogen and progesterone receptors (ESR1 and PGR) at concentrations of NSAIDs, respectively, hundreds and thousands of times lower than those predicted to occur in the plasma of wild fish in the UK. Previous research carried out on mammalian models has demonstrated that NSAIDs-mediated reduction of prostaglandin levels can lead to the down-regulation of the aromatase pathway and, in turn, decreased estrogen biosynthesis (Zhao *et al.,* 1996). Diclofenac displayed anti-estrogenic activity at receptor level *in vitro* (Klopčič *et al.,* 2018), whereas a study conducted on post-menopausal women also demonstrated that NSAID users had significantly lower serum estradiol concentrations than non-users (Hudson *et al.,* 2008). On the other hand, the evidence of a direct link between NSAIDs and the progesterone receptor are scarcer. NSAIDs administration and NSAID-mediated prostaglandin decrease has been associated with the inhibition of ovulation in both pre-clinical mammalian species and humans (Gaytán *et al.,* 2006; Stone *et al.,* 2002), although the direct involvement of the progesterone receptor remains unclear. These considerations are relevant to the ERA of NSAIDs, as these compounds can also inhibit reproductive activity in female fish (Lister and Van Der Kraak, 2008; Yokota *et al.,* 2016).

The AC50 value associated with the naproxen-mediated modulation of the PGR and the diclofenac-mediated modulation of the ESR1 were much lower than those associated with the inhibition of the drugs primary targets (COX-1 and COX-2). To facilitate the interpretation of their *in vivo* relevance, we compared those values with those associated with other potent pharmaceuticals that have the PGR and ESR as the primary targets. Runnalls *et al.* (2015) tested the effects of the synthetic progestin levonorgestrel (PGR agonist) and the synthetic estrogen ethinylestradiol (ESR agonist) on fish reproduction under chronic exposure conditions. Using these two compounds as the benchmark, it is possible to estimate the difference between the lowest ToxCast AC50 for the molecular initiating event and the drug plasma concentration that caused the statistically significant inhibition of egg production (effect size 30–40%). The latter was threefold higher for the levonorgestrel-PGR combination, and tenfold higher for the ethinylestradiol-ESR combination. Based on these pharmacodynamic considerations, the reported ToxCast data for the interactions between naproxen-PGR and diclofenac-ESR would suggest that these compounds could cause PGR- and ESR-mediated reproductive effects at plasma concentrations of 5 ng/mL and 1,500 ng/mL, respectively. No

reproductive toxicity studies have been carried for naproxen so far, but a few studies have been carried out with diclofenac. For example, Yokota *et al.* (2017) reported a water LOEC for reproductive effects of 37 µg/L diclofenac, corresponding to a predicted plasma concentration of 4500 ng/mL (prediction based on LogKow), which is only threefold higher than the above-mentioned prediction.

Collectively, this set of evidence indicate that pharmacodynamics-driven predictions may provide a valuable strategy to interpret the risk of mechanistic profiling data, although the inter-assay variability may represent a major confounding factor. For example, the ToxCast database contains 18 different assays which are able to the detect the perturbation of the PGR. Naproxen was only tested in one of those assays, in which it displayed positive activity. Similarly, 31 assays are available in the ToxCast database to detect the perturbation of the ESR1. Diclofenac was tested in 17 of those assays, displaying activity in three, and inactivity in 14. In addition to the inter-assay variability issue, some authors have also raised concerns about the reliability of the nuclear receptor assays used in the ToxCast programme, and in turn, the reliability of their associated AC50 values. For example, as discussed in Chapter 2, Janesick *et al.* (2016) identified a high percentage of false positives among chemicals classified as PPARγ agonists in ToxCast. These considerations, together with the high inter-study variability observed for COXs IC50s (Figure 3.3), reinforce the hypothesis that data generated from large-scale mechanistic profiling programmes are extremely valuable for generating testable hypotheses; whereas their direct application to drive the risk assessment process requires caution due to the high inter-assay variability of AC50s and IC50s.

Interpreting the *in vivo* relevance of the aforementioned *in vitro* mechanistic profiling data remains a major challenge in the field of toxicology. Corsi *et al.* (2019) tried to overcome this challenge by linking the ToxCast-informed bioactivity profile of a mixture of chemicals detected in the US Great Lakes with existing Adverse Outcome Pathways (AOPs). Furthermore, the ToxCast website itself links bioactivity data to existing AOPs whenever possible. In the present work, we observed that only a limited number of targets in our network was associated with AOPs in the AOPWiki. Although this approach may be a valuable strategy in the future, we concluded that the development stage of the AOPWiki is currently too preliminary to generate reliable *in vivo* predictions when applied to complex networks, such as the one generated for the 25 NSAIDs considered here. To overcome this challenge, we applied a different strategy by carrying out a zebrafish-specific target-to-phenotype association analysis for all those targets modulated at environmentally relevant concentrations of NSAIDs. This approach generated highly granular phenotypic predictions that could be used, for example, to guide the development of tailored *in vivo* experimental strategies.

Despite the successful application of the network pharmacology approach described here, there are some caveats that should be taken into consideration. Firstly, the NSAIDs bioactivity networks generated in this study are based on mammalian (largely human) data. Fish and human drug targets may display a different sensitivity to the same pharmaceutical compound. From a precautionary principal perspective, this factor may represent an issue only if the AC50s (or IC50s) for fish targets are significantly lower than the human ones; however, to our knowledge there is no evidence to support this hypothesis. A second limitation is that the target-to-phenotype association analysis is focused on zebrafish larvae and generates only qualitative predictions of the potential drug-induced phenotypes. These qualitative predictions cannot be used to infer effect magnitude, limiting the ability to directly inform the risk assessment process.

To overcome the latter limitation and provide a quantitative predictive model of NSAIDs-mediated effects in fish, we adopted a complementary predictive strategy centred on the primary targets of NSAIDs, rather than on their entire bioactivity network. In humans, NSAIDs exert their therapeutic action by inhibiting the enzymes COX-1 and/or COX-2, which are involved in the biotransformation of arachidonic acid into prostanoids. The biology of COX and prostanoids has been extensively reviewed by many authors (e.g. Grosser *et al.,* 2017; Ricciotti and FitzGerald, 2011), and it will not be discussed here. However, a basic comparative description of COXs functions in humans and fish is essential to appreciate the implications for the ERA of NSAIDs. COX-1 is constitutively expressed in most tissues and is involved in basal production of prostanoids. The latter play important physiological functions, including gastric epithelial cytoprotection (Grosser *et al.,* 2017). The perturbation of these physiological functions by non-selective NSAIDs may increase the risk of developing serious adverse effects, including gastrointestinal complications which are considered the most common NSAIDs-related adverse effects (Coxib and traditional NSAID Trialists' (CNT) Collaboration, 2013). On the other hand, COX-2 is generally not expressed under basal conditions, but it is rapidly upregulated in response to inflammation, and its products (e.g. prostaglandin E_2) potentiate the acute inflammatory response (Grosser *et al.,* 2017). This mechanistic observation justified the development of COX-2 selective inhibitors (Fitzgerald and Patrono, 2001). Acting only (or mainly) on the inducible COX-2, this sub-class of NSAIDs is indeed associated with a lower risk of gastrointestinal toxicity in the majority of studies (Conaghan, 2012; García Rodríguez and Barreales Tolosa, 2007). However, after clinical approval, it rapidly emerged that COX-2 selective inhibitors were also associated with higher incidence of cardiovascular adverse events (Mukherjee *et al.,* 2001). This unexpected scenario led to the withdrawal of rofecoxib and valdecoxib from the market in 2004 and 2005, respectively (Cotter and Wooltorton, 2005; Dieppe *et al.,* 2004). However, other COX-2

selective inhibitors (e.g. celecoxib) continue to be used in the clinic. Follow-up research demonstrated that COX-2 is not only upregulated during inflammation but is also involved in the production of prostanoids with homeostatic functions under basal conditions. For example, gastrointestinal mucosa, vasculature, and brain tissue have all been show to express COX-2 in absence of inflammation (Grosser *et al.,* 2017; Wallace and Devchand, 2005). COX-2-derived prostaglandin I_2 and E_2 are involved in the regulation of renal perfusion and blood pressure (Qi *et al.,* 2002), and prostaglandin I_2 is also involved in the antithrombotic mechanisms of the vessel wall (Grosser *et al.,* 2006). These functions mechanistically explain the increased risk of cardiovascular adverse effects (Grosser *et al.,* 2006). The review of the safety profile of NSAIDs has become the object of regulatory attention and frequent updates (for a review see Fanelli *et al.,* 2017). For example, the US Food and Drug Administration requested a boxed warning concerning the cardiovascular risk of NSAIDs in 2005; this warning was strengthened in 2015 to highlight that all non-aspirin NSAIDs (both COX-2 selective and non-selective) can increase the risk of heart attack and stroke (US Food and Drug Administration, 2018).

The lesson learnt from the human safety assessment of NSAIDs suggests that any attempt to define clear-cut safe exposure levels of these compounds for fish, with the currently available relatively small body of evidence, may be over-ambitious. COX-1 and COX-2 are also expressed in the zebrafish, and COX inhibitors suppress the formation of prostaglandins *in vivo* (Grosser *et al.,* 2002; Patel *et al.,* 2016). However, the interpretation of the phenotypic relevance of COX inhibition in fish is complicated by the whole-genome duplication that occurred in the teleost lineage after its divergence from the tetrapod lineage (Taylor *et al.,* 2003). Ishikawa *et al.* (2007) demonstrated that the genome of zebrafish contains two functional inducible isoforms of COX-2 genes, and that other fish species also contain alternate duplication and retention of COX-1 and COX-2. It is currently unknown if these duplication events also influence the species-specific pharmacological profile of NSAIDs. On the other hand, it is known that prostaglandins are involved in the regulation of regulatory-relevant phenotypes in teleost fish species, including development (Cha *et al.,* 2006; Grosser *et al.,* 2002) and reproduction (Stacey and Goetz, 1982; Takahashi *et al.,* 2018), but also immunity (Gómez-Abellán and Sepulcre, 2016), kidney function, and gill function (Choe *et al.,* 2006). Overall, this set of comparative pharmacological considerations justified the use of COX-1 inhibition as the key mechanistic parameter to interpret NSAIDs-mediated effects in our target-centred model.

From a mixture perspective, all NSAIDs act on COX-1 and COX-2, hence the most obvious approach was to consider the cumulative inhibition of the primary targets, especially COX-1, as the key event driving the toxicological risk. To do so, we expressed all NSAIDs in units of

diclofenac-equivalents, using the diclofenac COX-1 IC50 as the reference value for the equivalence calculation. This approach is conceptually similar to the calculation of estrogenic equivalents to express mixtures of estrogenic chemicals (i.e. using the potency of 17-beta estradiol as the reference value; Safe, 1998). Brian *et al.* (2005) were the first to demonstrate that the estrogenic equivalence model can predict the response of fish to estrogenic chemicals. Our work advances this concept one-step forward by explicitly considering the drug concentrations in the fish plasma, rather than in the surrounding water. This shift from external to internal concentrations is essential to enhance the predictive power of the model, as previous studies have demonstrated that pharmaceuticals with comparable *in vitro* potency can lead to very different *in vivo* risk, based on their specific uptake and pharmacokinetic profile (Margiotta-Casaluci *et al.,* 2016). It is important to consider however, that the fish plasma model, using LogKow, does not take into account the potential variability of pH in the surrounding water. The range of pH in surface waters can be highly variable and, as NSAIDs are ionisable compounds, these changes in pH are likely to significantly affect their pharmacokinetic profile (Valenti *et al.*, 2009). Thus, future efforts to predict the environmental risk of pharmaceuticals to aquatic wildlife, may want to consider the variability of pH within the environmental matrix of interest, as this is likely to change the exposure and risk profile of these ionisable compounds. Tanoue *et al.* (2017) suggest that the liposome-water partition coefficient (K_{lipw}), may be converted into the pH-dependent liposome-water coefficient (D_{lipw}), in order to more accurately estimate the bioconcentration of ionisable compounds. Incorporating this pH-dependent uptake measure into future efforts to predict the environmental risk of pharmaceuticals such as NSAIDs may further improve the accuracy of the assessment.

Integrating NSAIDs pharmacokinetic and pharmacodynamic considerations with the concept of pharmacological equivalence, we generated a powerful visual tool that summarises all the existing *in vivo* data concerning the chronic toxicity of NSAIDs in fish, as one single graph (Figure 3.7). This analysis revealed that 30% of effect data points retrieved from the scientific literature were predicted to occur at concentrations lower than the worst-case exposure scenario in the UK (highest average measured NSAIDs concentrations in WWTP effluents), whereas this percentage dropped to 9% when a more realistic exposure scenario is considered (i.e. measured NSAIDs concentrations in surface waters). The latter sub-set of data was originated from four out of 26 *in vivo* studies considered in the present work (Ji *et al.,* 2013; Mathias *et al.,* 2018; Morthorst *et al.,* 2018; Stancova *et al.,* 2015). It is important to note that the proposed framework is a dynamic model that can be updated as and when additional biological data becomes available. Another key feature of the model is the potential to adapt the environmental exposure threshold to other exposure scenarios of interest once

the concentrations of the individual components of the NSAIDs mixture are known. This flexible approach can facilitate the region-specific interpretation of the toxicological risk posed to fish by NSAIDs locally, and effectively support regulatory decision-making.

The robustness of ERA is directly affected by the quality of the underlying data. In recent years, a growing number of authors have expressed concern about the degree of quality and reproducibility of ecotoxicology studies (Harris and Sumpter, 2015; Martin *et al.,* 2019; Mebane *et al.,* 2019). NSAIDs – specifically diclofenac – have been the object of intense debate due to discrepancies in toxicological and histopathological findings, observed between academic studies (Hoeger *et al.,* 2005; Mehinto *et al.,* 2010; Schwaiger *et al.*, 2004; Triebskorn *et al.,* 2004) and industry studies (Memmert *et al.,* 2013). The controversy surrounding these discrepancies was fuelled by the fact that the outcomes of the four academic studies were used to justify the decision concerning diclofenac in Europe (i.e. its inclusion in the Watch List of emerging pollutants in 2015). A pathology working group was successively set up to independently review the histological sections from three of the studies that investigated the effects of diclofenac in trout (Hoeger *et al.,* 2005; Mehinto *et al.,* 2010; Memmert *et al.,* 2013; Wolf *et al.,* 2014). The pathology working group revealed that while some of the observed inter-study discrepancies were potentially due to the different experimental designs used in each study; the majority of inter-study variation was driven by issues of diagnostic interpretation (Wolf *et al.,* 2014). Some discrepancies have also been observed for ibuprofen. In this case, its impact on fish reproduction has been highlighted as of concern, with a lowest observed effect concentrations (LOEC) (i.e. for zebrafish) as low as 1 µg/L (Ji *et al.* 2013). On the other hand, Morthorst *et al.* (2013) observed no effects on zebrafish egg production up to 506 µg/L (Morthorst *et al.,* 2013), whereas a recent zebrafish short-term reproduction test set the LOEC, for the same endpoint, at 266 µg/L (Constantine *et al.,* 2020). Overall, the discrepancies discussed above represent a challenge for regulatory decision making. Our model does not contain a quality assessment of each study included in the analysis; however, this assessment could be carried out retrospectively by the end-user. This decision was justified by several reasons including the difficulty to set a univocal definition of ‘quality’ applicable to any context (e.g. academic vs industry, exploratory vs regulatory toxicology, etc.), and the risk of introducing undesired bias into the dataset. To demonstrate the positive value of retrospective analysis of specific data points in the model, we focused on one of the endpoints with the highest regulatory importance - egg production. We evaluated two important quantitative parameters: effect magnitude and effect direction. This analysis revealed a certain degree of inconsistency in the effects induced by ibuprofen, which sometimes caused a decrease in egg production and other times an increase. It is currently unknown if the observed discrepancy has a genuine biological explanation, or if it may be due

to methodological artefacts. In any case, it suggests that this type of evaluation should be taken into consideration during the risk assessment process.

It is important to consider that the human safety assessment of NSAIDs so far has been based on the results of hundreds of studies. For example, the meta-analysis published by the Coxib and traditional NSAID Trialists' (CNT) in 2013 identified 754 randomised trials involving more than 350,000 patients. Despite these numbers, the interpretation of the risk remains complex, and the discussion remains open (Coxib and traditional NSAID Trialists' (CNT) Collaboration, 2013). As a term of comparison, our COX-1-centred model portrays almost all existing data (to our knowledge) concerning the medium-to-long term effects of NSAIDs on fish. The model is based on 26 independent studies involving approximately 6,000 fish of several species at various life stages (mostly at early life stage). This number of animals already used to investigate the risk posed by NSAIDs in the aquatic environment is not negligible and raises the question whether additional *in vivo* ecotoxicity testing is needed. Our approach maximises the value of each *in vivo* study by integrating all data within a coherent predictive toxicology framework. For example, a very recent zebrafish short-term reproduction test involving 280 adult animals was carried out by Constantine *et al.* (2020). This study was not included in our dataset, and it was used to test the degree of concordance with the model displayed in Figure 3.7. Constantine *et al.* (2020) showed that 55 and 266 µg/L of ibuprofen caused 38% and 96% decrease of cumulative egg production, respectively (note: the effects at 55 µg/L were not statistically significant). Those exposure concentrations correspond to a plasma diclofenac-equivalents concentration of 53 and 258 ng/mL, which fall within the 30th percentile of the range of internal effect concentrations identified in our analysis (Figure 3.7). This agreement highlights the high predictive value of our model and its potential to support weight-of-evidence driven regulatory decision making.

Our analyses also highlight immunomodulation as a key endpoint of interest since our target-to-phenotype analysis highlights many specific immune-related predictions (Table 3.2), and the ‘immunomodulation’ phenotype represents one of the highest levels of risk in our multi-scale COX-1-centred model analysis (Figure 3.7). This endpoint also displays some of the highest levels of variation in our model since diclofenac-equivalent plasma effect concentrations range from around 0.097 to 110481.674 ng/mL (Figure 3.7). The immunomodulatory effects of NSAIDs, highlighted by our qualitative target-to-phenotype analysis and our quantitative multi-scale model, represent a key area of interest warranting further investigation.

3.6 Conclusions

In the present study we provide a pharmacology-informed workflow able to guide the incorporation of pharmacokinetics and pharmacodynamic considerations into the environmental risk assessment of NSAIDs and aid the implementation of predictive toxicology strategies, without the immediate need of performing additional animal testing. Our analyses highlighted that 19 out of 25 NSAIDs have been detected in the aquatic environment globally and demonstrated that the risk posed to fish by NSAIDs mixtures may not be negligible in situations of high population density (corresponding to high levels of drug consumption) and low dilution of WWTP effluents. Using the concept of pharmacological equivalence, we generated a multi-scale model able to guide the interpretation of the toxicological relevance of any given set of environmental concentrations of NSAIDs. We anticipate that this model could facilitate the interpretation of complex data and guide the regulatory decision-making process to better address the issue of both single NSAID and NSAIDs mixtures in the environment. On the other hand, the mechanistic, pharmacological, and biological complexity brought to light by the present work suggests that the clinical substitution of one NSAID with another - on the basis of the potential environmental risk - is far from simple and could have negative clinical implications, for example, by limiting the range of therapeutic options available to patients for the treatment of pain and inflammation.

3.7 References

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Chapter 4

In vivo characterisation of NSAIDs-mediated immune cell trafficking in the gastrointestinal system of healthy zebrafish larvae

4.1 Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are used daily by millions of people. Typically used to treat conditions involving inflammation and pain, NSAIDs have been linked to several adverse drug reactions (ADRs) in the clinic including gastrointestinal, renal, and cardiovascular complications. The exact mechanisms by which these drugs elicit adverse effects are largely unknown. However, several putative mechanisms have been suggested, some of which involve immunomodulatory processes. Here we show that a non-selective NSAID (diclofenac) and a COX-2 selective NSAID (meloxicam) have significantly different effects on immune cell trafficking within the gastrointestinal mucosa, and both modulate the expression of several immunomodulatory genes. Using transgenic zebrafish reporter lines and *in vivo* imaging techniques, we show that diclofenac exposure significantly upregulates the infiltration of neutrophils (*tg(MPX:EGFP)^{i114}*) and macrophages (*tg(MPEG1:mCherry-CAAX)^{gl26}*) into the mid/posterior region of the larval intestine (at 10 days post fertilisation). Meloxicam induced a significant upregulation of only macrophage infiltration in the lowest exposure concentration group. Differential expression analysis of whole larvae RNA-Seq revealed that diclofenac exposure significantly modulated the expression of 530 genes, whereas exposure to meloxicam yielded 193 differentially expressed genes (DEGs). Selectivity for COX-2 is thought to be gastro-protective, as COX-1 inhibition leads to dysregulation of the intestinal mucus barrier which protects the epithelium from luminal contents. However, our data suggests that several COX-1-independent mechanisms are also at play, including the downregulation of key anti-inflammatory/pro-resolving modulators (*anxa1b*, *anxa1c*, *anxa1d*, *lta4h, and loxl3b*), and several cell adhesion, ECM, and muscle related genes. Our combined imaging and transcriptomic data show that exposure to traditional NSAIDs (diclofenac) in the GI tract results in neutrophilic inflammation. These results provide further mechanistic insight into the adverse effects associated with prolonged NSAID exposure and demonstrate the importance of taking pharmacokinetic and pharmacodynamic properties of distinct NSAIDs into account.

4.2 Introduction

The class of non-steroidal anti-inflammatory drugs (NSAIDs) currently includes at least 25 distinct compounds which are available for human therapeutic use (Díaz-González and Sánchez-Madrid, 2015). Millions of people around the world use NSAIDs each day to treat a variety of health conditions involving fever, pain, and inflammation. In England alone, 15 million NSAIDs prescriptions were dispensed in 2014, in addition to the tens of millions of NSAIDs formulations purchased over-the-counter (Davis and Robson, 2016). Long-term therapeutic use of NSAIDs is associated with an increased risk of adverse drug reactions (ADRs) including gastrointestinal (GI), cardiovascular (CV), and renal complications. It has been reported that NSAID consumption is responsible for approximately 30% of ADR-related hospital admissions each year in the UK (Pirmohamed *et al.,* 2004). In general, it is well established that the risk of GI and CV complications is significantly increased upon ingestion of NSAIDs, irrespective of their affinity for the different isoforms of the primary drug target: cyclooxygenase 1 (COX-1) or cyclooxygenase 2 (COX-2) (Davis and Robson, 2016). However, the isoform selectivity profile of each NSAID is considered a key driver of the organ-specific relative risk, and this information is used to guide clinical decision making. For example, non-selective NSAIDs are more likely to induce GI complications such as ulceration or bleeding, compared to COX-2 selective inhibitors, whereas the latter have been associated with a higher risk of CV toxicity which has led to the withdrawal of rofecoxib and valdecoxib from the market (Davis and Robson, 2016). Although these general considerations may be clinically relevant, from a predictive toxicology perspective the comparison of the relative toxicity risk among NSAIDs is not simple. For example, although the risk for GI complications associated with COX-2 selective inhibitors might be lower, it is by no means negligible. The risk of GI bleed is increased by fourfold with non-selective NSAIDs, and threefold with COX-2 selective inhibitors (Masclee *et al.,* 2014).

Numerous NSAIDs are available on the market, with a diverse set of pharmacological features, which when considered in combination with the importance of patient-specific clinical history, suggest that a personalised NSAID selection approach could be the most desirable clinical scenario. Advancing the fundamental mechanistic understanding of NSAIDs toxicity is critically important to support future personalised medicine approaches. In this Chapter, as well as in Chapter 5, we will focus specifically on NSAIDs-mediated gastrointestinal toxicity, which is estimated to cause over 3,500 hospitalisations and 400 deaths per year in the United Kingdom (Hawkey and Langman, 2003).

Currently established modes of action, such as the inhibition of prostanoid synthesis through COX, can be causally linked to some of the adverse effects associated with NSAIDs. This

inhibition of COX function in the gastric mucosa is known to contribute to the development of serious GI complications (Dubois *et al.,* 2004). COX-1, constitutively expressed in most tissues, plays a protective role within the gastric mucosa due to its involvement in the synthesis of prostaglandin E_2. This essential prostaglandin enacts its effects through the four PGE_2 GPCR subtypes (EP1-EP4), including inhibition of GI contraction, stimulation of HCO_3 secretion, inhibition of intestinal motility, stimulation of mucus secretion, and stimulation of angiogenesis (Takeuchi and Amagase, 2018). On the other hand, inducible COX-2 acts predominantly as the pro-inflammatory mediator. This mechanistic hypothesis led to the development of COX-2 selective inhibitors (Fitzgerald and Patrono, 2001). Acting only (or mainly) on the inducible COX-2, this sub-class of NSAIDs is indeed associated with a lower risk of gastrointestinal toxicity in the majority of studies (Conaghan, 2012; García Rodríguez and Barreales Tolosa, 2007). However, the risk of gastrointestinal toxicity is not completely abrogated, suggesting the implication of other mechanisms of toxicity beyond COX-1 inhibition (Davis and Robson, 2016). One possible explanation for the gastrointestinal toxicity risk retained by COX-2 selective inhibitors is the discovery that COX-2 is not only upregulated during inflammation but is also involved in the production of prostanoids with homeostatic functions under basal conditions (i.e., in absence of inflammation) in various tissues, including the gastrointestinal mucosa (Grosser *et al.,* 2017; Wallace and Devchand, 2005). Moreover, it has been reported that COX-2 derived prostanoids may contribute to/be responsible for ulcer healing in the GI tract (Takeuchi and Amagase, 2018), and so COX-2 selective NSAIDs may actually impair this process leading to sustained damage and inflammation.

It appears likely that other mechanisms of toxicity also exist which, confounded with a depletion of endogenous prostanoids, lead to adverse effects within the GI tract. Several putative COX-independent mechanisms have been suggested, some of which may provide further insight into NSAIDs-induced toxicity. A review of mode of actions (MOAs) for diclofenac by Gan (2010) highlights multiple putative MOAs including inhibition of phospholipase A_2 (PLA_2), leukotriene synthesis and PPARγ, modulation of free AA, and stimulation of ATP-sensitive potassium channels via the L-arginine-NO-cGMP pathway. However, these mechanisms have been difficult to fully establish, and there remain unanswered questions due to contradictory evidence. For example, Gan (2010) suggests an inhibition of both PLA_2, and leukotriene synthesis, as putative mechanisms based on a combination of data sources, however, there appears to be some contradictory evidence. Early reports found that diclofenac did not directly inhibit PLA_2 (Ku *et al.,* 1986; Kothari *et al.,* 1987), whereas two later studies found that diclofenac, indomethacin, and ketoprofen do significantly inhibit PLA_2 (Singh *et al.,* 2006; Mäkelä, Kuusi and Schröder, 1997). Similarly, with regards to leukotriene synthesis, Gan concludes that diclofenac plays an inhibitory role based on the complementary findings

of two studies (Ku *et al.,* 1986; Kothari *et al.,* 1987). However, in 1993 Hudson and colleagues concluded that prolonged NSAIDs exposure induced a significant increase in the synthesis of leukotriene B4 (LTB_4) within the gastric mucosa of patients with rheumatoid arthritis. Other studies have highlighted the importance of immune components such as neutrophils in the systemic propagation of gastrointestinal damage, whereby their adhesion/infiltration is essential in the development of ulcer formation (Wallace, Keenan, and Granger, 1990). It is clear, however, that further work is necessary to fully uncover the complexities of the many potential COX-independent mechanisms associated with NSAIDs exposure.

4.3 Hypotheses, aims, and objectives

Here we hypothesise that innate immune cell trafficking into the intestinal mucosa represents an essential key event that, if sustained, anticipates the manifestation of significant tissue damage. Moreover, we hypothesise that the immunomodulatory effects of both non-selective and COX-2 selective NSAIDs are mediated by several COX-independent mechanisms. The biological plausibility of these hypotheses is supported by a large volume of evidence (Kucharzik *et al.,* 2001; Kirchner *et al.,* 1997; Wallace, 2008; Wallace, Keenan, and Granger, 1990), recently synthesised in an inflammation-related adverse outcome pathway (AOP) proposed by Villeneuve *et al.* (2018).

To test our hypotheses, we first aim to characterise the effects of NSAIDs exposure on the trafficking of two cellular innate immunity components, neutrophils, and macrophages, in the gastrointestinal tract of healthy zebrafish larvae. Secondly, we aim to advance the mechanistic understanding of NSAIDs-mediated effects beyond COX inhibition by determining transcriptomic responses in whole larvae.

To do this, we used two transgenic zebrafish reporter lines (*tg(MPX:EGFP)*^{i114} and *tg(MPEG1:mCherry-CAAX)*^{gl26}*)* that express fluorescent markers in neutrophils and macrophages, respectively. Thanks to the optical transparency of the zebrafish larvae, these strains can be used to observe and quantify, in real time, cellular trafficking using fluorescence microscopy. To determine the role played by COX isoform selectivity, zebrafish larvae were exposed for seven days to a non-selective NSAID (diclofenac) and a COX-2 selective NSAID (meloxicam). In order to advance our mechanistic understanding of NSAIDs-mediated effects, we conducted an RNA-Seq experiment to determine differential gene expression profiles from whole larvae.

4.4 Methods

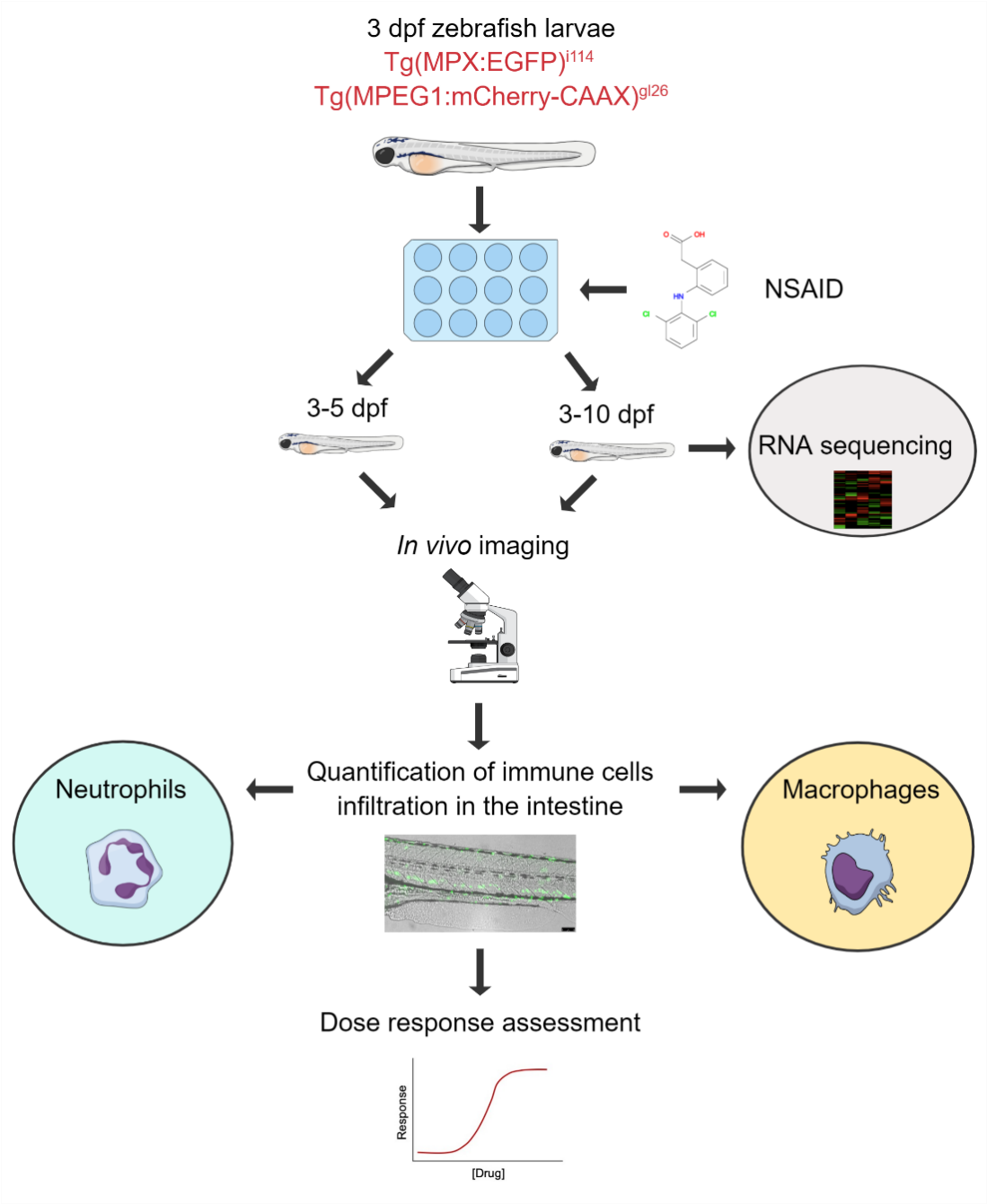

Figure 4.1. Methodological overview for Chapter 4 analyses.

4.4.1 Animal husbandry

Adult wildtype AB (WT AB), *tg(MPX:EGFP)*^{i114} and *tg(MPEG1:mCherry-CAAX)*^{gl26} zebrafish lines were maintained in flow through aquaria under optimal spawning conditions at Brunel University London (pH 7.4 ± 0.3; conductivity 300-1500 µS; temperature 27±1^{0}C). For each experiment, embryos were collected from five-to-eight breeding groups formed by two males

and four females. Embryos were subsequently rinsed with Tecniplast system water to remove any small debris. Using a Motic stereo microscope, dead, unfertilised, or poor quality embryos were removed. The remaining embryos were pooled and randomly allocated in Petri dishes with a maximum density of 60 embryos per dish. Embryos were maintained in Tecniplast system water at 27±1^{0}C. A complete water change was carried out every 24 hours to maintain high water quality until hatching (day three), when the larvae were used in the experiments.

4.4.2 Ethics and protocol reproducibility

Animals were treated in full accordance with the United Kingdom Animals (Scientific Procedures) Act regarding the use of animals in scientific procedures. All *in vivo* experiments involving protected zebrafish life stages were discussed at and approved by the Brunel Animal Welfare Ethical Review Body (AWERB) and carried out under relevant personal and project licences granted by the United Kingdom Home Office. All sections of this chapter adhere to the ARRIVE guidelines for reporting animal research (Kilkenny *et al.,* 2010). A completed ARRIVE guidelines checklist is included in the Supplementary Material (Appendix 4.1).

4.4.3 Preparation of stock solutions

All test compounds and reagents used were purchased from Sigma-Aldrich UK Ltd. Powdered diclofenac sodium salt (Sigma-Aldrich; D6899; CAS No 15307-79-6) and meloxicam sodium salt hydrate (Sigma-Aldrich; M3935; CAS No 71125-39-8) were weighed using a Sartorius Cubis microbalance, and dissolved in Tecniplast system water to achieve the desired master stock concentration. Once dissolved, the pH of each solution was adjusted to 7.4 ± 0.3. Similarly, powdered MS222 (Tricaine (Sigma-Aldrich; E10521; CAS No 886-86-2)) was weighed using a standard balance and dissolved in Tecniplast system water to generate a 4 g/L master stock (pH 7.4 ± 0.3). This master stock was used to generate working solutions of MS222 at 100 mg/L (anaesthesia) and 300 mg/L (euthanasia; Schedule 1 method). All master stocks were kept at 4^{0}C for a maximum of seven days.

4.4.4 Determination of maximum tolerated concentrations (MTC) (3 – 5 dpf)

To determine the maximum tolerated concentration of diclofenac and meloxicam, 3 dpf zebrafish larvae (WT AB) were exposed to a range of six concentrations, including a control group (clean system water; note: no solvents were used to dissolve the test compounds). The allocation of each treatment group to specific columns of 24-well plates was randomised. Each group included 16 larvae, which were loaded individually into single wells containing 1 ml of

exposure media. The exposures lasted for 48 hours, until the larvae reached 5 dpf. Diclofenac concentrations ranged from 0 to 33.4 mg/L (0, 2.09, 4.18, 8.35, 16.7 and 33.4 mg/L). Meloxicam concentrations ranged from 0 to 100 mg/L (0, 20, 40, 60, 80, and 100 mg/L). Throughout the experiments, 70% of the exposure media was replaced every 24 hours to maintain high water quality standards. The larvae were assessed twice a day for signs of toxicity or mortality. After 48 hours, the MTC was defined using a series of qualitative indicators of animal health as previously outlined in Winter *et al.* (2008), including loss of dorso-ventral balance, abnormal morphology, larval touch responsiveness using a seeker, and mortality indicated by the absence of heartbeat.

4.4.5 Pilot exposure (3 – 10 dpf)

Once the MTCs of diclofenac and meloxicam were determined in non-protected life stages, small-scale pilot studies were carried out to establish appropriate concentration ranges for medium exposure durations (seven days: 3 – 10 dpf). We aimed to ensure that the exposure concentrations selected for the final large-scale experiments were not associated with general toxicity and lethality. Diclofenac concentrations ranged from 0 to 8.4 mg/L (0, 0.525, 1.05, 2.1, 4.2, 8.4 mg/L), whereas meloxicam concentrations ranged from 0 to 80 mg/L (0, 10, 20, 40, 60, 80 mg/L). Exposure and control groups (clean systems water) were distributed randomly across 24-well plates, with 1 ml of medium and one larva per well. WT AB larvae were used for each pilot experiment and the number of larvae per group was 16 for the diclofenac pilot, and reduced to 8 replicates for the meloxicam pilot, as a refinement to reduce the number of animals used. 70% of the exposure media was replaced every 24 hours, and from 6 dpf larvae were fed Sparos Zebrafeed (<100 µM) daily. Larval health was assessed twice a day, and any larvae found to be showing signs of toxicity were culled via Schedule 1 (Animals (Scientific Procedures) Act 1986) overdose of MS222. The data generated during these pilot studies was modelled to establish LC50 and LC20 values. As control zebrafish larvae between 5 and 10 dpf can also display natural mortality in the range 10-30%, the compound-specific modelled LC20 was considered as the maximum tolerated concentration and was used to inform the experimental design of the final i*n vivo* experiments.

4.4.6 *In vivo* exposure experiments for the quantification of immune cell trafficking (3 – 10 dpf)

To characterise the effects of diclofenac and meloxicam on intestinal inflammation, two transgenic reporter lines were used to quantify the intestinal infiltration of neutrophils - *tg(MPX:EGFP)*^{i114} - and macrophages - *tg(MPEG1:mCherry-CAAX)*^{gl26} - via *in vivo* imaging

and fluorescence microscopy. Transmigration of immune cells into the intestine is a hallmark of intestinal inflammation, and a potential precursor to more severe toxicity. Hence, due to the gastrointestinal toxicity commonly associated with NSAIDs, we aim to quantify the numbers of immune cells within the gut following exposure to both diclofenac and meloxicam. In these experiments, diclofenac and meloxicam were tested at three different concentrations (diclofenac: 1.33, 2.66, 5.31 mg/L; meloxicam: 6.75, 13.5, 27 mg/L). Individual *tg(MPX:EGFP)*^{i114} or t*g(MPEG1:mCherry-CAAX)*^{gl26} larvae at 3 dpf were randomly allocated to individual wells of 24-well plates containing 1 ml of media (n = 18 per treatment group). Throughout the experiments, all plates were kept at 27±1^{0}C. The exposures lasted for 168 hours until 10 dpf with daily media replacement to ensure the maintenance of high water quality and feeding from 6 to 10 dpf (Zebrafeed <100 µM). Health was monitored twice every 24 hours, with any fish showing signs of toxicity culled via Schedule 1 overdose of MS222. All experiments were run in duplicate, and each compound was tested over two days; on each day, 18 larvae per treatment group underwent imaging. This meant that 36 fish per treatment group were used in each experiment; power calculations, using pilot data, estimated between 20 and 31 larvae (80 – 95% power, respectively) would be the required number of replicates in order to reach statistical significance.

4.4.7 Analytical quantification of drug concentrations in exposure media

To evaluate the potential degradation of the test compounds in the exposure set up, 5 mL of exposure media was collected from each treatment group at the end of two *in vivo* experiments. Concentrations of diclofenac and meloxicam were quantified with LC/MS-MS using standard methods (Boix *et al.,* 2015) (Table 4.1). Diclofenac concentrations were quantified in the media collected from three different treatment groups (1.33, 2.66, and 5.31 mg/L) as well as the control group (Tecniplast system water). Meloxicam concentrations were quantified in the media collected from the master stock solution (200 mg/L), due to a processing error with the working solution samples initially collected, and from a control sample (Tecniplast system water).

Table 4.1. Analytical quantification of diclofenac and meloxicam using LC/MS-MS.

LIQUID CHROMATOGRAPHY
Column – 4.6 x 150 mm
Column packing – Eclipse XD8-C18 (5 µm)
Column temperature – 40 °C
Injection volume – 20 µl
Effluent flow rate – 500 µl/min
Eluent A – HPLC grade of water + 0.1 % formic acid
Eluent B – HPLC grade of methanol
Mode of ionisation – Positive

Gradient					
Total time (min)	**Flow rate (µl/min)**	**A%**	**B%**	**C%**	**D%**
1	500	85	15	0	0
2	500	85	15	0	0
3	500	10	90	0	0
7	500	10	90	0	0
7.1	500	85	15	0	0
10	500	85	15	0	0

4.4.8 Quantification of immune cell trafficking by fluorescence microscopy

At the end of the exposure period, 10 dpf zebrafish larvae were anaesthetised using 100 mg/L of MS222. Once immobile, larvae were transferred to a solution of 10 mg/mL low melting point agarose/100 mg/L MS222 and mounted onto microscopy slides fitted with silicone isolating rings. Under a stereo microscope, larvae were orientated on their sides. Once successfully orientated, each slide was moved onto a dry bath (4°C) for approximately 20 seconds to facilitate the agarose semi-solidification, and subsequently transferred onto the stage of a Leica DMi8 inverted fluorescent microscope, where individual larvae were imaged at 10x magnification. Brightfield (BF), and either GFP (neutrophil reporter) or mCherry (macrophage reporter) filters were used to capture overlay and individual filter images of the mid/posterior gut region of each larva. Immediately after imaging, larvae were culled via Schedule 1 overdose of MS222 (300 mg/L).

4.4.9 Image analyses

Images were processed using the software ‘ImageJ’ (version 1.52a). Overlay images were generated for each larva using the raw individual filter images. The ‘Fire’ look-up table was used on all images to aid in the visual distinction of individual immune cells. Manual

quantification of immune cells within the mid/posterior gut region of each larva was performed, and then recorded using the software 'GraphPad Prism 8' (version 8.4.3).

4.4.10 Statistical analyses

Normality tests (D'Agostino & Pearson test) and parametric one-way ANOVA (analysis of variance), using GraphPad Prism 8 (version 8.4.3), were used to determine both the Gaussian distribution and statistical significance of the *in vivo* imaging data. Dunnett's test for multiple comparisons was used to assess the difference between the control group and each test group per experiment (adjusted P value; $\alpha = 0.05$).

4.4.11 *In vivo* exposures for the quantification of transcriptomic responses (3 – 10 dpf)

A dedicated *in vivo* exposure experiment was carried out to assess the effects of NSAIDs on the transcriptomic responses of zebrafish larvae. Zebrafish larvae were exposed to 1.33 mg/L, and 6.75 mg/L, of diclofenac and meloxicam respectively. The experiment also included a negative control group, which was exposed to Tecniplast system water. Zebrafish larvae (WT AB, 3 dpf) were randomly distributed across 24-well plates, with 1 ml of medium and one larva per well (n = 12 per treatment group). The exposure lasted for 168 hours from 3 – 10 dpf, with daily media replacement and feeding beginning at 6 dpf (Zebrafeed <100 µM). Health monitoring was performed twice a day, with any fish showing signs of toxicity culled via Schedule 1 overdose of MS222. At 10 dpf all fish were culled via Schedule 1 and transferred to Eppendorf tubes containing RNAlater (Sigma-Aldrich; R0901) in order to stabilise and preserve the RNA through immediate RNase inactivation. Samples were stored at 4^0C overnight, before being transferred to a -20^0C freezer.

4.4.12 RNA extraction, and purification

Individual zebrafish larvae were pooled to generate three biological replicates per treatment group (n=4 per replicate). The total RNA was extracted using the Qiagen RNeasy Mini Kit (QIAGEN; 74104). The digestion of potential genomic DNA contamination was carried out using the DNase Max Kit (QIAGEN; 15200-50). The concentration of total RNA in each sample was quantified by fluorometric assay using the Qubit RNA High Sensitivity Assay Kit (ThermoFisher; Q32852). Once the concentration was determined, all the samples were stored at -80^0C.

4.4.13 RNA library preparation and sequencing

RNA library preparation, sequencing, and differential gene expression analysis was carried out by Cambridge Genomic Services (CGS). RNA integrity and quality was determined using an Agilent 2100 Bioanalyzer prior to cDNA library preparation. cDNA libraries were prepared using the SMART-Seq low-input RNA library preparation kit. Libraries were then sequenced on an Illumina NextSeq 500 to 75 base pairs (bp). Quality control of raw reads was carried out using FastQC v0.11.4 (Andrews *et al.*, 2010), and low-quality bases and read adapters were trimmed from the reads using TrimGalore v0.5.0 (Krueger, 2013). Reads shorter than 20 bases long were discarded from the dataset. Read mapping was carried out using STAR v2.7.1 (Dobin *et al.*, 2013) and the Ensembl Danio_rerio GRCz11 (release 103) reference genome, using the annotated transcripts from the Ensembl Danio_rerio.GRCz11.103.gtf file. Estimates of gene expression were derived from the mapped reads using HTSeq count v0.6.1 (Anders, Pyl, and Wolfgang, 2015). Sample clustering was assessed using DESeq2 v1.24.1 (Love, Huber, and Anders, 2014) (R version 3.6.1). Principal Component Analysis (PCA) plots and heatmaps obtained from this analysis were based on normalised and rlog transformed counts, performed for all samples. Refer to Appendix 4.2 for all relevant figures, and summary tables.

4.4.14 Differential gene expression

Differential gene expression analysis was carried using edgeR v3.26.5 (Robinson, McCarthy, and Smyth, 2010) (R version 3.6.1) for the pairwise comparisons. The number of genes included in the analysis is selected so that at least half of the genes being tested have read counts higher than five counts per million (cpm). EdgeR is used to compute effective library sizes using the trimmed mean of M-values (TMM) normalisation, which accounts for sequencing depth and RNA composition. The exact test edgeR approach was used to make pairwise comparisons between groups, and P-values were adjusted for multiple testing via the FDR (Benjamini-Hochberg; cut-off at P = 0.05). Multidimensional scaling (MDS), scatter, common dispersion, and MA plots can be found in Appendix 4.2. The raw data can be found in Appendix 4.3 (https://figshare.com/s/f77c254ad15da7d64d5d.)

4.4.15 Functional annotation analyses

The differentially expressed genes (DEGs) from each pairwise comparison, were assessed for enrichment of known pathways and biological processes using DAVID (Database for Annotation, Visualisation, and Integrated Discovery) (Huang, Lempicki, and Sherman, 2008;

Huang, Sherman, and Lempicki, 2009). The total expressed genes from each comparison (14,297 – control vs diclofenac; 14,363 – control vs meloxicam) were used as the background against which the DEGs from each comparison were compared. Enrichment of all Gene Ontology (GO) categories, and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways, were assessed for each list of DEGs ('control vs diclofenac', and 'control vs meloxicam'). The 'Functional Annotation Chart' tool was used to identify significantly enriched annotations of interest (Benjamini-Hochberg; cut-off at P = 0.05). The 'Functional Annotation Chart' tool provides an enrichment analysis (modified Fisher Exact Test) to evaluate the most overrepresented annotation terms associated with our lists of DEGs, against each background. The results of the analyses can be found in Appendix 4.3 (https://figshare.com/s/f77c254ad15da7d64d5d).

4.5 Results

4.5.1 Measured water concentrations of diclofenac and meloxicam

Measured concentrations of diclofenac and meloxicam are displayed in Table 4.2. Diclofenac measured concentrations were ± 10% of nominal concentrations. On the other hand, meloxicam measured concentrations were approximately 30% higher than the nominal concentrations.

Table 4.2. Measured water concentrations of diclofenac and meloxicam.

Sample	Nominal concentration (mg/L)	Measured concentration (mg/L)
Diclofenac	0	<0.025
Diclofenac	0	<0.025
Diclofenac	0	<0.025
Diclofenac	5.31	4.880
Diclofenac	5.31	4.760
Diclofenac	5.31	4.800
Diclofenac	2.66	2.452
Diclofenac	2.66	2.488
Diclofenac	2.66	2.468
Diclofenac	1.33	1.512
Diclofenac	1.33	1.428
Diclofenac	1.33	1.420
Meloxicam	0	<0.05
Meloxicam	200	260
Meloxicam	200	269.6

4.5.2 Determination of maximum tolerated concentrations (MTC) (3 – 5 dpf)

After 48 hours of exposure to diclofenac all the larvae exposed to 33.4 mg/L and 16.7 mg/L were unresponsive with no obvious heartbeat. The larvae in the remaining treatment groups did not display any signs of toxicity. The experiment indicated a maximum tolerated concentration equal to 8 mg/L. Approximately 40% of larvae exposed to meloxicam at 100 mg/L for 48 hours were unresponsive with no obvious heartbeat. The larvae exposed to lower concentrations of meloxicam did not display any obvious sign of toxicity or mortality. Hence, the meloxicam maximum tolerated concentration was set at 80 mg/L. These concentrations were used to guide the design of the pilot longer-term exposure studies involving older life stages.

4.5.3 Pilot exposure study to determine the longer-term safety of diclofenac MTC (3 – 10 dpf)

A Kaplan Meier plot was generated using survival data from the pilot exposure to diclofenac (Figure 4.2). Using this data, we modelled the concentrations of diclofenac estimated to produce 50% and 20% mortality in 10 dpf larvae after seven days of exposure. These concentrations were 7.64 mg/L and 5.31 mg/L, respectively (Figure 4.3). The data revealed an important degree of inter-experiment variability as, unlike the pilot study, exposure to 8.4 mg/L caused no toxicity in previous experiments. Considering that zebrafish stocks kept at Brunel University London display a natural mortality of approximately 20% at day 10 dpf, the estimated LC20 (5.31 mg/L) was considered non-lethal and was used as the highest exposure concentration for the subsequent exposures studies.

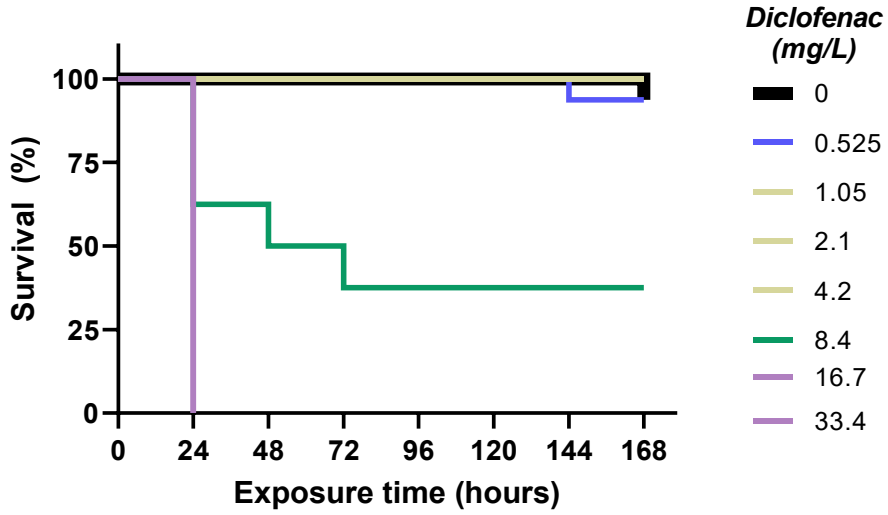

Figure 4.2. Kaplan Meier survival analysis of diclofenac exposed larvae. *3 – 10 dpf exposure period during which any larvae showing signs of toxicity were culled via schedule 1 overdose of MS222.*

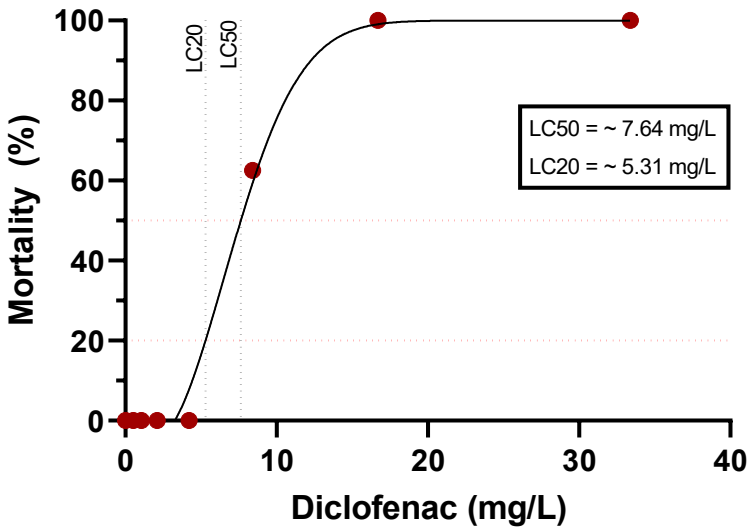

Figure 4.3. Non-linear regression modelling of LC50 and LC20 for diclofenac. *The pilot exposure data was used to model a best-fit curve to make an estimate of the concentrations required to induce 20% and 50% lethality. Red dotted lines indicate 20% and 50% response; black dotted lines represent the corresponding concentrations predicted to elicit 20% and 50% lethality.*

4.5.4 Pilot exposure study to determine the longer-term safety of meloxicam MTC (3 – 10 dpf)

A Kaplan Meier plot was generated using survival data from the pilot exposure to meloxicam (Figure 4.4). Using this data, we modelled the concentrations of meloxicam estimated to produce 50% and 20% mortality in 10 dpf larvae after seven days of exposure (Figure 4.5). These concentrations were 38.2 mg/L and 27.2 mg/L, respectively. In this case, the non-toxicity of the 5 dpf MTC was confirmed in the pilot experiment. However, larvae exposed to 80-60 mg/L displayed a time dependent increase of toxicity after the fourth day of exposure. Also in this case, the estimated LC20 (27.2 mg/L) was considered non-lethal and was used as the highest exposure concentration for the subsequent exposures studies.

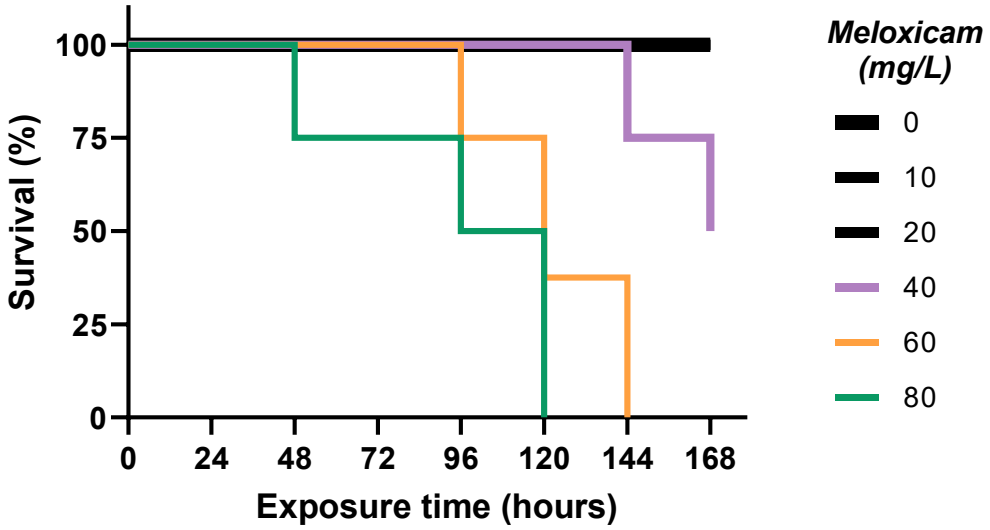

Figure 4.4. Kaplan Meier survival analysis of meloxicam exposed larvae. *3 – 10 dpf exposure period during which any larvae showing signs of toxicity were culled via schedule 1 overdose of MS222.*

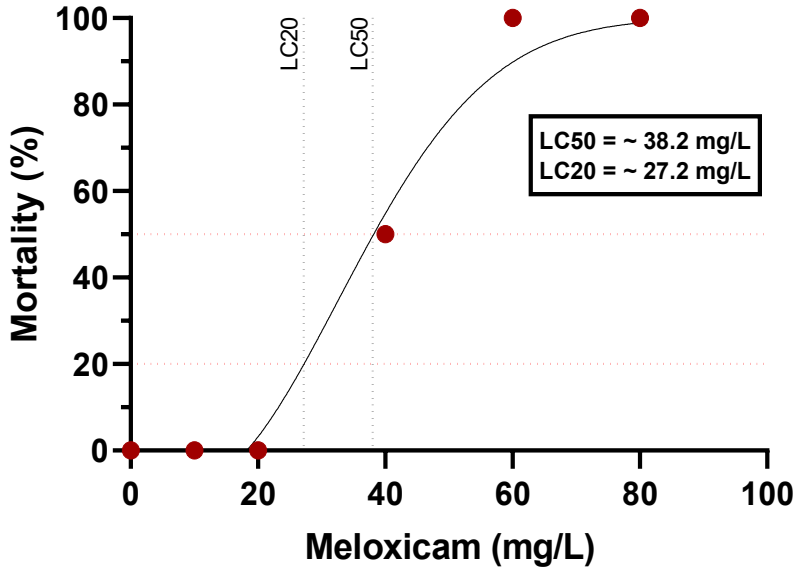

Figure 4.5. Non-linear regression modelling of LC50 and LC20 for diclofenac. *The pilot exposure data was used to model a best-fit curve to make an estimate of the concentrations required to induce 20% and 50% lethality. Red dotted lines indicate 20% and 50% response; black dotted lines represent the corresponding concentrations predicted to elicit 20% and 50% lethality.*

4.5.5 Quantification of NSAIDs-mediated immune cell trafficking in the intestine

Diclofenac exposure induced a significant infiltration of both neutrophils and macrophages into the mid/posterior intestinal region of 10 dpf zebrafish larvae exposed to diclofenac for seven

days (Figure 4.6). The mean number of neutrophils (MPX:EGFP reporter cells) infiltrating the GI mucosa in larvae exposed to 5.31 mg/L were significantly more than the number of cells observed in the control group (Ordinary one-way ANOVA; $P < 0.05$ adjusted for multiple comparisons). The number of replicates in each group for this experiment were 24 larvae (0, 1.33 mg/L, 2.66 mg/L) and 23 larvae (5.31 mg/L). Similarly, macrophage (MPEG1:mCherry reporter cells) intestinal infiltration showed a statistically significant upregulation in larvae exposed to 1.33 mg/L, 2.66 mg/L, and 5.31 mg/L, compared with controls (Ordinary one-way ANOVA, $P < 0.05$; $P < 0.01$, and $P < 0.05$, respectively; adjusted for multiple comparisons using Dunnett's). The number of replicates in each group for this experiment were 30 larvae (0, 1.33 mg/L, 5.31 mg/L) and 29 larvae (2.66 mg/L).

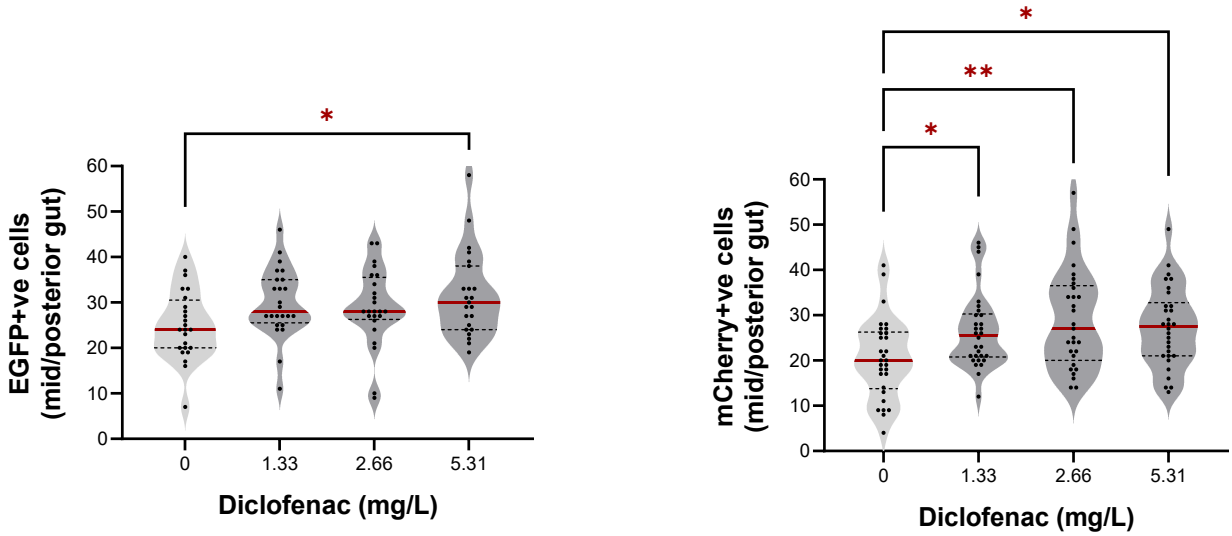

Figure 4.6. Quantification of neutrophils (EGFP+ve cells) and macrophages (mCherry+ve cells) in the mid/posterior gut of diclofenac exposed larvae at 10 dpf. *Red horizontal lines represent the mean number of cells per treatment group; the black dashed lines represent the quartiles; the red stars represent statistical significance (One-way ANOVA (Dunnett's multiple comparisons); * = P < 0.05; ** = P < 0.01). Neutrophil exposure: N = 24 (0, 1.33, 2.66 mg/L) and N = 23 (5.31 mg/L). Macrophage exposure: N = 30 (0, 1.33, 5.31 mg/L) and N = 29 (2.66 mg/L).*

Contrastingly, exposure to meloxicam did not induce any significant infiltration of neutrophils into the GI mucosa, compared with control fish (Figure 4.7). The number of replicates in each group for this experiment were 31, 34, 30 and 32 larvae (0, 6.75, 13.5, 27 mg/L, respectively). Exposure to 6.75 mg/L appeared to induce a significant upregulation of macrophage infiltration (Ordinary one-way ANOVA; $P = 0.0001$, adjusted for multiple comparisons using Dunnett's). However, larvae exposed to higher concentrations of meloxicam (13.5 mg/L and 27 mg/L) did not display a statistically significant infiltration, indicating a lack of dose-response concordance

(Figure 4.8). The number of replicates in each group for this experiment were 33, 26, 31 and 28 larvae (0, 6.75, 13.5, 27 mg/L, respectively).

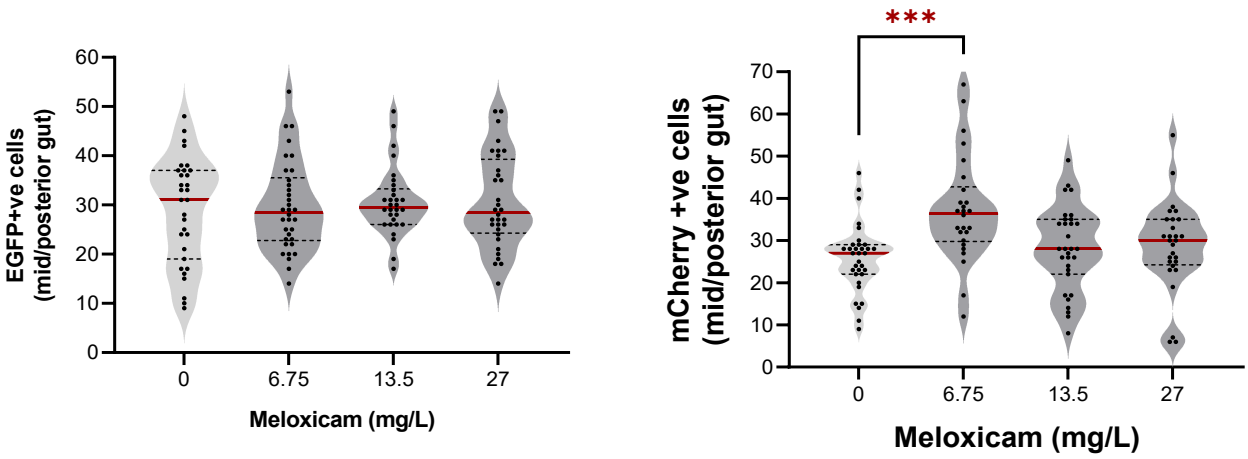

Figure 4.7. Quantification of neutrophils (EGFP+ve cells) and macrophages (mCherry+ve cells) in the mid/posterior gut of meloxicam exposed larvae at 10 dpf. *Red horizontal lines represent the mean number of cells per treatment group; black dashed lines represent the quartiles; red stars represent statistical significance (One-way ANOVA (Dunnett's multiple comparisons); **** = P < 0.0001). Neutrophil exposure: N = 31, 34, 30, 32 (0, 6.75, 13.5, 27 mg/L respectively). Macrophage exposure: N = 33, 26, 31, 28 (0, 6.75, 13.5, 27 mg/L respectively).*

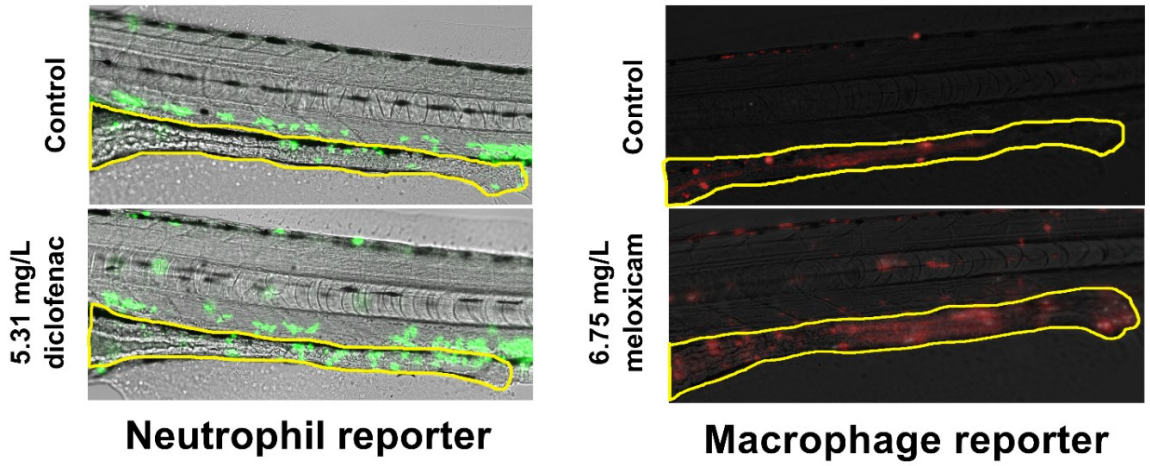

Figure 4.8. Overlay image examples of neutrophil (tg(MPX:EGFP)^{i114}) and macrophage (tg(MPEG1:mCherry-CAAX)^{gl26}) reporter cell quantification, respectively. *Yellow regions represent the area of quantification, relating to the mid/posterior gut of each larva.*

RNA-Seq: Differential gene expression analyses

Diclofenac and meloxicam exposure resulted in significant differential expression (FDR < 0.05) for a large number of genes, which is summarised in Table 4.3, and visualised in Figure 4.9 and Figure 4.10. After diclofenac treatment, 87% of differentially expressed genes (DEGs) were downregulated, and 13% were upregulated. On the other hand, after meloxicam treatment 89% of DEGs were downregulated and 11% were upregulated.

Table 4.3. Differential gene expression analyses summary. *Significant genes identified by using False Discovery Rate (FDR) to account for multiple comparisons.*

Group comparison	p<0.01 significant genes	p<0.05 significant genes	Total number of genes
CTRL__VS__DIC	277	530	14297
CTRL__VS__MEL	64	193	14363

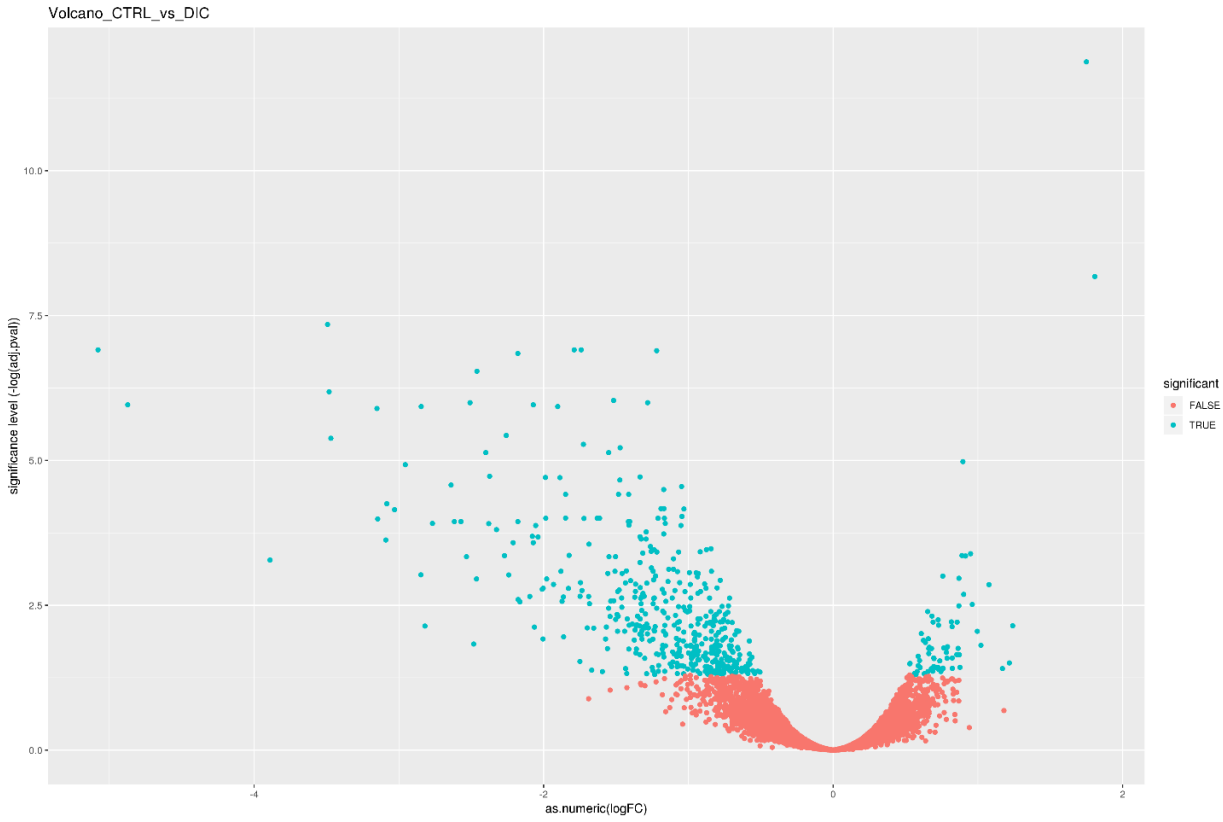

Figure 4.9. Volcano plot - control vs diclofenac. *Each data point represents a gene. Blue data points represent statistically significant DEGs (FDR ≤ 0.05), whereas red data points represent genes which are not differentially expressed.*

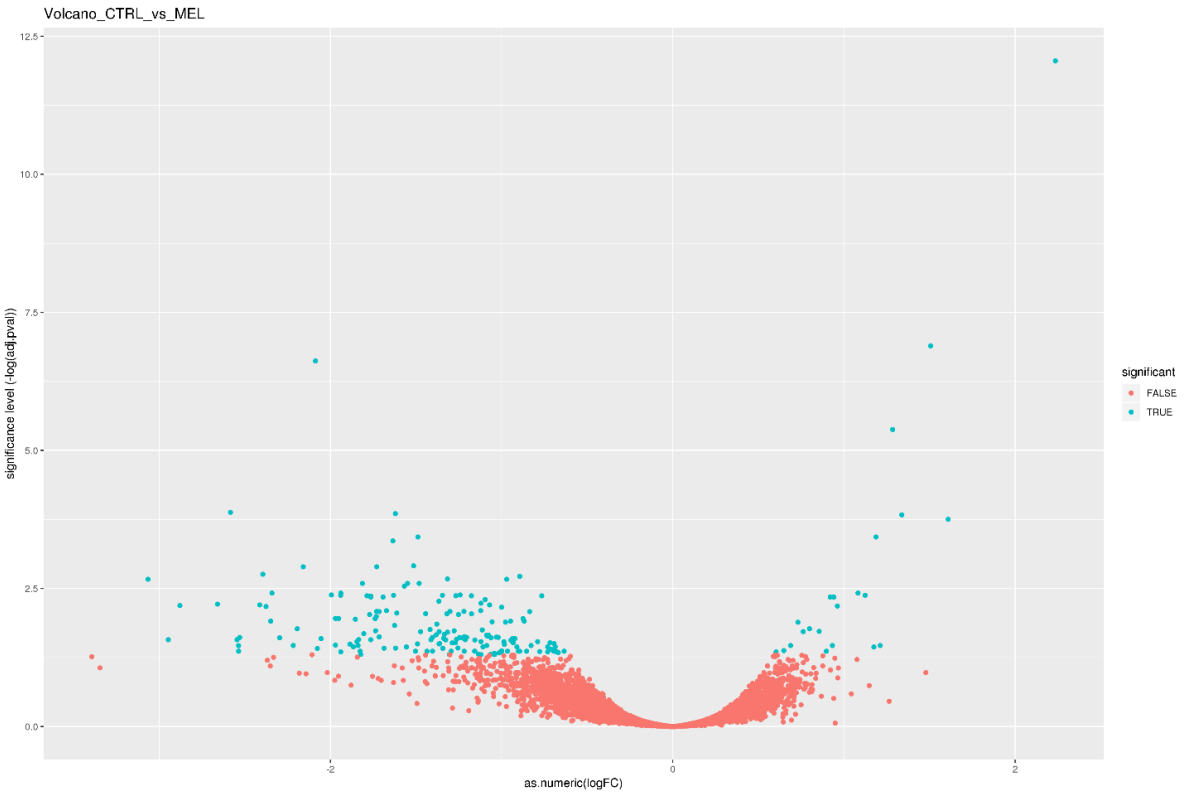

Figure 4.10. Volcano plot - control vs meloxicam. *Each data point represents a gene. Blue data points represent statistically significant DEGs (FDR ≤ 0.05), whereas red data points represent genes which are not differentially expressed.*

Diclofenac exposed larvae displayed 530 DEGs, of which 68 were upregulated (positive log_2FC), and 462 were downregulated (negative log_2FC). Larvae exposed to meloxicam showed a similar trend as 22 genes were upregulated, and 171 genes were downregulated, out of the 193 total DEGs. Interestingly, 155 of the DEGs in our analysis appear to be modulated by both diclofenac and meloxicam exposure.

4.5.7 Functional annotation analysis

Overrepresentation analysis of all GO terms and KEGG pathways using DAVID revealed 57 and 17 significantly enriched annotations (Benjamini-Hochberg P value ≤ 0.05), for the control vs diclofenac and control vs meloxicam pairwise comparisons, respectively (Figure 4.11 and Figure 4.12). Interestingly, 14 of these annotations are shared between both sets of data including: *extracellular matrix*, *extracellular space*, *extracellular region*, *extracellular matrix organization*, *extracellular matrix structural constituent*, *collagen trimer*, *skeletal system development*, *troponin complex*, *ECM-receptor interaction*, *Focal adhesion*, *Z disc*, *intermediate filament*, *calcium ion binding*, and *sarcoplasmic reticulum*.

Figure 4.11. Functional Annotation Enrichment analysis of all GO terms, and KEGG pathways, using DAVID for control vs diclofenac exposed zebrafish larvae. *The count represents the number of DEGs involved in the enrichment of each functional annotation. The colour represents the level of significance; darker = higher level of significance (Benjamini-Hochberg, P ≤ 0.05).*

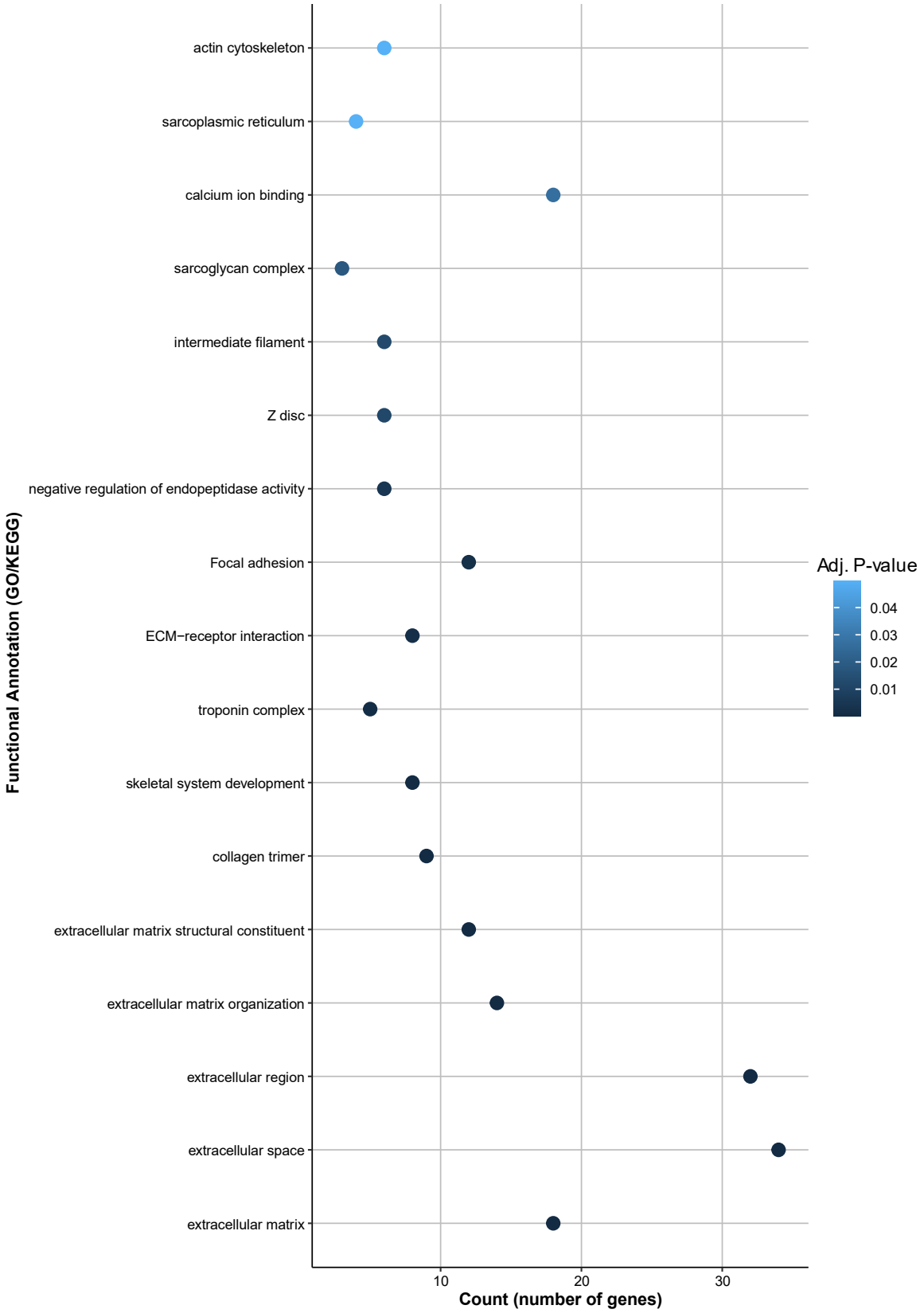

Figure 4.12. Functional Annotation Enrichment analysis of all GO terms, and KEGG pathways, using DAVID for control vs meloxicam exposed zebrafish larvae. *The count represents the number of DEGs involved in the enrichment of each functional annotation. The colour represents the level of significance; darker = higher level of significance (Benjamini-Hochberg, P ≤ 0.05).*

4.6 Discussion

Our data supports the notion that the immunomodulatory effects of NSAIDs extend beyond cyclooxygenase inhibition. The data generated in the present study supports the hypothesis that prolonged exposure to traditional NSAIDs, such as diclofenac, leads to intestinal epithelial damage through the dysfunction of several barrier-related proteins, in addition to the inhibition of COX-1 derived prostaglandins. An inflammatory response is induced following the intestinal damage, attracting neutrophils to the site of inflammation, via the release of cytokines and other inflammatory stimuli from intestinal epithelial cells (IECs). The infiltration of neutrophils into the damaged tissue is sustained due to the parallel downregulation of key anti-inflammatory and pro-resolution pathways. This combination of mechanisms leads to a sustained inflammation in the intestine. We propose that over time the non-resolving inflammation will lead to some of the adverse effects that we see in the clinical setting including ulceration, bleeding, and even perforation of the GI tract. Together our imaging and transcriptomic analysis provide robust evidence in support of this hypothesis. Our results highlight a significant increase in the infiltration of neutrophils and macrophages into the intestine of diclofenac-exposed larvae, combined with the significant modulation of key regulatory genes involved in maintaining intestinal barrier integrity (*claudin 1, integrin beta 1b.2, transforming growth factor beta-induced, alpha-tropomyosin, capping protein (actin filament) gelsolin-like b*) and inflammation (*annexin A1b, annexin A1c, annexin A1d, caspase b, CX chemokine ligand 34b, duplicate 11, lysyl oxidase-like 3b, leukotriene A4 hydrolase, PYD and CARD domain containing,* and *v-rel avian reticuloendotheliosis viral oncogene homolog*). The annexin A1 proteins are of particular interest due to their involvement in a number of critical biological processes, including the resolution of inflammation. Functional annotation analysis of DEGs, resulting from exposure to diclofenac, revealed the significant enrichment of GO term '*Inflammatory response' (GO:0006954)*. Interestingly, DEGs relating to meloxicam exposure did not result in the significant enrichment of any inflammation-related KEGG pathways or GO annotations.

4.6.1 Immune cell dynamics within the gastrointestinal tract of diclofenac and meloxicam exposed zebrafish larvae

It has been documented that COX-2 selective inhibitors, such as meloxicam, do not induce GI toxicity to the extent of traditional NSAIDs like diclofenac (Hawkey *et al*., 1998). Our data generally seems to support this notion; however, it is evident that meloxicam is likely to also modulate a number of COX-independent pathways, similar to diclofenac. The quantification of immune cell trafficking did not indicate a significant upregulation of immune cell infiltration into the intestine of meloxicam-exposed larvae. Only the lowest concentration of meloxicam

appeared to induce a significant increase in the number of macrophages residing in the gut. However, this observation is not supported by dose-response concordance, as no infiltration was observed at higher exposure concentrations. Conversely, exposure to diclofenac revealed a statistically significant upregulation of neutrophils and macrophages into the intestine of zebrafish larvae at 10 dpf. Only the top concentration of 5.31 mg/L diclofenac was able to induce a significant infiltration of neutrophils. However, all three exposure concentrations (1.33, 2.66, and 5.31 mg/L) of diclofenac were able to induce a significant upregulation of macrophages into the intestine at 10 dpf. On the whole, our data indeed suggests that the non-selective NSAID diclofenac is able to induce paradoxical inflammation within the GI tract, whereas the COX-2 selective NSAID meloxicam is unlikely to produce this inflammatory phenotype in the gut. It may be possible to hypothesise that a lack of COX-1 inhibition within IECs is sufficient for maintaining the integrity of the GI mucosa, preventing the infiltration of immune cells, and hence inflammation in the intestine. It is worth noting, however, that the protective role of COX-1 derived intestinal PGE_2 may not be sufficiently protective in the long term if COX-2 inhibition is also occurring. Sigthorsson *et al.* (2002) compared the effects of a traditional NSAID (indomethacin), a COX-1 selective inhibitor (SC-560), and a COX-2 selective inhibitor (celecoxib) on the small intestines of wild type, COX-1-, and COX-2-deficient mice. They concluded that the chronic inhibition of COX-2 is sufficient for significant damage of the intestine to occur, regardless of normal COX-1 derived PGE_2 levels in the gut. These findings suggest that COX-2 also plays a homeostatic role in maintaining the integrity of the intestinal epithelium, albeit to a lesser extent that COX-1. In fact, there is substantial evidence to support this hypothesis, indicating roles for COX-2 in mediating key components of mucosal defence, contributing to the resolution of GI inflammation, and facilitating ulcer healing (Wallace and Devchand, 2005). Previous academic research has demonstrated that COX-2 is able to not only initiate an acute inflammatory response, but also play a role in the resolution of inflammation through the production of PGE_2 and PGD_2 (Gilroy *et al*., 1999; Serhan *et al.*, 2007). Given that meloxicam is likely to modulate many similar genes and processes as diclofenac, it may be possible to hypothesise that both non-selective and COX-2 selective NSAIDs will lead to gastrointestinal toxicity via similar mechanisms; however, the toxicity induced by COX-2 selective NSAIDs becomes observable only with longer exposure periods. In fact, clinical research for meloxicam supports this as gastrointestinal adverse events are amongst the most frequently reported (Hosie, Distel, and Bluhmki, 1997). It may be possible that both diclofenac and meloxicam negatively modulate the resolution of inflammation through a number of pathways, leading eventually to toxicity.

4.6.1 Diclofenac-induced transcriptomic effects

Diclofenac exposure (1.33 mg/L) revealed statistically significant changes in the transcriptomic expression levels of 530 genes (FDR ≤ 0.05). The vast majority of these differentially expressed genes were downregulated (462 genes), with only a small number which were upregulated (68 genes). In order to interpret the biological significance of the data, a functional annotation enrichment analysis was performed using DAVID. Overrepresentation of all GO annotations and KEGG pathways was conducted using this list of 530 DEGs, compared against the background of 14,297 genes detected in the differential gene expression analysis. The 530 DEGs led to a statistically significant overrepresentation of 57 functional annotations, relating to a variety of biological processes. These functional annotations are displayed in Figure 4.11 where it is clear that effects on the extracellular matrix, structural constituents of the cell membrane, and receptor interactions are likely. Other areas of particular interest include functional annotations relating to muscle development, structure, and function for both skeletal and cardiac muscle. Additionally, diclofenac appears to have significant effects on the ocular system and the immune system. One of the most interesting functional annotations significantly enriched by diclofenac is the GO term *inflammatory response*. Nine differentially expressed genes were associated with the enrichment of this functional annotation including : *annexin A1b (anxa1b), annexin A1c (anxa1c), annexin A1d (anxa1d), caspase b (caspb), CX chemokine ligand 34b, duplicate 11 (cxl34b.11), lysyl oxidase-like 3b (loxl3b), leukotriene A4 hydrolase (lta4h), PYD and CARD domain containing (pycard),* and *v-rel avian reticuloendotheliosis viral oncogene homolog (rel)*. All nine of these genes were downregulated by diclofenac exposure. Investigating the characteristics of these genes, and their modulation, is of particular interest since the GI imaging data revealed the potential for inflammation-related effects from diclofenac exposure.

Downregulation of *lta4h* (*leukotriene A4 hydrolase*) by diclofenac is likely to result in a decrease in production of the pro-inflammatory mediator *leukotriene B4* (*LTB4*). This suggests that one of the putative mechanisms of diclofenac-induced gastrointestinal toxicity proposed by Hudson *et al*. (1993) (increased production of LTB4 via arachidonic acid substrate shunt) is unlikely to be accurate for diclofenac. In fact, it has been reported that the NSAID sulindac sulphide significantly downregulated the activity of LTA4H in HT-29 cells (human colon cancer cell line), which was exhibited by a decrease in LTB4 production (Guillen-Ahlers *et al.*, 2011). Furthermore, neutrophils have actually been implicated in the formation of gastric ulcers arising from exposure to indomethacin, with no observable changes in LTB4 synthesis (Wallace, Keenan, and Granger, 1990). Human LTA4H, however, has been shown to also demonstrate anti-inflammatory activity via its aminopeptidase active site by cleaving the

neutrophil chemoattractant Proline-Glycine-Proline (PGP) (Snelgrove *et al.*, 2010; Stsiapanava *et al.*, 2014). In addition to its aminopeptidase activity, it has been demonstrated that LTA4H is involved in the biosynthesis of the anti-inflammatory/pro-resolution mediator, resolvin E1. This positive mediator of resolution has been shown to reduce neutrophilic infiltration and proinflammatory cytokines, as well as upregulate macrophage phagocytosis of apoptotic neutrophils in mice (Oh *et al.*, 2011). Moreover, resolvin E1 has been shown to promote intestinal wound healing, through the migration and proliferation of intestinal epithelial cells (Quiros *et al.*, 2020). These data suggest that although downregulating the LTA4H enzyme will inhibit the production of the pro-inflammatory leukotriene LTB4, levels of PGP will remain high and levels of resolvin E1 will be suppressed, resulting in neutrophilic recruitment and ultimately persistent intestinal inflammation. Furthermore, the downregulation of *loxl3b* (*lysyl oxidase-like 3b*) may further contribute to an inflammatory phenotype since it has been implicated in the negative regulation of STAT3 signalling, which know to be a hallmark of intestinal inflammation (Ma *et al.*, 2017). In fact, direct inhibition of STAT3 has been shown to alleviate symptoms in inflammatory bowel disease models (Kasembeli *et al.*, 2018).

Downregulation of the annexin A1 genes (*anxa1b*, *anxa1c*, *anxa1d*) is also of particular interest, given their critical role in mediating the inflammatory response, and specifically the resolution of inflammation. Here we propose that the diclofenac-induced downregulation of *anxa1b*, *anxa1c*, and *anxa1d* has a very significant biological meaning. The suppression of these genes adds further weight to our hypothesis; that diclofenac exposure inhibits the resolution of inflammation. The annexin A1 proteins encoded by these genes have been well annotated in zebrafish (uniprot.org/uniprot/B8JLZ3) and are predicted to be involved in several immunomodulatory processes. Tissue expression analysis of the annexin A1 genes in zebrafish revealed that both *anxa1b* and *anxa1c* are expressed in numerous tissues, including epithelium (Farber *et al.*, 2003). The human ortholog of these genes, annexin A1 (ANXA1), has been well documented as a key endogenous anti-inflammatory signalling molecule, which is upregulated in response to inflammatory stimuli in order to resolve inflammation (Sugimoto *et al.*, 2016). Some of the earliest evidence has demonstrated that the activation and externalisation of ANXA1 from neutrophils negatively regulated the transendothelial migration of these cells *in vivo*, leading to clearance and the resolution of inflammation (Perretti *et al.*, 1996). The resolution of inflammation is an active process, which if left unregulated can lead to significant damage, and the annexin A1 proteins are critical components in restoring homeostasis to inflamed tissues. The migration and transendothelial infiltration of neutrophils may not only indicate the presence of tissue inflammation, but also the first essential phase in the resolution of inflammation. Following this, the clearance of neutrophils from the site of inflammation via either the induction of apoptosis and monocyte phagocytosis, or systemic

recirculation, should occur (Serhan *et al.*, 2007). It has been well documented that the generation of endogenous neutrophil-derived ANXA1 microparticles in the circulation is sufficient to inhibit cell adhesion of neutrophils and promote the resolution of inflammation (Dalli *et al.*, 2008). In fact, it has been demonstrated that an annexin A1 mimetic is able to improve experimental and indomethacin induced gastric damage in mice (Martin *et al.*, 2008), which increases the biological plausibility of NSAIDs-induced annexin A1 deficiency leading to GI toxicity. Persistent inflammation in the intestine, due to a lack of resolution, through the inhibition of ANXA1 may well be a realistic mechanism for the gastrointestinal toxicity associated with diclofenac, and perhaps even other NSAIDs. In fact, the modulation of annexin A1 by diclofenac has been reported in obese human subjects (van Erk *et al.*, 2010) which supports this putative mechanism of toxicity.

4.6.2 Meloxicam-induced transcriptomic effects

Meloxicam exposure (6.75 mg/L) revealed statistically significant changes in the transcriptomic expression levels of 193 genes (FDR ≤ 0.05). A similar trend to the DEGs induced by diclofenac exposure can be seen, as the vast majority of these genes were downregulated (171 genes), with only a small proportion which were upregulated (22 genes). Overrepresentation analysis of all GO annotations and KEGG pathways was also conducted using this list of 193 DEGs, compared against the background of 14,363 genes detected in the differential gene expression analysis. The 193 DEGs produced a statistically significant overrepresentation of 17 functional annotations, relating to a variety of biological processes. These functional annotations are displayed in Figure 4.12 where it is clear that effects on the extracellular matrix, structural constituents of the cell membrane, receptor interactions, and muscle development and function are likely. This molecular level analysis, combined with our in vivo imaging analyses, suggest that meloxicam may be less promiscuous than diclofenac, in terms of COX-independent mechanisms of toxicity. This narrative also seems to fit with the data generated in Chapter 2 and Chapter 3, which indicate that meloxicam is likely to interact with many fewer targets than diclofenac. Interestingly, the functional enrichment analysis for meloxicam DEGs did not reveal a statistically significant overrepresentation of any immune or inflammation-related annotations. This is in contrast to the analysis regarding diclofenac-induced DEGs, and seems to be in relative agreement with our *in vivo* imaging analyses, further suggesting a perhaps increased tolerability of meloxicam compared to diclofenac. Furthermore, out of the 17 functional annotations enriched my meloxicam exposure, 14 of these are shared with the enriched annotations associated with diclofenac. It may be unsurprising that these two compounds share a number of transcriptomic effects, since they

belong to the same class of drug. Exploring these similarities may help to identify mechanistic commonalities that NSAIDs share as a class.

4.6.3 Comparing transcriptomic effects of both diclofenac and meloxicam

Our transcriptomic analyses led to the overrepresentation of the following functional annotations: *extracellular matrix, extracellular space, extracellular region, extracellular matrix organization, extracellular matrix structural constituent, collagen trimer, skeletal system development, troponin complex, ECM-receptor interaction, Focal adhesion, Z disc, intermediate filament, calcium ion binding,* and *sarcoplasmic reticulum*. Some of these shared functional annotations stem from the downregulation of genes such as *alpha-tropomyosin (tpma), integrin beta 1b.2 (itgb1b.2), transforming growth factor beta-induced (tgfbi), gelsolin b* (*gsnb*), *capping protein (actin filament) gelsolin-like b* (*capgb*), *profilin 1 (pfn1), caveolin 3 (cav3),* and numerous collagen proteins. Disrupting the homeostatic regulation of such genes may significantly contribute towards a plethora of adverse effects. Although many of these genes are not well characterised in zebrafish, their human orthologs may provide some insight into their probable function, with regards to adverse effects. TPM1, the human ortholog of *tpma*, is associated with actin filament binding and stabilising the cytoskeleton in non-muscle cells for example (uniprot.org/uniprot/P09493#function; Gunning *et al*., 2005). The other orthologs (of *itgb1b.2, tgfbi, gsnb, capgb,* and *pfn1*) are associated with, amongst other things, integrin signalling which plays a vital role in maintaining the health of intestinal epithelial cells (IECs). Integrin signalling in the intestinal epithelium can regulate adhesive junctions, focal adhesion, ECM protein interactions, and even pathogen recognition (Beaulieu, 1999; Thinwa *et al.*, 2014). It has been found that beta 1 integrins are essential modulators of homeostasis in the intestinal epithelium through regulating proliferation via the Hedgehog (Hh) signalling pathway (Jones *et al.*, 2006). The downregulation of several other ECM related genes, such as collagens and matrix metallopeptidases, may add to further de-stabilise the intestinal barrier as these proteins all contribute to the normal functioning of the intestine. Downregulation of collagen I biosynthesis in IECs by the NSAID indomethacin, for example, has been associated with mucosal injury in rats (Edogawa *et al.*, 2014). Notably, both diclofenac and meloxicam also downregulate *claudin 1* (*cldn1*) expression, which may significantly contribute to de-stabilisation of the intestinal barrier, since claudins are essential foundational components of the TJ complex. In fact, claudins enact this stabilisation of the TJ through linkage with the actin cytoskeleton by binding to scaffolding proteins (Umeda *et al.*, 2006). Thus, it is clear that suppression of any one of the aforementioned genes or processes has the capacity to cause significant disruption to the functioning of the mucosal barrier. Together, the downregulation of all of these genes, in addition to the inhibition of COX-1 and

COX-2 derived prostaglandins suggests a significant increase in the likelihood of intestinal barrier compromise, through increased permeability of the intestinal epithelium, and subsequent exposure to luminal contents. Furthermore, the vast majority of these genes would likely be essential in repairing the damaged epithelium, and so sustained downregulation would probably inhibit this complex process. Moreover, if we consider the added inflammatory effects which stem from diclofenac exposure, it may be unsurprising that gastrointestinal toxicity associated with NSAIDs is so common.

Interestingly, diclofenac and meloxicam also appear to have probable effects on muscle function. The relevant shared functional annotations enriched by both sets of DEGs include *troponin complex, Z disc, intermediate filament, calcium ion binding,* and *sarcoplasmic reticulum*. The significant enrichment of these annotations suggest that both diclofenac and meloxicam are highly likely to significantly affect the normal functioning of muscle cells including skeletal, cardiac, and smooth muscle. In fact, there is plenty of evidence in the literature to support this notion. NSAIDs have been shown to have significant effects on skeletal muscle regeneration, by inhibiting muscle protein synthesis rates (Bamman, 2007). Baek *et al* (2010) demonstrated that diclofenac and meloxicam are both able to significantly alter the expression levels of various cardiac muscle movement, membrane organisation, and stress-related proteins in primary cardiomyocytes. Muñoz and colleagues (2011) found that NSAIDs are able to inhibit vascular smooth muscle cell proliferation through the disruption of normal calcium (Ca^{2+}) clearance by mitochondria within these cells. This dysregulation of normal vascular smooth muscle cell proliferation can significantly contribute towards serious cardiovascular pathologies such as atherosclerosis (Brooks *et al.*, 2003). Clearly the modulation of these COX-independent mechanisms may lead to significant pathologies including, but not limited to, cardiovascular adverse events. This might be somewhat unsurprising considering the long-established link between NSAIDs administration and cardiovascular toxicity, especially with COX-2-selective NSAIDs (Davis and Robson, 2016). Our data here may provide further mechanistic rationale for cardiovascular toxicity associated with NSAIDs exposure, beyond theories based solely upon COX inhibition and prostanoid signalling pathways.

4.6.4 Exploring the systemic effects of NSAIDs on the GI tract

Clearly, damage or compromise of the intestinal mucosa from exposure to NSAIDs does not only arise from topical mechanisms of action. There is a wealth of evidence which demonstrates the systemic effects of NSAIDs administration including ulcer formation from injectable ketorolac and intravenous aspirin, and even from enteric-coated drugs and pro-

drugs (Wallace, 2008). Although the most likely cause of the systemic effects of NSAIDs stem initially from the inhibition of COX-derived prostaglandins, there is data to suggest the involvement of neutrophils in the propagation of GI inflammation. Damage to the GI epithelium is more than likely to induce neutrophil transepithelial migration, due to the release of inflammatory stimuli from the damaged IECs, leading to inflammation in the intestine. In fact, analysis of the colonic mucosa from patients with active inflammatory bowel disease (IBD) revealed that the downregulation of claudin-1 expression in IECs was associated with the infiltration neutrophils (Kucharzik *et al.*, 2001). Furthermore, it has been demonstrated that exposure to the NSAIDs indomethacin and naproxen leads to an increase in neutrophil adhesion to the mesenteric vasculature (Kirchner *et al*., 1997). Not only this, but data from neutropenic rats revealed that indomethacin-induced enteropathy was dependent on the presence of neutrophils (Wallace, 2008; Wallace, Keenan, and Granger, 1990). Our fluorescent imaging data reveals a statistically significant increase in the number of neutrophils in the mid/posterior gut of larval zebrafish exposed to diclofenac, which is in agreement with the available data for indomethacin. Larvae exposed to meloxicam, however, did not show any increase in neutrophil transendothelial migration into the intestine, compared with control fish. The increased numbers of macrophages in the intestine of diclofenac exposed larvae (and perhaps also meloxicam, although it appears less likely given the insignificance of the top two concentration groups) may well be an added hallmark of intestinal inflammation. The biological plausibility of monocytes migrating to sites of intestinal inflammation and differentiating into activated macrophages is high. It has been well reported that intestinal macrophages perform a variety of functions which include mediating host interaction with the microbiota, managing inflammation, modulating T cells, and facilitating wound repair (Wang *et al.,* 2019). However, our transgenic reporter line does not differentiate between resident intestinal macrophages and migrating monocytes, or between the numerous different types of macrophages, and so it is difficult to ascertain the exact biological significance of the data. Overall, this data is in agreement with the assumption that COX-2 selective NSAIDs are less likely to induce GI toxicity, at least in the short-term, and that neutrophilic infiltration into the GI mucosa is a key marker of intestinal inflammation. Our transcriptomic analysis adds further weight to the likelihood of this inflammation, as diclofenac significantly altered the expression of key inflammation-related genes, which significantly enriched the functional annotation *inflammatory response*. It is important to note, however, that the RNA-Seq analysis is based on expression levels from all tissues within the entire organism, whereas the *in vivo* imaging data is focused solely on the gastrointestinal tract. Thus, it is not possible to directly associate the statistically significant changes in gene expression, at the organism level, with the phenotypic effects observed within a single tissue.

4.7 Study limitations and future refinements

NSAID selection in the present study was a considerably long and arduous process due to significant technical difficulties with stock solution preparation. Irreproducible protocols from the literature and inaccurate information regarding water and solvent solubilities meant that trialling the COX-2 selective inhibitors celecoxib, rofecoxib, valdecoxib and parecoxib was unsuccessful. Although time consuming, it became clear that these compounds would not dissolve easily without using high concentrations of solvent. However, an important factor influencing our NSAIDs selection was that we wanted to avoid using any solvents in the dissolution of our compounds wherever possible. It has been well documented that solvents, in particular DMSO, are able to significantly alter the transcriptomic and epigenetic landscape of exposed tissues, even at low 'non-toxic' concentrations (Verheijen ***et al.,*** 2019). Thus, our ideal scenario was to find two compounds, a non-selective and a COX-2 selective NSAID, which were highly water soluble. The sodium salt derivatives of diclofenac and meloxicam represented ideal test compounds from both a practical, and more importantly, a biological perspective.

One limitation of the *in vivo* imaging data generated in the present study is that it is not possible to establish which of the immune cells in the gut of each larvae were resident prior to exposure, and which cells underwent the transendothelial migration into the mucosa as a result of exposure. This means that we see a lot of variability between biological replicates, since individual larvae possess varying numbers of tissue resident immune cells, which means that a large number of replicates are needed to generate sufficient statistical power. A refinement to this limitation could come from using an alternate neutrophil reporter line (*tg(mpx:Gal4/UAS:Kaede)*) which contains a photoconvertible Kaede protein. This means that neutrophils expressing the Kaede protein can be selectively photoconverted using a specific wavelength of light stimulation, resulting in a change of fluorescence from green to red light (Ellett ***et al.,*** 2015). Thus, we could use this reporter to fine our imaging experiments by photoconverting transgenic neutrophils in the intestine directly prior to commencing the exposure. This would mean that imaging at 10 dpf would reveal a number of red fluorescent neutrophils in the intestine, which were present at the start of exposure, and also a number of green fluorescent neutrophils which have infiltrated the intestine over the exposure course.

The RNA-Seq investigation was based on a "3 biological replicates x 3 treatment group design". The use of three biological replicates per treatment is common amongst most published RNA-Seq studies involving chemical exposure and zebrafish larvae. However, our results indicate that a larger sample size may increase the overall confidence of the procedure. Some of the analysis metrics generated, such as sample clustering and correlation heatmaps,

highlighted a potential discrepancy with the sample 'CTRL_1_S1', as it did not correlate overly well with the other control samples and did not cluster well either. Another layer of complexity, which affects the interpretation of the biological relevance of the transcriptomic profile associated with this sample, is the fact that each biological replicate was formed by 4 larvae. This decision was driven by previous experimental observations that four larvae per sample would allow the extraction of enough total RNA to allow a standard cDNA library preparation and sequencing run (>500 ng RNA). Due to ethical considerations of reduction and refinement we chose to proceed with four larvae per sample, as we estimated that we would be able to extract more than enough RNA, whilst mitigating excessive use of larvae at 10 dpf. However, the total RNA concentration of three out of 12 samples was below this threshold and required the use of a more expensive low-input cDNA library preparation step for the whole sample batch. The presence of one true biological outlier among the four larvae may, in principle, affect the overall analysis. This may suggest that an optimisation of the pool size would be required to ensure the dilution of possible biological outlier effects within each sample pool. Another factor which is likely to influence the sample clustering and correlation heatmaps, is the fact that one week old larvae have been shown to have significantly more complex transcriptomic signatures than earlier life stages (Yang *et al.*, 2013). This increased complex is likely to result in increased variation between individuals and may be unsurprising since the expression profiles of early life stages are likely to be tightly regulated by developmental processes. This consideration adds weight to the idea that increasing pool size per sample would help to dilute individual biological variation between intra-group samples.

4.8 Conclusions

Here we show that the deleterious effects of non-selective NSAIDs, such as diclofenac, on the gastrointestinal tract may be driven by a neutrophil-dependent process, through the downregulation of key anti-inflammatory and pro-resolving mediators. Our *in vivo* imaging data supports this hypothesis, which is further strengthened by our transcriptomic analysis, whereby we confirm the upregulation of infiltrating neutrophils in the gut with the concurrent downregulation of several key inflammatory mediators. Modulation of these key genes may well be sufficient to inhibit the resolution of inflammation in the gut, following both direct (topical mechanisms) and systemic (inhibition of COX-1 derived prostaglandins) damage to the epithelium by non-selective NSAIDs. The COX-2 selective NSAID meloxicam did not appear to induce the same phenotype as diclofenac, as we found no obvious increase in neutrophilic infiltration into the GI tract. However, several of the same genes and processes do appear to be modulated. It is possible to hypothesise that the initiation of intestinal damage caused by

meloxicam could be temporally delayed due to the gastro-protective effects of COX-1 derived prostaglandins. This may indicate that longer exposure times would have been required to detect intestinal inflammation attributed to COX-2 selective NSAIDs. Furthermore, our transcriptomic analyses highlighted a number of other COX-independent mechanisms of action for both diclofenac and meloxicam. Further analysis and data-mining may provide significant insight into the mechanisms underlying the cardiovascular toxicities associated with NSAIDs.

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Chapter 5

Modelling the immunomodulatory effects of NSAIDs in inflamed zebrafish larvae

5.1 Abstract

Chronic use of NSAIDs is common in patients requiring relief from inflammation and pain. It is evident that long-term therapeutic use of NSAIDs significantly increases the risk of adverse events in patients, however, for most people there exists little alternative. Generating a better understanding of NSAIDs effects on tissues commonly associated with NSAIDs-induced pathologies may aid healthcare professionals in prescribing the most appropriate drugs, with a more personalised approach. For example, it is currently unclear whether it is safe to prescribe NSAIDs to patients suffering from inflammatory bowel disease (IBD), since enteropathy represents one of the most common NSAIDs-induced pathologies. The data generated in Chapter 4 demonstrates that exposure to diclofenac induces an inflammatory phenotype in the intestine of healthy zebrafish. Here we predict that the immunomodulatory effects of NSAIDs in the intestine are dependent upon the inflammation status of the intestinal mucosa. We used dextran sodium sulphate (DSS) (0.1 g/L) to reproducibly induce neutrophilic inflammation in the mid/posterior gut of transgenic zebrafish larvae (*tg(MPX:EGFP)*^{i114}) at 10 days post fertilisation. Using this model of intestinal inflammation, we found that co-exposure of DSS with either diclofenac or meloxicam was sufficient to rescue this inflammatory phenotype. Therefore, our data suggests that the immunomodulatory effects of NSAIDs in the intestine are reliant on the health status of the tissue, suggesting a potentially protective role of COX-2 in the GI mucosa.

5.2 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat a wide variety of medical conditions involving inflammation. For example, the database DrugCentral (University of New Mexico, US) indicates that the popular NSAID diclofenac is indicated to treat 18 different conditions ranging from rheumatoid arthritis to ocular pain. It is possible to argue that the "inflammatory" state of patients with different diseases will display a high degree of variability, ranging from systemic inflammation to highly localised inflammation. This implies that, once administered, an NSAID will act on both healthy and inflamed tissues, and that the topology of these tissues will be different in individual patients, and different conditions. By targeting inflamed tissues, NSAIDs will elicit their therapeutic effect. On the other hand, in healthy tissues NSAIDs may cause a disruption of homeostatic mechanisms and trigger toxicity pathways. A typical example of this scenario can be seen in patients with rheumatoid arthritis (RA) or osteoarthritis (OA) who are normally co-prescribed NSAIDs in order to relieve pain and inflammation in their joints. In addition to the positive therapeutic effect on their joints, these patients also experience a significantly higher risk of developing small intestine injury because of chronic NSAIDs administration (Tacheci *et al.*, 2016). Moreover, patients with inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis (UC), have a higher risk of developing RA (Chen *et al.*, 2020). This potential disease co-occurrence leads to the observation that some patients with RA will take NSAIDs while having a "healthy" gastrointestinal system, whereas in others NSAIDs will act on an "inflamed" gastrointestinal system. Predicting the effects of NSAIDs in these scenarios is not as straightforward as one would imagine. For example, in the specific instances mentioned above, it is unclear whether NSAIDs administration will exacerbate their disease, have a beneficial effect, or have no effect at all. Contrary to popular opinion, there is substantial evidence to suggest that NSAIDs may not exacerbate IBD phenotypes and may in fact be protective in UC patients (Hensley and Beales, 2015). However, there remains uncertainty with some groups reporting that NSAIDs are indeed capable of exacerbating IBD phenotypes (Okayama *et al.*, 2007; Singh *et al.*, 2004). To add further layers of complexity to this clinical challenge, several studies have indicated that both aspirin and non-aspirin NSAIDs may decrease the risk of developing colorectal cancer (a type of cancer with a strong inflammatory component) (Barry, Fedirko and Baron, 2019; Dulai *et al.,* 2016). Overall, these clinical observations indicate that considering the disease state of the tissue/organ of interest is essential in order to understand the multi-scale modes-of-action of NSAIDs, and enhance both safety and efficacy assessment.

In Chapter 4 we established that NSAIDs, in particular non-selective NSAIDs such as diclofenac, are capable of inducing inflammatory phenotypes in the intestine of healthy zebrafish. It is unclear, however, whether this paradoxical inflammation (potentially leading to

gastrointestinal toxicity) occurs both in healthy and inflamed intestines. The arising questions are: would existing inflammation in the gastrointestinal system be further exacerbated by NSAIDs administration? And do the effects of NSAIDs differ depending upon the inflammation status of the tissue? The GI tract represents the ideal tissue to help explore these questions since enteropathy represents one of the most common NSAIDs-induced pathologies, and since we are able to effectively model intestinal inflammation in the zebrafish larvae (Brugman, 2016). Dextran sodium sulphate (DSS) is a chemical which has been used to robustly induce intestinal inflammation in both murine and larval zebrafish models (Eichele and Kharbanda, 2017; Oehlers *et al.*, 2013). This inflammation is characterised by an increased infiltration of neutrophils into the intestine of larval zebrafish similar to the inflammatory phenotype observed with exposure to diclofenac, as described in Chapter 4.

Determining the role of NSAIDs in inflamed tissues, such as the intestine which is normally associated with NSAIDs-induced pathology, will be critical to informing the safe administration of NSAIDs in patients suffering from chronic illnesses. For example, determining NSAIDs safety profile in patients suffering with IBD would significantly aid in the prescription of the most appropriate anti-inflammatory medication, as it is currently unclear whether NSAIDs will exacerbate or ameliorate disease phenotypes in these patients (Moninuola *et al.*, 2018). It is evident that NSAIDs are capable of considerably disrupting homeostatic mechanisms in healthy tissues, which leads to toxic phenotypes, and that simultaneously NSAIDs are effective at relieving inflammation and pain in some inflamed tissues. What remains unclear, is determining the role of NSAIDs in inflamed tissues that are typically associated with significant NSAIDs-induced pathology. This is vitally important for patients suffering from IBD, for example, since alternative anti-inflammatory therapies often come with significant health risks. For instance, taking opioids to treat pain or using corticosteroids to treat inflammation might well be effective (Mitsuyama *et al.*, 1998), but the side effects of using such drugs often significantly outweigh the benefits. Especially if we consider treating mild common conditions, such as back pain or headaches, it is not realistic that someone would even consider taking opioid or steroidal medication, let alone be prescribed it. However, if it is not clear whether taking NSAIDs is safe, or even which NSAIDs might be safer than others, then what options exist for IBD patients requiring relief from inflammation and pain?

5.3 Hypotheses, aims, and objectives

Here we hypothesise that the immunomodulatory effects of NSAIDs in the intestine will differ, depending upon the inflammation status of the GI tract. We predict that both diclofenac and meloxicam will exert anti-inflammatory effects in the intestine of inflamed zebrafish larvae, characterised by the abrogation of neutrophilic infiltration within the gut.

We aim to address this hypothesis through a series of larval exposures to assess the effects of NSAIDs on neutrophil and macrophage trafficking within the intestine of inflamed zebrafish. We aim to validate the use of dextran sodium sulphate (DSS) for inducing intestinal inflammation, in the form of neutrophilic infiltration into the gut. We believe that this data will provide us with further insight into the immunomodulatory effects of NSAIDs within the GI tract. As in Chapter 4, the two NSAIDs tested here were selected on the basis of the pharmacological profile, as diclofenac is a dual COX inhibitor whereas meloxicam is a more selective COX-2 inhibitor.

To address our aims we exposed both neutrophil (t*g(MPX:EGFP)*^{i114}) and macrophage (*tg(MPEG1:mCherry-CAAX)*^{gl26}) reporter lines to diclofenac, meloxicam, DSS independently, and DSS in combination with each NSAID. These exposure conditions allowed us to assess the reproducibility of the data generated in Chapter 4 and evaluate whether either diclofenac or meloxicam are able to rescue intestinal inflammation in DSS-exposed larvae.

5.4 Methods

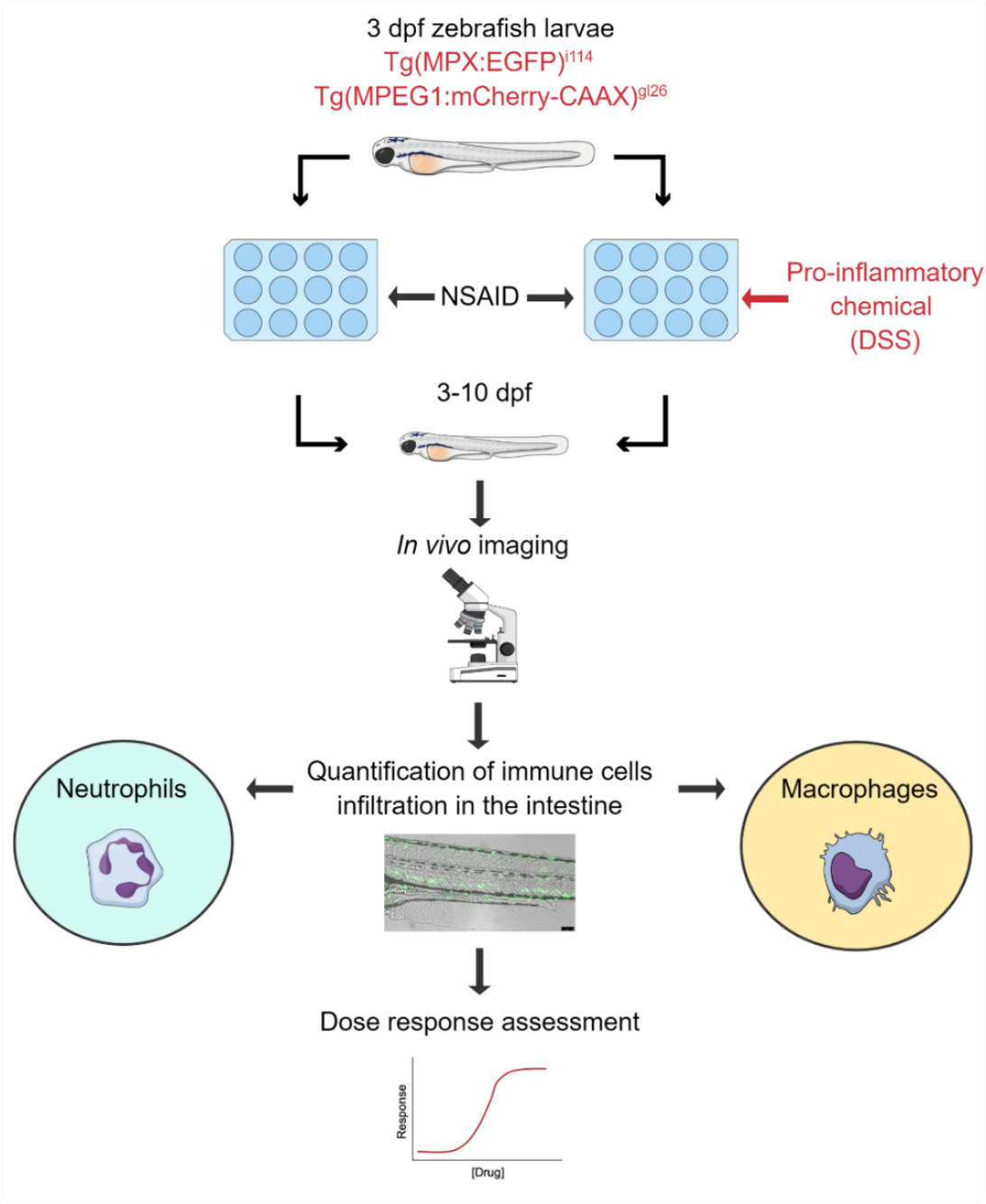

Figure 5.1. Methodological overview/vision for Chapter 5 experiments.

5.4.1 Animal husbandry

Adult wildtype AB (WT AB), *tg(MPX:EGFP)^{i114}* and *tg(MPEG1:mCherry-CAAX)^{gl26}* zebrafish lines were maintained in flow through aquaria under optimal spawning conditions at Brunel University London (pH 7.4 ± 0.3; conductivity 300-1500 µS; temperature 27±1^{0}C). For each experiment, embryos were collected from five to eight breeding groups formed by two males and four females. Embryos were subsequently rinsed with Tecniplast system water to remove

any small debris. Using a Motic stereo microscope, dead, unfertilised, or of poor-quality embryos were removed. The remaining embryos were pooled and randomly allocated in Petri dishes with a maximum density of 60 embryos per dish. Embryos were maintained in Tecniplast system water at 27±1^{0}C. A complete water change was carried out every 24 hours to maintain high water quality until hatching (3 dpf), when the larvae were used in the experiments.

5.4.2 Ethics and protocol reproducibility

Animals were treated in full accordance with the United Kingdom Animals (Scientific Procedures) Act regarding the use of animals in scientific procedures. All *in vivo* experiments involving protected zebrafish life stages were discussed at and approved by the Brunel Animal Welfare Ethical Review Body (AWERB) and carried out under relevant personal and project licences granted by the United Kingdom Home Office. All sections of this Chapter adhere to the ARRIVE Guidelines for reporting animal research (Kilkenny et al., 2010). A completed ARRIVE guidelines checklist is included in the Supplementary Material (Appendix 5.1).

5.4.3 Preparation of stock solutions

Powdered diclofenac sodium salt (Sigma-Aldrich; D6899; CAS No 15307-79-6), meloxicam sodium salt hydrate (Sigma-Aldrich; M3935; CAS No 71125-39-8), and dextran sodium sulphate (MP Biomedical; 9011-18-1; colitis grade (36 – 50 kDa)) were weighed using a Sartorius Cubis microbalance, and dissolved in Tecniplast system water to achieve the desired master stock concentration. Once dissolved, the pH was adjusted to 7.4 ± 0.3. Similarly, powdered MS222 (Tricaine (Sigma-Aldrich; E10521; CAS No 886-86-2)) was weighed using a standard balance and dissolved in Tecniplast system water to generate a 4 g/L master stock (pH 7.4 ± 0.3). This master stock was used to generate working solutions of MS222 at 100 mg/L (anaesthesia) and 300 mg/L (euthanasia; Schedule 1 method). All master stocks were kept at 4^{0}C for a maximum of seven days.

5.4.4 Determination of maximum tolerated concentration of DSS (MTC) (3 – 5 dpf)

MTC experiments for diclofenac and meloxicam were carried out prior to working with DSS and are detailed in Chapter 4. To determine the maximum tolerated concentration of DSS, 3 dpf zebrafish larvae (WT AB) were exposed to a range of four concentrations, including a control group (Tecniplast system water). The allocation of each treatment group to specific columns of 24-well plates was randomised. Each group included 21 larvae, which were loaded

individually into single wells containing 1 ml of exposure media. The exposures lasted for 48 hours, until larvae reached 5 dpf. DSS concentrations included 0, 2.5, 4.5, and 9 g/L. Throughout the experiments, 70% of the exposure media was replaced every 24 hours to maintain high water quality standards. The larvae were assessed twice daily for signs of toxicity or mortality. After 48 hours, the MTC was defined using a series of qualitative indicators of animal health as previously outlined in Winter *et al.* (2008), including loss of dorso-ventral balance, abnormal morphology, larval touch responsiveness using a seeker, and mortality indicated by the absence of heartbeat.

5.4.5 DSS model validation by fluorescent microscopy of neutrophil trafficking at 5 dpf

Following the MTC, a model validation experiment was run for 48 hours of exposure with a concentration range of 0 – 2.5 g/L DSS (0, 0.65, 1.25, 2.25 g/L), using the transgenic neutrophil reporter line *tg(MPX:EGFP)*^{i114}, in order to assess intestinal inflammation at 5 dpf. The allocation of each treatment group to specific columns of 24-well plates was randomised. Each group included 21 larvae, which were loaded individually into single wells containing 1 ml of exposure media. The exposures lasted for 48 hours, until larvae reached 5 dpf. Throughout the experiments, 70% of the exposure media was replaced every 24 hours to maintain high water quality standards. The larvae were assessed twice daily for signs of toxicity or mortality. At 5 dpf fluorescent *in vivo* imaging was carried out using a Leica DMi8 inverted fluorescent microscope on the mid/posterior intestine of each larvae (described below under 'Quantification of immune cell trafficking by fluorescence microscopy'). Image processing and statistical analysis was performed as described below under 'Image analysis' and 'Statistical analyses'.

5.4.6 Longer-term pilot exposure to assess safety of DSS (3 – 10 dpf)

Pilot exposures for diclofenac and meloxicam were carried out prior to working with DSS and are detailed in Chapter 4. Once a maximum tolerated concentration of DSS was determined in non-protected life stages, a small-scale pilot study was carried out to establish an appropriate concentration for 168 hours of exposure. We aimed to find a concentration which induces neutrophilic inflammation in the gut and is not associated with general toxicity or lethality. Initially, DSS concentrations ranged from 0 to 1.3 g/L (0, 0.325, 0.65, and 1.3 g/L), which were randomly distributed across 24-well plates, with 1 ml of medium and one larva per well. WT AB larvae were used, and the number of larvae per group was 12. However, by 48 hours it became apparent that each exposure concentration was inducing noticeable levels of

toxicity, which would likely lead to mortality, and so the experiment was terminated via Schedule 1 overdose of MS222 (300 mg/L) (Animals (Scientific Procedures) Act 1986). The pilot exposure was repeated with an adjusted concentration range of 0 to 0.1 g/L (0, 0.001, 0.0033, 0.01, 0.033, and 0.1 g/L). Exposure medium and WT AB larvae at 3 dpf were distributed randomly across 24-well plates, with 1 ml of medium and one larva per well. The number of larvae per group was 21. 70% of the exposure media was replaced every 24 hours, and from 6 dpf larvae were fed Sparos Zebrafeed (<100 µM) daily. Larval health was assessed twice a day, and any larvae found to be showing signs of toxicity were culled via Schedule 1 overdose of MS222. At 10 dpf, the pilot experiment was terminated via Schedule 1 overdose of MS222, and the top concentration demonstrating no signs of general toxicity or lethality was used to inform the design of the final *in vivo* experiments.

5.4.7 DSS model validation by fluorescent microscopy of neutrophil trafficking at 13 dpf

Longer-term exposure experiments were carried out to validate the DSS-induced model of intestinal inflammation, characterised by increased neutrophilic infiltration into the mid/posterior intestine. We used *tg(MPX:EGFP)*^{i114} larvae at 3 dpf and a concentration range of 0 to 0.1 g/L (0, 0.001, 0.0033, 0.01, 0.033, and 0.1 g/L). 1 ml of medium and one larva were distributed randomly across 24-well plates. Each group consisted of 28 larvae. 70% of the exposure media was replaced every 24 hours, and from 6 dpf larvae were fed Sparos Zebrafeed (<100 µM) daily. Larval health was assessed twice a day, and any larvae found to be showing signs of toxicity were culled via Schedule 1 overdose of MS222. At 13 dpf, larvae were imaged using the Leica DMi8 inverted fluorescent microscope, in order to quantify neutrophils within the intestine, as described below under 'Quantification of immune cell trafficking by fluorescence microscopy'. Image processing and data analysis was carried out as described below under 'Image analysis'. The top concentration of DSS which demonstrated a statistically significant upregulation of neutrophilic infiltration within the mid/posterior intestine was selected for use in the final *in vivo* experiments.

5.4.8 *In vivo* exposure experiments for the quantification of immune cell trafficking (3 – 10 dpf)

To characterise the effects of diclofenac and meloxicam on the inflamed intestine of zebrafish larvae, two transgenic reporter lines were used to quantify the intestinal infiltration of neutrophils – *tg(MPX:EGPF)*^{i114} – and macrophages – *tg(MPEG1:mCherry-CAAX)*^{gl26} – via fluorescent microscopy and *in vivo* imaging. Diclofenac and meloxicam were tested at six

concentrations, including a control group (clean Tecniplast system water). Diclofenac test groups included: **1**.) control (Tecniplast system water); **2**.) DSS (0.1 g/L); **3**.) diclofenac (1.33 mg/L); **4**.) diclofenac (0.67 mg/L) + DSS (0.1 g/L); **5**.) diclofenac (1.33 mg/L) + DSS (0.1 g/L); and **6**.) diclofenac (2.66 mg/L) + DSS (0.1 g/L). Meloxicam test groups included: **1**.) control (Tecniplast system water); **2**.) DSS (0.1 g/L); **3**.) meloxicam (6.75 mg/L); **4**.) meloxicam (3.38 mg/L) + DSS (0.1 g/L); **5**.) meloxicam (6.75 mg/L) + DSS (0.1 g/L); and **6**.) meloxicam (13.5 mg/L) + DSS (0.1 g/L). Individual *tg(MPX:EGFP)^{i114}* or *tg(MPEG1:mCherry-CAAX)^{gl26}* larvae at 3 dpf were randomly allocated to individual wells of 24-well plates containing 1 ml of media (n = 18 per treatment group). Throughout the experiments, all plates were kept at 27±1^{0}C. The exposures lasted for 168 hours until 10 dpf with daily media replacement (70%) to ensure the maintenance of high water quality and feeding from 6 to 10 dpf (Zebrafeed <100 µM). Health was monitored twice every 24 hours, with any fish showing signs of toxicity culled via Schedule 1 overdose of MS222. All experiments were run in duplicate, and each compound was tested over two days; on each day, 18 larvae per treatment group underwent imaging. This meant that 36 larvae per treatment group were used in total for each experiment; power calculations, using pilot data, estimated between 20 and 31 larvae (80 – 95% power, respectively) would be the required number of replicates in order to reach statistical significance.

5.4.9 Quantification of immune cell trafficking by fluorescence microscopy

At the end of the exposure period, 10 dpf zebrafish larvae were anaesthetised using 100 mg/L of MS222. Once immobile, larvae were transferred to a solution of 10 mg/mL low melting point agarose/100 mg/L MS222 and mounted onto microscopy slides fitted with silicone isolating rings. Under a stereo microscope, larvae were orientated on their sides. Once successfully orientated, each slide was moved onto a dry bath (4^{0}C) for approximately 20 seconds to facilitate the agarose semi-solidification, and subsequently transferred onto the stage of a Leica DMi8 inverted fluorescent microscope, where individual larvae were imaged at 10x magnification. Brightfield (BF), and either GFP (neutrophil reporter) or mCherry (macrophage reporter) filters were used to capture overlay and individual filter images of the mid/posterior gut region of each larva. Immediately after imaging, larvae were culled via Schedule 1 overdose of MS222 (300 mg/L).

5.4.10 Image analysis

Images were processed using the software ‘ImageJ’ (version 1.52a). Overlay images were generated for each larva using the raw individual filter images. The ‘Fire’ look-up table was used on all images to aid in the visual distinction of individual immune cells. Manual

quantification of immune cells within the mid/posterior gut region of each larva was performed, and then recorded using the software 'GraphPad Prism 8' (version 8.4.3).

5.4.11 Statistical analyses

Normality tests (D'Agostino & Pearson test) and parametric one-way ANOVA (analysis of variance), using 'GraphPad Prism 8' (version 8.4.3), were used to determine both the Gaussian distribution and statistical significance of the in vivo imaging data. Dunnett's test for multiple comparisons was used to assess the difference between the control group and each test group per experiment (adjusted P value; $\alpha = 0.05$).

5.4.12 *In vivo* imaging control group analyses

Water control groups from Chapter 4 and Chapter 5 *in vivo* imaging experiments were compared using parametric one-way ANOVA (Tukey's multiple comparisons) to assess the variability between controls over time. DSS control groups were similarly compared using non-parametric T-test's (Mann-Whitney) in order to better understand the variability between these groups over time, and between experiments. Non-parametric T-tests were used as each comparison was between only two groups. Non-parametric tests were used if the data was deemed non-normal using D'Agostino & Pearson's normality test.

5.5 Results

5.5.1 Determination of DSS maximum tolerated concentration (MTC) (3 – 5 dpf)

After 48 hours of exposure to 9, 4.5, and 2.5 g/L of DSS, 95.5%, 59.1% and 31.3% of the larvae in these test groups were unresponsive with no obvious heartbeat, respectively. The remaining larvae in these test groups appeared normal and were responsive to seeker. No toxicity or mortality was detected in the control group, and larvae were freely responsive to seeker stimulus. These data were surprising since typical concentrations in larval models of DSS-induced colitis from the literature range from 2.5 to 5 g/L (Oehlers *et al.*, 2013).

5.5.2 DSS model validation (3 – 5 dpf)

Due to the surprisingly high levels of toxicity induced by the concentrations of DSS in the previous experiment, we adjusted the concentration range in order to successfully establish a maximum tolerated concentration of DSS for 48 hours exposure (Figure 5.2). In the test group

exposed to 2.25 g/L DSS, 22.7% of larvae were found to be unresponsive with no obvious heartbeat after 48 hours. All of the larvae in the remaining test groups (0, 0.65, and 1.25 g/L DSS) were healthy with no obvious signs of toxicity. Therefore, the maximum tolerated concentration of DSS was set at 1.25 g/L. After 48 hours of exposure larvae were imaged using fluorescent microscopy, in order to establish whether increased infiltration of neutrophils in the intestine had occurred. Test groups 1.25 g/L and 2.25 g/L produced a statistically significant upregulation of neutrophil infiltration into the mid/posterior intestine of transgenic larvae (*tg(MPX:EGFP)*^{i114}) at 5 dpf (One-way ANOVA; P<0.05 and P<0.001, respectively (Dunnett's multiple comparisons). Although test group 0.65 g/L was not found to be statistically significant using ANOVA, a multiple comparisons test for linear trend was found to be significant from left to right (P<0.05).

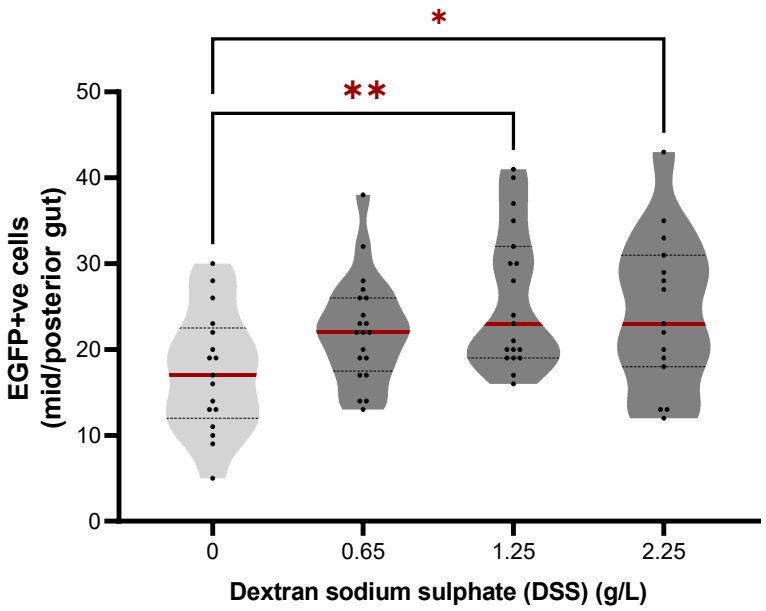

Figure 5.2. DSS model validation from 3 – 5 dpf. *Red horizontal lines represent the mean number of immune cells per group; black dashed lines represent the quartiles; red stars indicate the level of statistical significance of the treatment groups compared with control group (* = P < 0.05; ** = P < 0.001 (one-way ANOVA; Dunnett's multiple comparisons)). N = 17, 20, 19, 15 (0 – 2.25 g/L, respectively).*

5.5.3 Pilot exposure to determine the longer-term safety of DSS (3 – 10 dpf)

A Kaplan Meier plot was generated using survival data from the pilot exposure to diclofenac (Figure 5.3). After 96 hours of exposure two larvae in the control group showed signs of sickness, and after 120 hours one larva in the 0.001 g/L DSS group also showed signs of toxicity, and so these larvae were immediately culled via Schedule 1 overdose of MS222. The remaining larvae in all test groups appeared healthy and responsive to stimulus, showing no signs of toxicity up to 168 hours of exposure (3 – 10 dpf). These data suggested that 0.1 g/L DSS was an appropriate concentration for the final *in vivo* exposure experiments, since there were no obvious signs of toxicity in this test group at 10 dpf.

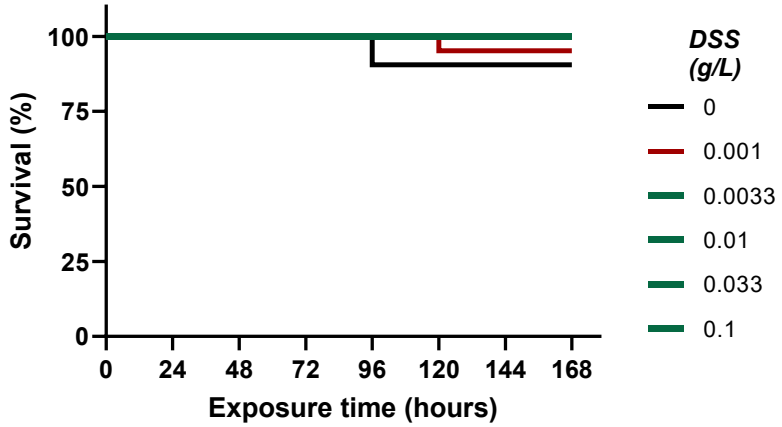

Figure 5.3. Kaplan Meier survival analysis for DSS exposed larvae. *3 – 10 dpf exposure period during which any larvae showing signs of toxicity were culled via schedule 1 overdose of MS222.*

5.5.4 Validation of DSS-induced intestinal inflammation (3 – 13 dpf)

Fluorescent microscopy and image analysis revealed that 0.01, 0.033, and 0.1 g/L concentrations of DSS induced a statistically significant increase of neutrophilic infiltration into the mid and posterior gut of transgenic larvae (*tg(MPX:EGFP)*^{i114}) at 13 dpf (Figure 5.4). These data confirm that 0.1 g/L of DSS represents an appropriate concentration for the induction of intestinal inflammation. Hence, we selected 0.1 g/L of DSS for use in the following large-scale *in vivo* exposure experiments.

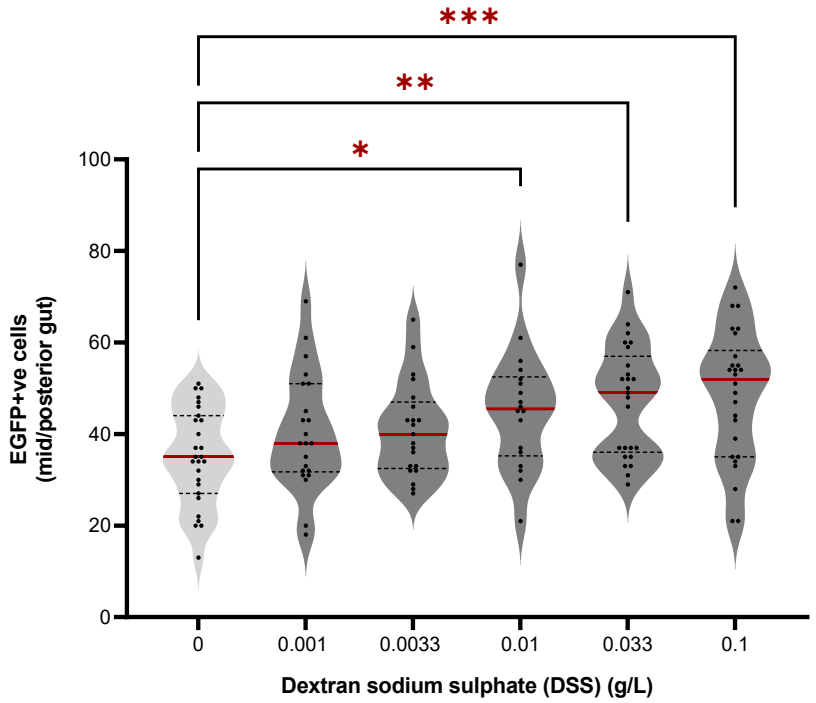

Figure 5.4. Validation of DSS-induced intestinal inflammation at 13 dpf. *Red horizontal lines represent the mean number of immune cells per group; black dashed lines represent the quartiles; red stars indicate the level of statistical significance of the treatment groups compared with control group (one-way ANOVA (Dunnett's multiple comparisons) * = P < 0.05; ** = P < 0.01; *** = P < 0.001). N = 27, 22, 21, 18, 25, 26 (0 – 0.1 g/L, respectively).*

5.5.5 Quantification of NSAIDs-mediated immune cell trafficking in the intestine of inflamed larvae

Larvae exposed to DSS (0.1 g/L) demonstrated a statistically significant upregulation of neutrophils (MPX:EGFP reporter cells) into the mid/posterior intestine at 10 dpf, compared with control fish (one-way ANOVA (Dunnett's multiple comparisons; $P < 0.05$) (Figure 5.6). In the first experiment in Figure 5.5, DSS did not produce a statistically significant up-regulation of neutrophils into the intestine, although a similar trend of increase was seen. This was surprising considering the pilot data results suggesting statistically significant effects from concentrations of DSS as low as 0.01 g/L (Figure 5.4). DSS also appeared to have no statistically significant effect on the numbers of macrophages (mCherry +ve reporter cells) infiltrating the intestine (Figure 5.7, and Figure 5.8). The mean number of neutrophils infiltrating the GI mucosa in larvae exposed to 1.33 mg/L diclofenac was not significantly higher than in the control group (one-way ANOVA (Dunnett's multiple comparisons)) (Figure 5.5), which supports the data generated in Chapter 4. Diclofenac at 1.33 mg/L was selected for the Chapter 5 experiments based on the originally analysed Chapter 4 data, which suggested that 1.33 mg/L would be sufficient for inducing intestinal inflammation. However, re-analysis of the Chapter 4 data suggested that 5.31 mg/L would have been a more appropriate concentration to use. There was no statistically significant effect on the numbers of macrophages residing within the intestine of our larvae exposed to diclofenac (1.33 mg/L) (Figure 5.7), which is in conflict with the data produced in Chapter 4. Equally, exposure to meloxicam (6.75 mg/L) did not produce any statistically significant effects on immune cell trafficking into the mid/posterior intestine, compared with control larvae (Figure 5.6, and Figure 5.8). Larvae exposed to diclofenac (0.67, 1.33, and 2.66 mg/L) combined with 0.1 g/L DSS, showed no statistically significant effects on neutrophil trafficking within the intestine, when compared with DSS (0.1 g/L), or diclofenac (1.33 mg/L) alone (Figure 5.5). Similarly, a co-exposure of meloxicam (3.38, and 6.75 mg/L) and DSS (0.1 g/L) did not result in any significant increase or decrease in neutrophilic infiltration, compared with either DSS (0.1 g/L) or meloxicam (6.75 mg/L) alone (Figure 5.6). The irreproducibility of diclofenac and meloxicam to induce effects on macrophage migration into the intestine, and the lack of DSS-induced effects on macrophage numbers, indicates that macrophages may not be reliable indicators of intestinal inflammation (Figure 5.7, and Figure 5.8).

It is important to note that several larvae exposed to 'meloxicam (13.5 mg/L) + DSS (0.1 g/L)' showed unexpected signs of toxicity prior to imaging at 10 dpf during the first experiment with meloxicam. Therefore, the larvae in this test group were not used for imaging and they were immediately culled via schedule 1 overdose of MS222. This test group was not used for any further experiments.

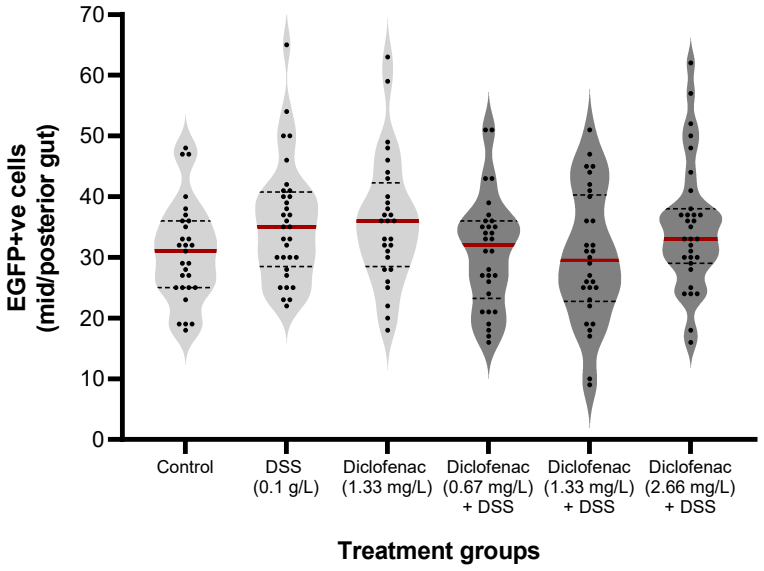

Figure 5.5. Quantification of diclofenac-mediated neutrophil trafficking in the intestine of inflamed larvae. *Red horizontal lines represent the mean number of immune cells per treatment group; black dashed lines represent the quartiles. One-way ANOVA was used to assess for statistical significance of the data. N = 29, 32, 28, 30, 30, 31 (treatment groups, left to right).*

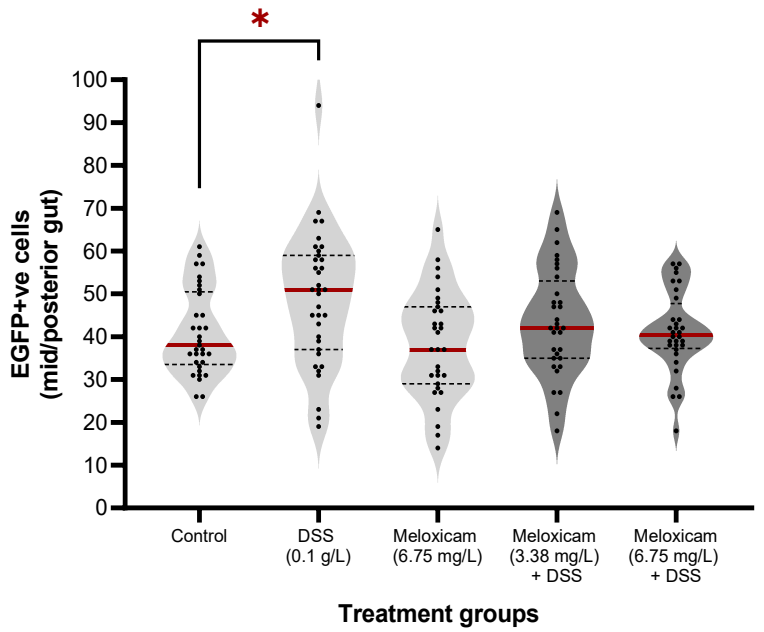

Figure 5.6. Quantification of meloxicam-mediated neutrophil trafficking in the intestine of inflamed larvae. *Red horizontal lines represent the mean number of immune cells per treatment group; black dashed lines represent the quartiles; red stars indicate the level of statistical significance of the treatment groups compared with the control group (One-way ANOVA (Dunnett's multiple comparisons); * = P < 0.05). N = 33, 35, 31, 32, 32 (treatment groups, left to right).*

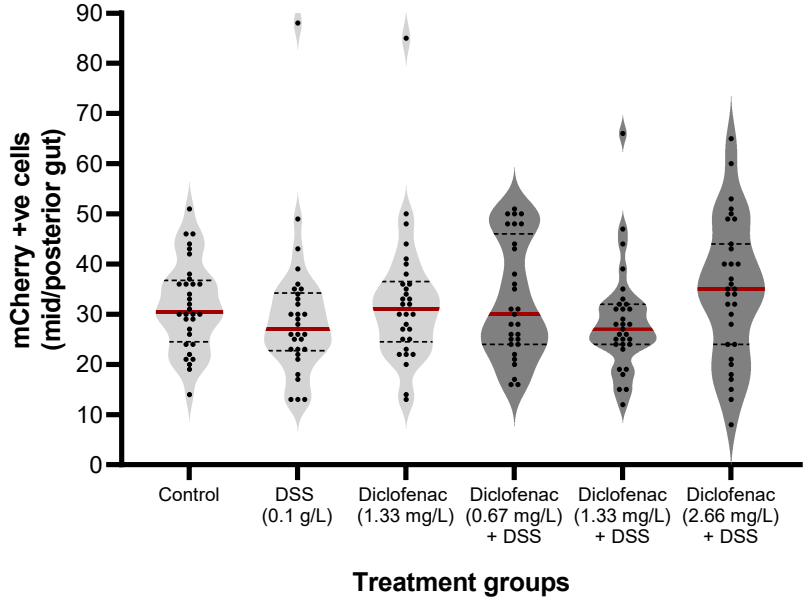

Figure 5.7. Quantification of diclofenac-mediated macrophage trafficking in the intestine of inflamed larvae. *Red horizontal lines represent the mean number of immune cells per treatment group; black dashed lines represent the quartiles. Non-parametric one-way ANOVA performed, as majority of treatment groups not normally distributed (only 'Control' and 'Diclofenac (2.66mg/L)+DSS' normally distributed). N = 32, 30, 30, 29, 31, 31 (Treatment groups, left to right).*

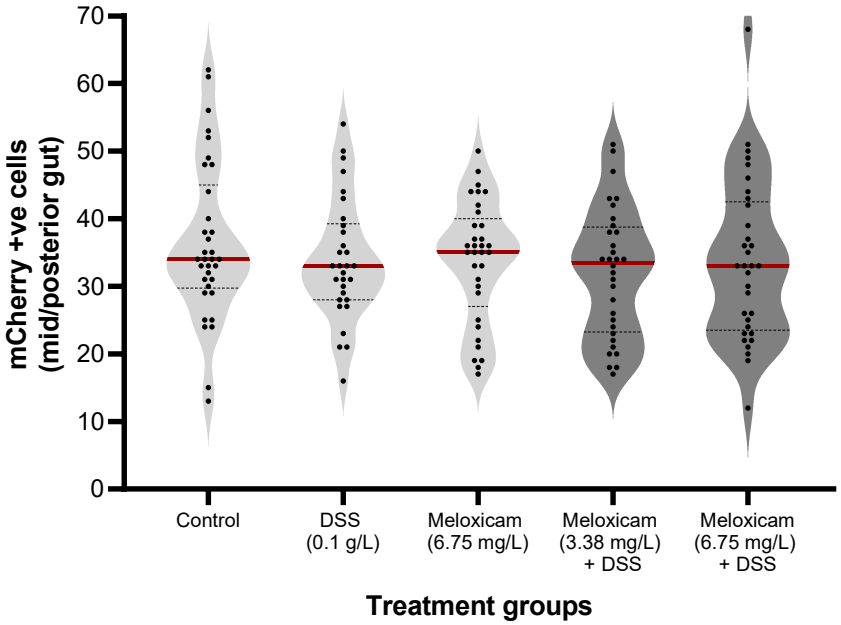

Figure 5.8. Quantification of meloxicam-mediated macrophage trafficking in the intestine of inflamed larvae. *Red horizontal lines represent the mean number of immune cells per treatment group; black dashed lines represent the quartiles. Ordinary one-way ANOVA used to assess the data for statistical significance. N = 34, 30, 33, 32, 33 (Treatment groups, left to right).*

5.5.6 *In* vivo imaging control group analyses

The water control groups from each of the imaging experiments (Chapter 4 and 5) were compared using one-way ANOVA, in order to understand the variability between controls over time and between experiments. Figure 5.9 (A) represents the four control groups from the imaging experiments using the neutrophil reporter line (tg(MPX:EGFP)^{i114}) in order of time, from earliest to latest experiment. It is clear to see a trend of increasing median numbers of neutrophils within the gut of the control fish over time. The normally distributed data revealed a statistically significant difference between the control groups (one-way ANOVA; P<0.0001), with Tukey's multiple comparison test revealing a significant difference between control groups one, two, and three, with control group 4 (Adjusted P value <0.0001, P<0.0001, P<0.0002, respectively). Figure 5.9 (B) represents the four control groups from the imaging experiments using macrophage reporter line (tg(MPEG1:mCherry-CAAX)^{gl26}) in order of time, from earliest to latest experiment. It is clear to see a trend of increasing median numbers of neutrophils within the gut of the control fish over time. The normally distributed data revealed a statistically significant difference between the control groups (one-way ANOVA; P<0.0001), with Tukey's multiple comparison test revealing a significant difference between control groups one and three, one and four, and two and four (adjusted P value < 0.0001).

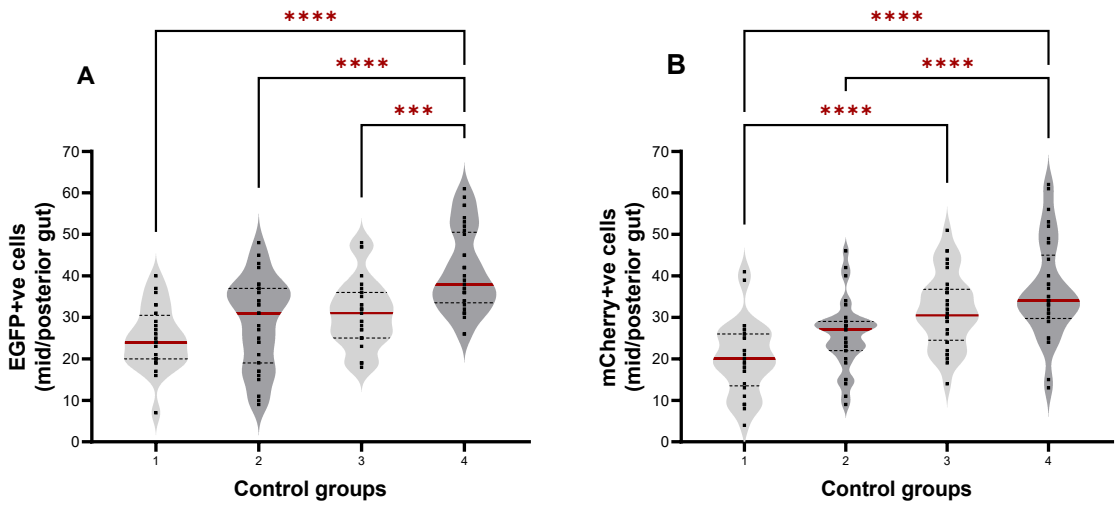

Figure 5.9. In vivo imaging water control group analyses of (A) neutrophil (tg(MPX:EGFP)^{i114}) and (B) macrophage (tg(MPEG1:mCherry-CAAX)^{gl26}) reporter lines. *Red horizontal lines represent the mean number of cells per treatment group; the black dashed lines represent the quartiles; the red stars represent statistical significance (One-way ANOVA (Tukey's multiple comparisons); *** = P < 0.0002; **** = P < 0.0001). (A) N = 24, 31, 29, 33; (B) N = 29, 32, 32, 34.*

The DSS control groups from the *in vivo* imaging experiments in Chapter 5 were compared, in order to better understand the variability between experiments (Figure 5.10). It is clear that between the experiments there is a statistically significant difference in the numbers of immune cells infiltrating the mid/posterior intestine between experiments, indicating a high level of variability in the control groups.

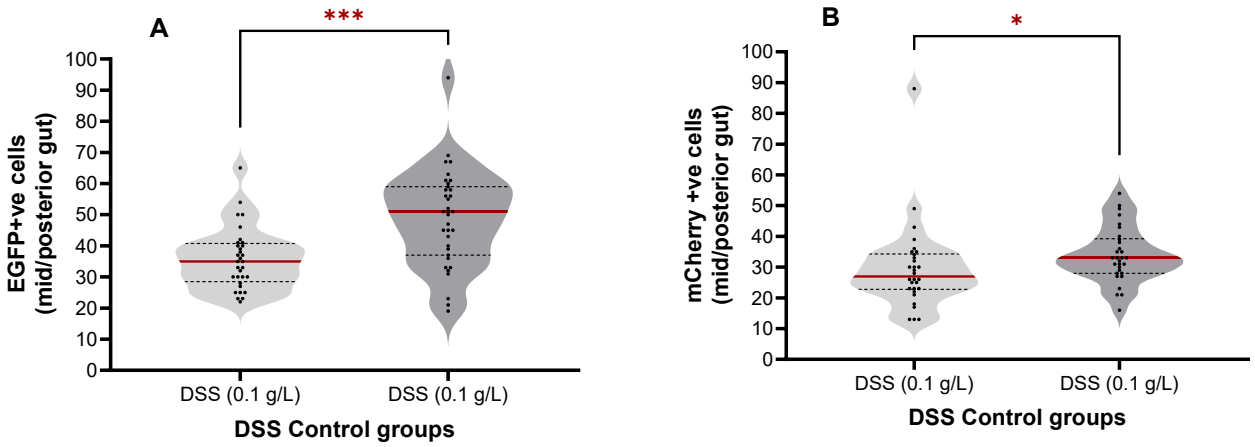

Figure 5.10. *In vivo* imaging DSS control group analyses of (A) neutrophil (*tg(MPX:EGFP)^{i114}*) and (B) macrophage (*tg(MPEG1:mCherry-CAAX)^{gl26}*) reporter lines. *Red horizontal lines represent the mean number of cells per treatment group; the black dashed lines represent the quartiles; the red stars represent statistical significance (Non-parametric T-test (Mann-Whitney); * = P < 0.05; *** = P < 0.0001). (A) N = 32 & 35; (B) N = 30.*

5.6 Discussion

The aim of our study was to try and characterise the immunomodulatory effects of NSAIDs in inflamed tissues. It is well known that NSAIDs are capable of inducing significant adverse effects in the GI tract, kidney, liver, and cardiovascular system. However, they are also effective at alleviating pain and inflammation at specific damage sites. We believe that problems arise due to the systemic exposure associated with NSAIDs administration, whereby homeostasis in 'healthy' tissues is disrupted through the inhibition of constitutive prostaglandin synthesis, and the additional downregulation of important pro-resolution and anti-inflammatory proteins. Perhaps unsurprisingly, considering their purpose, NSAIDs seem to afford protection to tissues in a state of inflammation, including the intestine. Although this may seem obvious, NSAIDs are known to induce severe GI complications in the clinic and we have previously demonstrated that diclofenac exposure leads to neutrophilic inflammation in the intestine, in addition to modulation of several key mediators of inflammation (refer to Chapter 4 results for details). In this Chapter we show that the colitis-inducing chemical DSS is able to induce neutrophilic inflammation in the mid/posterior intestine of zebrafish larvae at 10 dpf (Figure

5.4). However, our original experimental design for this Chapter was based on previously analysed data in Chapter 4 which suggested that 1.33 mg/L of diclofenac would be sufficient to induce intestinal inflammation. After re-analysis of the data it appears that 1.33 mg/L diclofenac does not significantly upregulate the infiltration of neutrophils into the intestine, and 5.31 mg/L would have been the appropriate concentration to use. The current experimental design for Chapter 5 using increased concentrations of diclofenac may have led to some statistically significant data being produced. There is a trend of increased neutrophilic infiltration into the intestine of zebrafish exposed to 1.33 mg/L diclofenac, however, the threshold for statistical significance was not met. Nevertheless, it is worth discussing the effects of NSAIDs on inflamed tissues from sources in the literature, in order to gain a better understanding of what those effects might have looked like.

Since NSAIDs are prescribed, or bought over the counter, to treat a multitude of inflammatory conditions it is inevitable that exposure to both healthy and inflamed tissues will occur in these patients. For example, rheumatoid arthritis (RA) and osteoarthritis (OA) patients are often prescribed NSAIDs to relieve pain and inflammation in their diseased tissues. However, NSAIDs formulations and delivery methods are not currently targeted to specific tissues (Crofford, 2013), although topical NSAIDs are the least systemic application, and thus probably the least toxic (Derry, Moore, and Rabbie, 2012). Nonetheless, there remains a level of systemic exposure in non-inflamed tissues which may result in adversity in cases of patients with increased risk factors and chronic exposure. It is evident that patients taking NSAIDs chronically, such as in cases of RA and OA, often present with a significant risk of serious gastrointestinal adverse events (Tacheci *et al.*, 2016). Moreover, there is also evidence to suggest that NSAIDs may actually increase TNF-α production in the synovial joints of RA patients, exacerbating inflammation in their diseased tissue (Page *et al.,* 2010). Clearly there exists uncertainty as to whether NSAIDs are in fact beneficial to RA patients, or whether they are likely to exacerbate their disease. Drawing parallels with the uncertainty surrounded NSAIDs effects in RA patients, questions surrounding NSAIDs use and the implications for IBD patients have been under debate in the literature for many years. There are many examples of experimental data which suggest that NSAIDs exacerbate IBD phenotypes in various animal models, and also plenty of other evidence to the contrary. For example, one study demonstrated that DSS-induced colonic lesions in rats were exacerbated through the administration of either non-selective, COX-1 selective, or COX-2 selective NSAIDs (Okayama *et al.*, 2007). However, one review determined that it is unclear whether the use of NSAIDs by patients with IBD increases the risk of inducing flare ups, or indeed whether COX-2 selective drugs provide a safer alternative (Klein and Eliakim, 2010). Following this, a case-controlled study examining the association between the use of NSAIDs and flare up of IBD

concluded that NSAIDs were not associated with any increased risk of IBD relapse and may actually be protective in patients with ulcerative colitis (UC) (Hensley and Beales, 2015). Furthermore, three years later a systematic meta-analysis concluded that no reliable association between NSAIDs use and the risk of exacerbating IBD could be found in the literature (Moninuola *et al.*, 2018). It is clear that more work is needed to identify the mechanisms at play to inform safety decisions about appropriate NSAIDs selection.

The mechanism by which DSS induces intestinal inflammation is generally thought to arise from disruption of the intestinal epithelial cell (IEC) lining, which leads to luminal contents entering the mucosa and initiating an inflammatory response. Hence, DSS does not directly induce intestinal inflammation but rather directly injures IECs, and destabilises the mucus layer, which inevitably leads to disruption of intestinal barrier function (Eichele and Kharbanda, 2017). In zebrafish larval models of colitis a concentration of 0.5% (5 g/L) is typically used for an exposure period of 3 – 6 dpf yielding a mortality rate of around 10% (Oehlers *et al.*, 2013). Our long-term exposure period of 3 – 10 dpf meant we were able to use a significantly lower concentration of DSS (0.01%; 0.1 g/L), which allowed us to steer clear of mortality whilst maintaining a reproducible level of intestinal inflammation. Moreover, since our aim was not to specifically model colitis or generate intestinal ulceration, but simply to induce tissue-specific inflammation, we were able to use much lower concentrations of DSS than typical models of colitis. Interestingly, murine models of DSS-induced colitis use doses between 2 – 5% in their drinking water, although comparative doses in zebrafish larvae are incredibly toxic and lead to significant levels of mortality (Oehlers *et al.*, 2013). This discrepancy could be due to the exposure route of each model since whole larval immersion means that multiple tissues are exposed, whereas the murine models provide direct exposure to the GI tract through the drinking water. However, it is certainly worth considering what concentrations between ten- or even hundred-fold higher than larval models may have on murine models at the systemic level. Do such high concentrations of DSS lead to undetected toxicity in tissues beyond the gastrointestinal tract in these murine models? Due to the acute nature of several studies, incredibly high concentrations of DSS are used to rapidly induce significant lesions/ulcerations and bleeding. However, the downside of using such high concentrations is that mortality rates also increase. Animals with such severe symptoms, on the brink of mortality, are surely not representative of the human disease state. Hence, attempting to model the effects of drugs like NSAIDs in animals so close to death, with symptoms so severe they are past the point of return, seems like a fruitless exercise. This could perhaps explain the discrepancy between some of the data in the literature mentioned previously. For example, Okayama *et al.* (2007) carried out exposures using 2.5% DSS in rats over a period of six days, resulting in severe lesions which were exacerbated by NSAIDs administration. Whereas the human case-

controlled study carried out by Hensley and Beales (2015) found that NSAIDs did not exacerbate IBD and may in fact be protective to UC patients. If we consider that the DSS-exposed rats could be significantly unwell, beyond the IBD-like phenotypes, it may be unsurprising that the colitis phenotypes are exacerbated by further chemical assault in the form of NSAIDs administration. This rationale helped inform our DSS concentration selection, since our aim was to simply model neutrophilic inflammation in the intestine in order to assess the immunomodulatory effects of NSAIDs in inflamed tissue and stay well clear of mortality.

It is unclear how NSAIDs may help to rescue inflammation in inflamed tissues at the mechanistic level. However, it stands to reason that if the inducible COX-2 isoform is upregulated in response to inflammatory stimuli, it may provide a level of tissue protection attributed to the homeostatic and pro-resolving functions associated with COX-2 (Wallace and Devchand, 2005). There is some evidence in the literature to support this theory, whereby aspirin administered to a rat model of gastritis actually reduced gastric damage and decreased the adherence of leukocytes within mesenteric venules (Souza *et al.*, 2003). Perhaps if a higher concentration of diclofenac was used in our exposure experiments, we may have been able to see a similar statistically significant trend. Although it is worth noting that the COX-2 selective NSAID rofecoxib actually exacerbated gastritis in that study, however, direct comparison between NSAIDs effects within the stomach and within the intestine should be made with caution. Nonetheless, this data may have important implications for patients suffering from chronic inflammatory conditions, such as RA or IBD, who require long-term therapeutic relief from inflammation and pain. Together the evidence suggests that, at least for IBD patients, administration of NSAIDs may produce less GI toxicity than in healthy patients. Perhaps paradoxically the upregulation of COX-2 in the GI mucosa provides a sufficient level of protection from NSAIDs-induced damage in the gut.

5.6.1 Control group analyses

In order the better understand some of the variability between the water and DSS control groups between experiments, we compared the numbers of infiltrating neutrophils and macrophages from each *in vivo* imaging experiment over time (Figure 5.9 and Figure 5.10). Interestingly, both the water controls and DSS controls demonstrated a statistically significant increase in the numbers of immune cells residing within the mid/posterior intestine of zebrafish larvae at 10 dpf over time. These increases appear to complement each other, and it appears likely from these analyses that what might be a considered a baseline value in one experiment, may not be an accurate baseline from which to assess subsequent experiments from. These results highlight the importance of the control groups, as it is clear that it may not be possible

to identify a threshold value for the number of immune cells considered to induce inflammation within the intestine. This baseline value is likely to change significantly from exposure to exposure. It is not clear what factors might affect these changes in baseline, perhaps the age of the breeding stock has an impact, or perhaps the different breeding pairs lead to offspring with varied levels of GFP expression.

5.6.2 Study limitations and future refinements

One limitation could be that we ran co-exposures of DSS with each NSAID, rather than sequential exposures (i.e. exposure to DSS followed by exposure to NSAID). Although this is common practice, it means we cannot be fully certain that either NSAID would have reversed DSS-induced inflammation in the intestine, or whether they actually prevented the initiation of neutrophilic inflammation in the first place. It may be plausible that NSAIDs are able to prevent the initial damage to the mucosal barrier, which is normally induced by DSS, however it is unclear how this might work. Perhaps the combination of chemicals interact with each other in a specific way rendering them unable to exert their toxic effects on IECs, or perhaps DSS behaves as expected and NSAIDs subsequently rescue this phenotype. Interestingly, the top co-exposure group concentration in the meloxicam experiments (13.5 mg/L + DSS 0.1 g/L) was not used due to toxicity at 10 dpf. Although we were unable to image these fish, it suggests that perhaps mixture effects beyond the GI tract increased the level of toxicity in these larvae. Due to the increased cardiovascular risk factors associated with COX-2 selective NSAIDs, it may be possible to hypothesise that the additional DSS insult exacerbated these effects, leading to toxicity. However, this is only speculation since no analysis was possible on these samples. Nevertheless, further work would be needed to fully establish the processes involved.

Another limitation revealed by our imaging data concerns the use of the transgenic macrophage reporter strain in assessing the effects of NSAIDs in the intestine. We found that the data generated in Chapter 4 using the *tg(MPEG1:mCherry-CAAX)*^{gl26} reporter line was not reproducible in the subsequent experiments carried out in this chapter. The macrophage reporter experiments did not reproduce any levels of significance in the repeat test groups (diclofenac 1.33 mg/L, and meloxicam 6.75 mg/L). These data suggest that monocyte/macrophage trafficking may not be a reliable hallmark of NSAIDS-induced GI inflammation, or in fact DSS-induced GI inflammation. Additionally, it seems that in order to assess the effects of diclofenac on an inflamed intestine, using our current experimental design, an increase in the concentration is required. In Chapter 4, our re-analysed data revealed that 5.31 mg/L would have been a sufficient concentration of diclofenac to induce a

statistically significant upregulation of neutrophils into the intestine. However, when designing the experiments for Chapter 5, the decision to use 1.33 mg/L diclofenac was based upon the principle of using the lowest observable effect concentration, in order to reduce the severity of the exposures where possible. Unfortunately, due to the initial inappropriately analysed Chapter 4 data, 1.33 mg/L was deemed to show a statistically significant effect and so this concentration was selected for use in the Chapter 5 experiments.

Similar to Chapter 4, one future refinement for these types of exposures could be to use an alternate neutrophil reporter line (*tg(mpx:Gal4/UAS:Kaede)*) which contains a photoconvertible Kaede protein. The use of this transgenic line would drastically reduce the variability within each exposure group, and may also reduce the variability between control 'baseline' values over time also. This refinement would increase the granularity of our *in vivo* imaging experiments, and may also result in a reductions of the number of replicates required to achieve a statistically significant result.

5.7 Conclusions

Our data was not about to definitively assess the effects of diclofenac on an inflamed intestine as the concentration selected for these exposure experiments was inappropriate. In order to refine the results of this Chapter and provide a more appropriate assessment of diclofenac's effects within an inflamed GI tract, a concentration of at least 5.31 mg/L should be used. Nevertheless, our data demonstrates a trend of increasing numbers of neutrophils within the intestine of larvae exposed to 1.33 mg/L diclofenac, and we are able to show that 0.1 g/L of DSS is likely to be a viable method of robustly inducing neutrophilic inflammation in the GI tract of zebrafish larvae at 10 dpf.

5.8 References

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Chapter 6

Final discussion

6.1 Project summary and overview

The aim of this research project was to advance the understanding of the biological mechanisms underlying the multi-scale effects of non-steroidal anti-inflammatory drugs (NSAIDs), with a particular focus on immune- and inflammation-mediated effects in the gastrointestinal (GI) tract. The two cornerstones of drug development are efficacy and safety assessment. Whereas efficacy is assessed by quantifying a small set of therapeutically relevant endpoints, the aim of safety assessment is to exclude every other effect across all the major organs and systems. The complexity of this biological challenge explains why despite the financial investment of hundreds of millions of pounds, and the development of sophisticated technologies, drug-mediated toxicity remains a major source of attrition during the drug development process; leading to failure of a high percentage of development projects at both pre-clinical and clinical stages (Cook *et al.,* 2014; Morgan *et al.,* 2018; Weaver and Valentin, 2019). Traditionally, regulatory-relevant *in vivo* toxicity testing is largely focused on apical endpoints and does not offer the flexibility to adapt study design by implementing mode-of-action predictions and considerations. With the development of highly precise mechanistic profiling methods and human-relevant *in vitro* assays, the modern drug development process is rapidly overcoming this limitation by increasing Its reliance on predictive, mechanistic, and systems toxicology (ICH M3(R2), S1A,S2(R1),S5(R2), S7A, S7B, S8; Gintant, Sager, and Stockbridge, 2016). For example, between 2005 and 2010, 82% of drug development projects at AstraZeneca were closed at pre-clinical stages due to unpredicted toxicity. Safety issues were not limited to pre-clinical testing as they were also responsible for the closure of 62% of Phase 1, 35% Phase 2a, and 12% Phase 2b clinical projects (Cook *et al.,* 2014). At that stage, AstraZeneca started the implementation of the so-called 5R's framework (Right Target, Right Tissue, Right Safety, Right Patient, and Right Commercial Potential) (Morgan *et al.,* 2018). The 5R's framework is based on an explicit integration of mechanistic considerations at multiple levels throughout the overall process, paying particular attention to the physiological role of both primary and secondary drug targets in both health and disease (Morgan *et al.,* 2018). Following such implementation, between 2012 and 2016, safety issues represented the reason for closure of 50%, 38.5%, and 8% of189rojectcts at pre-clinical, clinical Phase 1, and clinical Phase 2 stages, respectively (*versus* 82%, 62% and 35% of the previous five years). This success highlights the importance of integrating mechanistic considerations, not

just for toxicological studies in general, but also for regulatory toxicity testing. Although this trend is rapidly becoming a mainstream approach in the field of drug/chemical safety assessment for humans (Aronson *et al.,* 2018; Legler *et al.,* 2020), this is not the case in the field of environmental safety assessment, which remains solidly centred on a small set of regulatory *in vivo* tests based on apical endpoints (development, growth, reproduction, and mortality). This limited scope leaves very little room for the implementation of mode-of-action driven approaches into the process pipeline. This PhD project embraces the modern predictive vision based on mechanistic and mode-of-action considerations of NSAIDs. Overall, this project has generated a novel workflow of integrated *in silico* and *in vivo* approaches for assessing NSAIDs safety, and for exploring their adverse effects from both a phenotypic and a mechanistic perspective.

In Chapter 2 and Chapter 3, we demonstrated the value of using existing mechanistic data in order to generate *in silico* predictions of the hazards and risks associated with exposure to both individual NSAIDs and their mixtures, relevant for both humans and wildlife. Mechanistic profiling of existing experimental data in Chapter 2, at multiple levels of biological and functional organisation, aided us in generating data-driven hypotheses and designing the experimental *in vivo* phases of the project. Novel data mining and integration of mechanistic data from several biological databases also allowed us to generate qualitative and quantitative predictions of NSAIDs effects. For example, curated gene expression data from the Comparative Toxicogenomics Database (CTD) was extracted for over 20 NSAIDs, which gave us a qualitative understanding of the molecular footprint of each NSAID. These NSAID-gene interactions were then used to identify potential pathway enrichments and disease associations that are likely to occur from significant perturbation of each set of NSAID-gene interactions. These analyses enabled us to qualitatively assess the potential effects of each NSAID from a molecular level, up to the phenotypic level of interaction. Immunomodulation was apparent at each level of biological organisation amongst the majority of the NSAIDs within the CTD, highlighting a key avenue of research warranting further investigation. In order to assess the risks of drug-target interactions occurring at therapeutically relevant concentrations of NSAIDs, we modelled the quantitative bioactivity data extracted from the ToxCast and ChEMBL databases, against the available human Cmax data from the literature. These analyses highlighted the risk of each NSAID modulating targets at the protein level, at therapeutic exposure concentrations. Many of these targets also happen to be key immunomodulatory proteins including NSAIDs primary targets PTGS1 (COX-1; cyclooxygenase 1) and PTGS2 (COX-2; cyclooxygenase 2), and many secondary targets such as cytokines, interleukins, chemokines, and other important immune system components. This consistent emphasis on immunomodulation from there aforementioned

analyses, at different levels of biological organisation, led us to explore the potential immune-mediated effects of NSAIDs further. Experimental data was mined from a publication by Kidd *et al.* (2016), in order to perform a comparative analysis of the predicted immunomodulatory activity of NSAIDs. Our analysis of the data revealed that distinct NSAIDs may vary significantly in their ability to modulate components of the immune system, providing a qualitative overview of the immunomodulatory potential of each drug. Quantitative information was associated with each NSAIDs' immune cell interactions in the form of an 'immunomod score'. We utilised this quantitative data to carry out a hierarchical clustering analysis in order to identify similarities and differences amongst NSAIDs' effects on immune cell state transitions. This clustering exercise led to the identification of six main clusters ranging from highly active drugs to low activity drugs. This analysis gave us a good idea of which NSAIDs are likely to interact with the immune system, and thus provided us with a strong rationale for NSAIDs selection in our *in vivo* study design. Perhaps unsurprisingly there seems to be a high correlation between the level of immunomodulatory activity and the level of known toxicity associated with each NSAID, since a number of the drugs within the highly active drugs clusters have been withdrawn from the market due to severe adverse drug reactions.

It became clear from our Chapter 2 analyses that, among other endpoints, immunomodulation represents a key mode-of-action warranting further investigation. In Chapter 3 we described the application and expansion of the data-driven approach used in the previous chapter to evaluate NSAIDs-mediated effects specifically in fish species, and to assess the toxicological risk associated with NSAIDs mixtures in the environment. Firstly, we demonstrated the utility of the combined mechanistic dataset generated from ToxCast and ChEMBL. We developed an interaction network for a mixture of 25 NSAIDs, highlighting the promiscuity of these drugs to modulate targets independent of COX-1 or COX-2. The complexity of this hazard network was then refined by applying a realistic internal exposure scenario, generating a risk-based bioactivity network. By using the concentrations of NSAIDs predicted to be present in the blood of wild fish in the UK (highest average measured concentrations in UK wastewater treatment plant (WWTP) treated effluents) – predicted by applying the Fish Plasma Model (FPM) – we filtered each drug-target interaction by AC50 value. The resulting risk-based network displayed only those drug-target interactions which are predicted to occur at this defined exposure scenario. This mechanistic risk-based network gave us an indication of the targets most likely to be modulated under realistic exposure conditions and included COX-1 and COX-2, as well as CXCL8 (C-X-C motif chemokine ligand 8), CXCR1 (C-X-C motif chemokine receptor 1), CCL2 (C-C motif chemokine ligand 2), MMP1 (matrix metallopeptidase 1), ESR1 (oestrogen receptor 1), and PGR (progesterone receptor). Each interaction was also coupled with a risk ratio which enabled us to demonstrate which NSAIDs are driving the risk of each

target modulation (when multiple drugs interact with the same target), and also gave us an idea of the level of risk. For example, the two steroid receptors PGR and ESR1 represent the targets with the highest risk, as blood concentrations of naproxen and diclofenac were predicted to be 15,375-fold and 321-fold higher than their associated AC50 values, respectively. Using this risk-based bioactivity network we performed a gene-to-phenotype association analysis by data mining available databases. Perturbation of the eight targets in our network resulted in a long list of highly specific zebrafish phenotypes. This indicated that significant modulation of these targets could result in profound effects on the cardiovascular system, immune system, liver, pancreas, kidneys, general development, growth, and reproduction. Although this phenotypic anchoring analysis could not provide quantitative indications of the likelihood of each phenotype occurring, it provides very specific predictions that were useful for hypothesis generation in the subsequent *in vivo* phases of the project.

In order to provide a quantitative estimation of the toxicological risk posed by NSAIDs to fish, in the second part of Chapter 3 we described the development and generation of a multi-scale COX-1-centred model, based on 151 data points generated in 26 *in vivo* studies, carried out using 10 different fish species. This model displayed the range of blood concentrations of NSAIDs (expressed as diclofenac-equivalents, using diclofenac human COX-1 IC50 as the reference value) associated with statistically significant adverse phenotypes retrieved from the literature (151 data points), which were categorised into nine types of effect at three different levels of biological organisation. We were then able to overlay the predicted levels of NSAIDs concentrations in the blood of wild fish in the UK, which enables an immediate estimation of the risk associated with exposure to this specific mixture of NSAIDs. Using this concept of pharmacological equivalence enabled us to guide the interpretation of the toxicological relevance of environmental concentrations of NSAIDs, through a visual dynamic model. We anticipate that this model could facilitate in the interpretation of complex data and aid the regulatory decision making process for both single NSAIDS, and mixtures of NSAIDs, in the environment. Interestingly, one of the nine phenotypic endpoints in the model displaying the highest level of risk was ‘immunomodulation’. This also appeared to be the endpoint with highest level of variability, with data points ranging from around 0.097 to 110481.674 ng/mL diclofenac-equivalent predicted blood concentrations. Our Chapter 3 analyses provided quantitative estimations of the risks associated with environmental concentrations of NSAIDs at the mechanistic and phenotypic levels, using both existing mechanistic data and phenotypic effect data. The value of these pharmacology-informed workflows is such that they remove the immediate requirement for additional animal testing to assess the risks associated with environmentally-relevant concentrations of NSAIDs. Ultimately, we were able to determine that the risk posed to fish by environmental mixtures of NSAIDs may not be negligible, in cases

of high drug consumption and low dilution of WWTP effluents. Overall, these Chapter 3 analyses supported the hypothesis generated in Chapter 2 by highlighting immunomodulation as one of the most sensitive endpoints, indicating the highest level of risk, warranting further investigation.

In Chapter 4 we described a novel experimental approach based on the use of transgenic zebrafish larvae to validate our data-driven hypothesis generated in Chapter 2 and Chapter 3. In order to further our knowledge of NSAIDs-mediated effects, and unravel the role played by immunomodulation in the manifestation of GI toxicity, we exposed healthy transgenic zebrafish larvae to a non-selective NSAID (diclofenac) and a COX-2 selective NSAID (meloxicam). Neutrophil and macrophage reporter strains enabled us to visualise these innate immune cell dynamics within the GI tract in real time, via fluorescent microscopy and imaging techniques. These novel analyses confirmed a statistically significant role for neutrophils and macrophages in the pathogenesis of diclofenac-induced GI toxicity (5.31 mg/L). The data concerning meloxicam was somewhat ambiguous, with only the lowest tested concentration of meloxicam showing a statistically significant upregulation of macrophages into the intestine of exposed larvae. However, this result was not supported by dose-response concordance and should be considered with caution. In order to explore the mechanistic effects of each NSAID we performed whole larvae RNA-Seq and differential gene expression analysis. Diclofenac exposure resulted in a total of 530 statistically significant differentially expressed genes (DEGs), whereas meloxicam exposure yielded 193 DEGs. Functional enrichment analyses of all Gene Ontology and KEGG annotations revealed several shared potential effects of both diclofenac and meloxicam. However, these analyses also highlighted some key differences in mechanistic effects between these two NSAIDs. For example diclofenac, and not meloxicam, led to the significant overrepresentation of the GO term *inflammatory response*, highlighting the potential for this NSAID to disrupt normal inflammatory responses. Some of the most interesting genes enriching this annotation include anti-inflammatory annexin A1 genes (*anxa1b, anxa1c,* and *anxa1d*), as well as *lta4h and loxl3b*. Several other mediators of cell adhesion, ECM organisation, and muscle function were also modulated by each NSAID, however, the downregulation of the annexin A1 genes in particular represents a potentially significant novel key event in the pathogenesis of NSAIDs in the GI tract. We predict that the downregulation of key anti-inflammatory and pro-resolving genes (*anxa1b, anxa1c, anxa1d,* and *lta4h*) by NSAIDs contributes significantly to their toxicity through inhibiting the resolution of inflammation, leading to persistent neutrophil infiltration in the damaged GI tract. Non-selective NSAIDs such as diclofenac are more likely to induce GI damage through both topical and systemic effects, leading to the initial neutrophilic response in the GI mucosa, which subsequently remains persistent through the inhibition of COX-2, and downregulation

of the annexin A1 genes. Although we did not see an inflammatory phenotype in the intestine of meloxicam exposed larvae, we did see that many of the same genes were modulated as with diclofenac exposure. We predict that a more chronic exposure period would probably lead to many of the same phenotypes seen in diclofenac exposed larvae. It is clear that the selective inhibition of COX-2 provides some level of protection, in terms of NSAIDs-induced GI inflammation. Overall our data from Chapter 4 supports the notion that the immunomodulatory effects of NSAIDs extend beyond COX inhibition. Although the focus of this research project was to explore the immunomodulatory effects of NSAIDs, our transcriptomic data suggests that the mechanisms underlying the cardiovascular toxicity associated with NSAIDs may stem from significant effects on cardiac and/or vascular smooth muscle cell function. This data and theory is supported by evidence from the literature, which shows the NSAIDs are able to significantly alter the normal functioning of skeletal, cardiac, and smooth muscle cell types.

In a realistic clinical scenario a patient would only consume, or be prescribed, NSAIDs to treat conditions of inflammation and/or pain. This raises questions about the relevance of using 'healthy' models to assess the toxicological effects of drugs like NSAIDs. On the other hand, if a patient suffering with an inflammatory condition is administered NSAIDs, this will inevitably result in the exposure of both healthy and inflamed tissues. Thus, it is clear that determining the role that NSAIDs play in specific tissues, under conditions of both health and disease, is vital to better understanding their toxicological effects. In Chapter 5 we addressed this issue by validating the use of a chemically-induced model of GI inflammation, which is commonly used to model colitis-like phenotypes in zebrafish. Larval immersion in 0.1 g/L dextran sodium sulphate (DSS) proved to be a robust method of inducing intestinal inflammation up to 13 dpf. This inflammation was characterised by a statistically significant upregulation of neutrophil infiltration into the GI mucosa. The initial design of the experiments in Chapter 5 was based upon data initially analysed inappropriately in Chapter 4, from which we selected 1.33 mg/L diclofenac as the concentration able to induce intestinal inflammation. However, re-analysis of the data with correct statistics revealed that 5.31 mg/L would have been the appropriate concentration to use in these Chapter 5 experiments. This would explain the lack of statistically significant results obtained in this chapter, as concentrations too low to detect an effect were used; even though positive trends of increase were still observed. This suggests that had a sufficient concentration of 5.31 mg/L diclofenac been used, the positive trend may have increased such that the threshold for statistical significance might have been reached.

6.2 Clinical considerations

It is well understood that GI toxicity still represents a major safety liability associated with NSAIDs use (Wallace, 1997; Bindu, Mazumder, and Bandyopadhyay, 2020). Considering the global clinical importance of NSAIDs for the treatment of inflammation and pain, it is important to try and better our understanding of the toxicological effects of NSAIDs in the GI tract. Although progress towards uncovering the exact mechanisms of NSAIDs-induced GI toxicity has been made, their pathogenesis is still not completely understood (Wallace, 2008). In Chapter 2 it became clear that NSAIDs effects may significantly extend beyond COX inhibition, as our *in silico* analyses highlighted the wealth of available mechanistic data for NSAIDs at multiple levels of biological and functional organisation. In terms of clinically-relevant information, our qualitative analysis of the CTD data highlighted the types of genes, pathways, and diseases likely to be perturbed by each NSAID. This information may be useful as an initial screen to assess the differences in safety profile between NSAIDs, as the data infers each drugs' level of promiscuity and the types of effects which may come as a result of long-term therapeutic use. Moving from hazard to risk assessment, in terms of safety, requires the incorporation of quantitative information into the analysis to determine the likelihood of each event occurring. The ToxCast/ChEMBL data analysis provided us with mechanistic information about NSAIDs effects at the functional protein level, with AC50 values associated with each drug-target interaction. By overlaying the relevant human Cmax of each NSAID, we were able to quantitatively assess the risk of each drug-target interaction occurring at therapeutically-relevant concentrations. These analysis highlight the value of using mechanistic data to assess risk, as we are able to rapidly assess which NSAIDs are most likely to have off-target effects, and hence which NSAIDs represent the highest level of risk at therapeutic concentrations. Another benefit of our novel computational workflow stems from the potential to guide more informed decision making for prescribing NSAIDs, leaning towards a personalised medicine approach. For example, there is currently no mechanistic rationale for healthcare professionals to follow when prescribing non-selective NSAIDs for pain/inflammation management. Therefore, deciding between either ibuprofen or diclofenac, for example, is based often on anecdotal clinical experience. Although clinical guidelines exist including recommendations on NSAIDs administration for general practitioners (GPs), these include only vague guidance based largely on empirical evidence. However, our *in silico* data analysis suggests that a therapeutic concentration of ibuprofen is likely to interact with 23 targets including the COX proteins (out of 26 total; 88%), whereas diclofenac is likely to interact with only 12 targets including COXs (out of 38 total; 32%). In this scenario, prescribing ibuprofen instead of diclofenac may increase the level of risk to the patient, as a significantly higher number of secondary targets are modulated by ibuprofen. Another example of

benefitting NSAIDs selection could be when prescribing NSAIDs to patients suffering with rheumatoid arthritis; it would be beneficial to choose an NSAID which is not likely to interact with TNF-α, as this pro-inflammatory cytokine is known to be upregulated in their diseased joints (Page *et al.*, 2010). Hence, predictive *in silico* analyses may provide a mechanistic rationale for the selection of a particular NSAID over another in this treatment scenario. The methodology used by Kidd *et al.* (2016) is an excellent example of the huge potential for this type of approach. Our data mining of this predictive *in vitro* immunomodulatory data further indicated the potential for distinct NSAIDs to directly modulate cells of the immune system. It is clear that developing these types of predictive *in silico* approaches further and integrating them into the safety testing process may allow for the characterisation of drug immunomodulatory potential prior to *in vivo* testing, improving the overall process.

Our novel *in vivo* data has contributed to furthering our mechanistic knowledge of NSAIDs immunomodulatory activity in the intestine, highlighting the differences between non-selective and COX-2 selective NSAIDs, and demonstrating the significance of tissue inflammation status in modulating NSAIDs effects. On the whole, our data confirms that neutrophilic inflammation in the GI mucosa represents a key event in the pathogenesis of non-selective NSAIDs-induced enteropathy. We also shed light on a novel putative mechanism for NSAIDs toxicity in the GI tract. The non-selective NSAID diclofenac downregulates annexin A1 genes (*anxa1b, anxa1c, and anxa1d*), as well as other mediators of inflammation/resolution (*lta4h, and loxl3b*). Modulation of these key genes may well be sufficient to inhibit the resolution of inflammation in the gut, following both direct (topical mechanisms) and systemic (inhibition of COX-1 derived prostaglandins) damage to the epithelium by non-selective NSAIDs. Of particular interest are the annexin A1 genes, as human annexin A1 is known to be a potent anti-inflammatory mediator responsible for the clearance of neutrophils, through inhibiting focal adhesion (and thus transendothelial migration) and inducing apoptosis (Sugimoto *et al.*, 2016). Thus, modulating the expression of these genes may well explain the persistent inflammatory phenotype associated with diclofenac exposure in the intestine of healthy zebrafish larvae. Although we know that COX-2 selective drugs present a lower level of risk, in terms of GI toxicity, the risk is by no means negligible (Masclee *et al.*, 2014). In fact, Sigthorsson *et al.* (2002) found that chronic inhibition of COX-2 by celecoxib was sufficient for significant damage of the intestine to occur, regardless of normal levels of COX-1 derived PGE_2 present in the gut. Even though meloxicam exposed larvae did not display the same inflammatory phenotype as diclofenac exposed fish, it is evident that meloxicam is likely to also modulate a number of COX-independent pathways, similar to diclofenac. Hence, we predict that a more chronic exposure period would ultimately result in similar GI inflammation. Adding weight to this prediction and increasing the likelihood that NSAIDs do inhibit the

resolution of inflammation in the intestine, comes from the further characterisation of COX-2 in the gut. As well as acting as an inducible pro-inflammatory mediator, COX-2 has been recognised as a key component of mucosal defence, which contributes to the resolution of GI inflammation, and regulates ulcer healing (Wallace and Devchand, 2005). Thus, it is biologically plausible that the inhibition of COX-2 with concurrent downregulation of annexin A1 expression, and the modulation of several other inflammatory/pro-resolving genes, will lead to persistent GI inflammation. Our data suggests that perhaps targeting the resolution of inflammation as a strategy to mitigate NSAIDs-induced enteropathy could provide an avenue for novel therapies to combat GI toxicity. For example, developing an agonist of the annexin A1 receptor (FPR2) to take in combination with non-selective NSAIDs may successfully mitigate the immunomodulatory effects of NSAIDs in the GI tract. In fact, strategies to develop small molecule agonists targeting the FPR2 receptor, in order to promote the resolution of inflammation, are already ongoing with treatment areas including rheumatoid arthritis and inflammatory bowel disease (IBD) (Corminboeuf and Leroy, 2015). Glucocorticoids, which are often prescribed to rheumatoid arthritis patients, are known to mediate their effects through the annexin A1 pathway. However, it is well established that they interfere with gastric ulcer healing and increase the risk of GI perforation (Martin *et al.*, 2008). Since annexin A1 has been shown to promote ulcer healing and mucosal defence in experimental and indomethacin induced models of gastric damage, it is likely that glucocorticoids enact these detrimental effects through annexin A1-indpendent mechanisms (Martin *et al.*, 2008). Thus, using an alternative to glucocorticoids would be necessary to alleviate NSAIDs-induced enteropathy through the annexin A1 pathway. Since it has been previously documented that indomethacin-induced gastric ulceration is a neutrophil-dependent process (Wallace, Keenan, and Granger, 1990) it is reasonable that targeting pro-resolution pathways, such as the annexin A1/FPR2 pathway, represents a viable route for development of novel therapeutics. For patients who rely on the long-term therapeutic use of NSAIDs for inflammation and pain management, it is clear that current formulations and combinatory drug treatments to mitigate NSAIDs-induced GI toxicity are not sufficiently protective. For example, gastric bleeding and ulceration are not significantly reduced when NSAIDs are enteric-coated to prevent topical contact toxicity, or even when administered as pro-drugs which are inactive until metabolised (Wallace, 2008). Co-administration of NSAIDs with proton pump inhibitors (PPIs) has demonstrated a significant reduction in gastric toxicity (Abraham *et al*., 2008), however, this protection does not extend to the intestine which remains vulnerable to NSAIDs' effects. It is clear that for patients who depend on NSAIDs there is a significant need for alternative methods to mitigate NSAIDs-induced GI toxicity, and our data suggests that a novel avenue for targeted therapies may include the resolution of inflammation through the annexin A1/FPR2 pathway.

An advantage to using zebrafish larvae to study immune cell dynamics in the intestine, over murine models, is that we are able to rapidly image the gut in real time whilst the organism is still alive. Using the rat model, researchers are required to sacrifice the animals, then surgically remove, and homogenise the intestinal tissue; protein content is then estimated in the supernatant using spectrophotometric assay, before calculating an estimate of MPO (myeloperoxidase) activity from the data (Okayama *et al.*, 2007). Clearly the advantage of the zebrafish model here is the ability to directly quantify the number of immune cells in the intestine, without having to make an estimate of neutrophilic activity from an indirect back-calculation. Another advantage to using zebrafish in this comparison, is the fact that we were able to use a much larger sample size (N = ~ 36 larvae per group in total) compared with murine studies where typically N = four to six animals per group is acceptable (Okayama *et al.*, 2007). Another important factor to consider is the concentration of DSS used to induce colitis in murine models. Typically between 2 and 5% is administered, however, Oehlers *et al.* (2013) state that comparative doses of DSS in zebrafish larval models are incredibly toxic and significantly lethal. It may well be plausible that using such concentrations of DSS in murine models leads to severe irreversible damage in the gut, which is not likely to be improved by subsequent assault with NSAIDs. However, the aim of our study was not to specifically model colitis, but to simply induce tissue-specific inflammation which meant we could use much lower (non-lethal) concentrations of DSS.

6.3 Environmental considerations

The current environmental risk assessment (ERA) process is mechanistically agnostic and focuses solely on traditional fate and exposure predictions, with individual compounds level of toxicity experimentally determined using simple tests focusing on apical endpoints (Lee and Choi, 2019). The implementation of pharmacological and mechanistic considerations into the ERA of pharmaceuticals can aid in the understanding of the risk and allow the integration of modern predictive toxicology approaches. Although numerous studies have experimentally demonstrated the positive impact of this type of approach (Margiotta-Casaluci *et al.*, 2014; Margiotta-Casaluci *et al.*, 2016; Valenti *et al.*, 2012) the ERA process remains unchanged. Furthermore, the lack of a mechanistic rationale behind the ERA of individual compounds prevents the application of predictive approaches for the assessment of potential environmental mixtures. The current ERA process has no capacity to assess the effects of mixtures of compounds, which represents a very real and significant limitation of the process. Since the number of exposure scenarios that may occur globally are virtually endless, this suggests that simplistic experimental determination of the risk is impossible to achieve. Thus,

the future implementation of predictive toxicology approaches will be vital to reach environmental protection goals (Marmon, Owen and Margiotta-Casaluci, 2021).

Chapter 3 describes the development of an innovative pharmacology-informed framework, that integrates both drug pharmacokinetic and pharmacodynamic features, in order to inform the ERA of NSAIDs mixtures in the environment. Two distinct predictive *in silico* approaches were described which use both existing mechanistic and phenotypic effect data to assess the risk posed to fish by an environmentally-relevant mixture of NSAIDs. It is important to note that both of our predictive approaches consider predicted internal concentrations based on the FPM, which significantly enhances the predictive power of the model. Previous studies have demonstrated drugs with comparable *in vitro* potency can have very different levels of risk, based on their specific uptake and pharmacokinetic profile (Margiotta-Casaluci *et al.*, 2016). The first approach is based on the combined ToxCast/ChEMBL dataset generated in Chapter 2. Utilising this mechanistic data, we were able to generate a bioactivity network for 25 NSAIDs, highlighting protein targets which are shared amongst each NSAID as well as targets modulated by individual drugs. This hazard-based assessment formed a complex network, highlighting the modulatory potential of each NSAID and for the mixture as a whole. This mechanistic analysis revealed that the 25 NSAIDs have a wide range of mechanisms of action beyond COX inhibition. In order to make an assessment of the risk to wild fish in the aquatic environment we applied a realistic exposure scenario to the network. Each drug-target interaction AC50 value was filtered based on the concentrations of NSAIDs predicted to be present in the blood of wild fish in UK rivers. The exposure scenario used was the highest average measured concentrations of NSAIDs in UK WWTP treated effluents, which were transformed into predicted internal blood concentrations using the fish plasma model (FPM). The original hazard-based bioactivity network could then be filtered by AC50, resulting in a drastic reduction in the complexity of the network. The 25 NSAID mixture with 507 total interactions was reduced to a risk-based network of four NSAIDs (diclofenac, naproxen, ibuprofen, and mefenamic acid) with 12 interactions. This mechanistic assessment of the risk to wild fish in the UK highlights the targets most likely to be modulated by environmental concentrations of NSAIDs, and also highlights the risk ratio of each NSAID modulating each interaction. It is clear that naproxen and diclofenac represent the most significant drivers of risk, since their predicted blood concentrations are 15,375-fold and 321-fold higher than the AC50 values of PGR and ESR1, respectively. These high values were driven by the low ToxCast AC50s reported for naproxen-PGR and diclofenac-ESR1, which were 0.007 nM and 0.5 nM, respectively. In order to move from functional protein level of interaction to apical level phenotype prediction, we used a variety of biological databases to perform target-to-phenotype association analysis. Unfortunately, this meant moving away from risk assessment,

as it was not possible to quantitatively assess the likelihood of each phenotype occurring, or in fact the effect magnitude. Nonetheless, we were able to generate a long list of zebrafish-specific phenotypes associated with modulation of each target from the risk-based bioactivity network. These highly granular qualitative predictions were used to guide the development of the tailored *in vivo* experiments carried out in Chapter 4 of this project. For example, some of the phenotype associations generated include '*abnormal leukocyte migration*', '*abnormal macrophage chemotaxis*', and '*decreased neutrophil number*'.

In order to make a quantitative estimation of the risk posed by NSAIDs mixtures in the UK, at multiple levels of biological organisation, our second approach generated a multi-scale model centred on COX-1 rather than on the entire mechanistic bioactivity network of NSAIDs. As all NSAIDs act on COX-1 and/or COX-2, we considered the cumulative effects of the environmental mixture of NSAIDs in the UK on COX-1 as the key event driving the toxicological risk, using diclofenac as the reference compound. This powerful visual tool summarises all of the existing *in vivo* data concerning the chronic toxicity of NSAIDs in fish, as a single plot. Our model revealed that 30% of effect data points were predicted to occur at internal concentrations lower than the worst-case exposure scenario in the UK, which drops to 9% when a more realistic exposure threshold is applied. Interestingly, immunomodulation was once again highlighted as potentially significant endpoint, as it presented with the highest level of risk. Overall this model enables a dynamic prediction of the risk to fish species in the UK associated with exposure to a mixture of NSAIDs. The utility of this predictive model is such that any exposure scenario concerning NSAIDs, or any other class of compound with a shared mode of action, can be assessed to determine the situation-specific risk. The threshold of predicted internal environmental mixture concentration would simply move along the axis accordingly, to match a new exposure scenario.

We predict that the implementation of predictive toxicology approaches, such as the one we described in Chapter 3, will be essential to better inform regulatory decision making for NSAIDs and other pharmaceutical contaminants. We demonstrated the potential for this type of approach by providing a pharmacology-informed workflow able to guide the incorporation of pharmacokinetic and pharmacodynamic features into the ERA of NSAIDs, removing the immediate need for performing additional animal testing.

6.4 Project limitations and future research priorities

6.4.1 Chapter 2

One challenge that comes from using biological databases such as the CTD that use automated, or semi-automated, approaches to extract data from various sources is quantification of confidence and uncertainty in the datasets. For example, we considered the 'interaction count' as an indirect marker of reproducibility, to estimate confidence in each drug-gene interaction from the CTD, as this value indicates the number of times this interaction has been observed in the literature. Using this logic, drugs like indomethacin, which have some of the highest interaction counts per interaction, give us high levels of confidence in the data. However, NSAIDs like carprofen and tenoxicam which only display one drug-gene interaction, and an interaction count of one, suggest a low level of confidence in the data. Although low interaction counts and numbers of drug-gene interactions do not infer high levels of confidence, it is important to note that this does not necessarily mean a drug is less mechanistically active or promiscuous. The CTD is a database curated from the literature, meaning it is entirely dependent upon the quantity and quality of available research describing the mechanistic effects of these drugs. Moreover, not all drugs have been fully curated in the database which may also contribute to the lack of interaction data available for some of the seemingly 'least active' drugs. Thus, for drugs with a low number of interactions and interaction counts it is plausible that an absence of evidence is not evidence of absence. It may well be that some NSAIDs simply have a higher volume of research associated with them, which can have an impact on the reliability of the CTD. For example, comparing the amount of available data for the NSAIDs ibuprofen and tenoxicam in PubMed revealed that 11,279 papers have been published studying ibuprofen in the last 20 years, whereas only 294 exist for tenoxicam. This example alone highlights the enormous discrepancy in the amount of available data between different drugs. Another important factor to consider when interpreting the data from the CTD is that the drug-gene interactions are not associated with any quantitative data, which means it is not possible to predict at what concentrations of drug these interactions are likely to take place. It is also unclear whether the drug-gene interaction represents an up- or downregulation of expression.

Similar considerations are relevant for the functional *in vitro* bioactivity data generated retrieved from ToxCast and ChEMBL. Quite simply, if the chemical has not been tested, then the data will not be available in the database. With regards to ToxCast, one NSAID may display activity for a particular target, and another NSAID may not. However, this does not necessarily mean that the latter lacks the ability to modulate the same target. It may mean that this particular NSAID has not been tested in one of the specific *in vitro* assays required to assess

this interaction. For example, the data from ToxCast states that naproxen interacts with PGR (progesterone receptor) and that meloxicam does not. However, it is not clear whether meloxicam has been tested in one of the 18 assays designed to detect PGR activity, or whether it was simply 'inactive' in one of these assays. Aside from issues surrounding the quantity of data, inter-assay variability may represent a major confounding factor. ToxCast contains 31 assays which detect the modulation of ESR1 (estrogen receptor 1), and diclofenac was tested in 17 of those assays of which only three returned a positive result, meaning it was 'inactive' in 14 of those assays. Yet, all that is apparent in the bioactivity dataset retrieved from ToxCast is that diclofenac is able to modulate ESR1, even with the weight of evidence leaning in favour of inactivity. Clearly a certain level of uncertainty may be associated with each drug-target interaction, which has previous come under scrutiny in the literature. For example, Janesick *et al.* (2016) identified a high percentage of false positives among the chemicals identified as PPARγ agonists in ToxCast. Overall, these considerations suggest that data generated from large-scale mechanistic profiling initiatives are extremely valuable for generating testable hypotheses. However, using this type of data to drive risk assessment directly may require more caution due to uncertainties surround the high levels of inter assay variability.

6.4.2 Chapter 3

The same considerations outlined for the use of the combined ToxCast/ChEMBL dataset in Chapter 2 are relevant for this same dataset used in Chapter 3. We applied mixture toxicology network concepts to this mechanistic data, and successively incorporated NSAIDs pharmacokinetic and pharmacodynamic features, in order to assess the risks to wild fish species in the UK. Despite the successful application of this network pharmacology approach, there are some considerations worth taking into account. Firstly, the data generated from ToxCast and ChEMBL are mammalian data, most of which is human. It is possible that drug targets display different levels of sensitivity between fish and humans, when exposed to the same compound. However, this may only be an issue in terms of our analyses if the AC50s for fish targets are significantly lower than their respective human AC50 value. To our knowledge there does not seem to be any evidence to support this hypothesis, but nonetheless it is worth taking into account. Secondly, the phenotypic anchoring analysis, which linked the eight targets in the risk-based bioactivity network to zebrafish-specific adverse phenotypes, generates only qualitative predictions. This means that we are unable to infer the effect size of each prediction, which limits the ability of this analysis to directly inform the ERA process.

Some important factors to consider relating to the multi-scale COX-1-centered pharmacology-informed model in Chapter 3, and to the robustness of the ERA process in general, stem from quality of the underlying data. These risk assessment approaches are directly affected by the data used, and in recent years concerns have been raised over the degree of quality and reproducibility of ecotoxicological studies (Harris and Sumpter, 2015; Martin *et al.*, 2019; Mebane *et al.,* 2019). For example, among NSAIDs diclofenac in particular has been the object of intense scientific debate due to discrepancies in toxicological and histopathological findings between various academic studies, and one industry study (Hoeger *et al.,* 2005; Mehinto *et al.*, 2010; Schwaiger *et al.*, 2004; Triebskorn *et al.*, 2004; Memmert *et al*., 2013). Controversy was sparked regarding these discrepancies as the outcomes of the four academic studies were used to justify the decision to add diclofenac to the European Union Watch List of emerging pollutants in 2015. Subsequently, an independent histological review of the samples from three of these academic studies revealed that some of the discrepancies were due to experimental design, however the majority of inter-study variation was driven by issues of diagnostic interpretation (Wolf *et al.*, 2014). This example of diclofenac alone highlights the current difficulties with the ERA process, whereby regulators have to make decisions based on a limited amount of experimental data which may be low quality, unreliable, or irreproducible. Clearly inter-study discrepancies represent a real challenge for regulatory decision-making. Our model does not contain a quality assessment of each study included in the analysis, as it is difficult to set an unequivocal definition of 'quality' applicable to any context, and there is a risk of introducing bias into the model. The major advantage of our model, however, is the ability to assess inter-study variability in a clear visual model which allows direct inter-study comparison. In order to demonstrate some of the variability between data points in the model, we focused on one of the endpoints with the highest regulatory relevance – egg production (which falls under the umbrella of 'reproduction' in our model). By assessing effect magnitude and effect direction our analysis revealed some inconsistencies in the effects between studies using ibuprofen, which caused a decrease in egg production in some studies and an increase in others. These types of discrepancies suggest that this type of retrospective analysis should be taken into consideration during the risk assessment process.

The utility of our pharmacology-informed multi-scale model centred on COX-1 is such that we plan to hopefully develop this dynamic model into a commercial software app. Our workflow can be readily adapted to fit any environmental exposure scenario concerning NSAIDs mixtures, or in fact modified to assess any other class of compounds with a shared mode-of-action. Hence, we see a huge potential for the use of this model by industry and regulators

alike, in order to help rapidly interpret complex sets of biological data within a regulatory-relevant context.

6.4.3 Chapter 4

The first significant obstacle which became apparent during the work in Chapter 4 was NSAID selection, which was a considerably lengthy and laborious task due to technical difficulties with stock solution preparation for a number of NSAIDs. Inaccurate water and solvent solubility information, combined with irreproducible protocols from the literature, meant that trialling celecoxib, rofecoxib, valdecoxib, and parecoxib was unsuccessful. It became abundantly clear that these compounds would not dissolve without using high concentrations of solvent, which are likely to be toxic to zebrafish larvae. This was an important consideration which influenced our NSAIDs selection process, as we decided to avoid using solvents where possible. Although solvent use is common practice when dissolving some compounds, there is a wealth of data which suggests that solvents like DMSO are able to significantly alter the transcriptomic and epigenetic landscape of biological tissues, even at low 'non-toxic' concentrations (Verheijen *et al.,* 2019). After much trial and error, we were able to identify diclofenac sodium salt and meloxicam sodium salt hydrate as our test compounds, as these represented ideal candidates from both a practical and a biological perspective.

In terms of the *in vivo* imaging experiments, one limitation of our model is that we are not able to determine which of the immune cells within the gut tissue at 10 dpf migrated into the GI mucosa as a result of exposure, and which cells were resident in the gut prior to exposure. This limitation means that we see a lot of variability between biological replicates, since individual larvae possess varying levels of tissue-resident immune cells. This means that a large number of replicates are required to generate enough statistical power. Refinement of this limitation for future studies could come in the form of using an alternate neutrophil reporter line, for example. The tg(mpx:Gal4/UAS:Kaede) reporter line contains a photoconvertible Kaede protein which can be selectively converted prior to exposure. This conversion, for example in the GI tract specifically, would convert the resident cells to red, whilst any migrating neutrophils from outside the gut would still fluoresce green (Ellett *et al.,* 2015). This solution would make it inherently clear which cells have infiltrated the GI mucosa as a result of exposure at 10 dpf.

Our RNA-Seq experiment was based on a common design amongst most published RNA-Seq studies involving chemical exposure and zebrafish larvae, whereby we compared three treatment groups with three biological replicates. Our results suggest that perhaps a larger sample size would increase the overall confidence in the data analysis. For example, sample

clustering using PCA and the correlation heatmaps highlighted a potential discrepancy with sample 'CTRL_1_S1', as it did not appear to cluster or correlate well with the other control samples. Another factor to consider is the number of pooled larvae which formed each biological replicate. Our experiment included four larvae per replicate, which was driven by pilot data suggesting that this would give us a sufficient quantity of RNA for a standard input cDNA preparation and sequencing run. We chose not to exceed this number of larvae due to ethical considerations, as 10 dpf larvae are protected animals, and we believed we would extract sufficient RNA. However, the amount of total RNA for three of our 12 samples was below the required threshold for standard input sequencing, and so we proceeded with the ultra-low input alternative. It is plausible that if one of the four larvae in sample 'CTRL_1_S1' was a true biological outlier, then this could skew the overall analysis. These considerations suggest that optimisation of pool size for each sample may be significantly beneficial, in order to dilute intra-group variability. Another consideration which may explain this variability between samples of the same treatment group, is that the transcriptomic signatures of one week old larvae become significantly more complex than earlier life stages (Yang *et al.,* 2013). This may not be surprising considering that embryonic and early life stages have tightly regulated transcriptomic expression patterns due to the essential developmental processes involved. This significant complexity at 10 dpf, combined with our relatively small sample size, may explain why the PCA sample clustering and correlation heatmaps demonstrated poor clustering and correlation between control samples. Hence, using a bigger pool size per sample may also help to dilute the variation in between samples of the same treatment group in the future. Alternatively, RNA extracted from specific organs/tissues (rather than from the whole larvae) may provide a clearer discrimination between treated animals and controls.

Lastly, a key limitation to note is that the transcriptomics analyses utilised whole larvae, whereas the imaging data focused solely on the gastrointestinal tract. This means that although comparisons may be made, and links hypothesised, we are unable to definitively make association between the *in vivo* imaging and transcriptomic analyses.

6.4.4 Chapter 5

Our imaging data in this chapter revealed an important limitation concerning the use of the transgenic macrophage reporter line (*tg(MPEG1:mCherry-CAAX)*^{gl26}). Unfortunately, the Chapter 4 data using this strain was not reproducible in our Chapter 5 experiments. For example, diclofenac exposure led to a significant upregulation of macrophage infiltration into the intestine of zebrafish larvae at 10 dpf in all three treatment groups (1.33, 2.66, and 5.31 mg/L), in our Chapter 4 study. However, the 1.33 mg/L diclofenac treatment group in Chapter

5 was not reproducible and did not demonstrate significant upregulation of monocyte infiltration into the intestine compared with control fish. A similar story is apparent with meloxicam whereby the highly significant increase in infiltration of monocytes in the lowest exposure group in Chapter 4 was irreproducible in the repeat test group in Chapter 5 (6.75 mg/L meloxicam). The inter-study irreproducibility of the macrophage reporter line suggests that perhaps monocyte/macrophage trafficking may not be a reliable biomarker of NSAIDs-induced GI inflammation, or indeed DSS-induced GI inflammation.

Another potential limitation relates to our study design, as we decided to run co-exposures of DSS with each NSAID, rather than a sequential exposure (i.e. exposure to DSS followed by exposure to NSAID). This means that we would be unable to fully ascertain whether either NSAID rescued DSS-induced intestinal inflammation, or whether they prevented the initial neutrophilic response to DSS in the first place. It is possible that NSAIDs prevent DSS-induced damage to the GI mucosa, however, it is not exactly clear how this might work. It could be that the mixture of chemicals react with one another, rendering them unable to exert their toxic effects of IECs, or it could just be that NSAIDs do in fact recuse the DSS-induced neutrophilic inflammation in the intestine. The concept of mixture effects may not be unrealistic as the top co-exposure concentration group of meloxicam + DSS (13.5 mg/L + 0.1 g/L, respectively) demonstrated toxicity at 10 dpf. In Chapter 4 up to 27 mg/L meloxicam showed no signs of obvious toxicity and represented our top concentration group. However, a combination of meloxicam and DSS appeared to increase toxicity in these fish beyond the GI tract. Although we terminated the exposure and did not image these fish for ethical reasons, it suggests that mixture effects led to significant toxicity, the mechanisms of which are unclear. Perhaps due to the increased cardiovascular risk associated with COX-2 selective inhibitors, it may be plausible that further chemical insult with DSS exacerbated these affects, leading to toxicity. However, this is only speculation since no analysis was possible on these samples. Nonetheless, it is clear that further work would be necessary to fully establish the mechanisms at play.

Finally, the biggest limitation of the work in this Chapter was the decision to use 1.33 mg/L diclofenac in our experiments, and not 5.31 mg/L. Initially, this decision was based upon incorrectly analysed Chapter 4 data suggesting that 1.33 mg/L diclofenac was able to produce statistically significant effects of neutrophilic infiltration into the mid/posterior intestine. However, re-analysis of the Chapter 4 data with appropriate statistics revealed that this concentration is too low to produce a significant effect, and 5.31 mg/L diclofenac would have been the appropriate selection for the Chapter 5 experiments.

6.4.5 Future research priorities

Future research should focus initially on reassessing the effects of diclofenac on an inflamed GI tract, using a more appropriate concentration of at least 5.31 mg/L. Following this, I feel that the focus should shift towards the potential link between non-selective NSAIDs, such as diclofenac, and their likely effects on the Annexin A1 pathway in particular. Confirmation of this link through qPCR would be a great first step towards verifying significant modulation of this gene at the level of transcription. Ideally, this would be a tissue-specific analysis which would add to the weight-of-evidence implicating this mechanism of action in the pathogenesis of NSAID-induced GI toxicity. In parallel, I believe that refining the *in vivo* imaging experiments through the use of a Kaede transgenic neutrophil reporter would add significant value to this type of analysis, and significantly reducing the variability seen between samples.

6.5 Concluding remarks

The overall aim of this research project was to further our mechanistic understanding of NSAIDs effects. The evidence presented in this thesis demonstrates the successful development and application of novel *in silico* workflows, and novel *in vivo* experiments, which further our mechanistic knowledge of NSAIDs effects. We explored the key clinical and environmental health implications of NSAIDs use through a number of approaches. Firstly we mined existing experimental data to model multi-level effects, which highlighted the significance of immunomodulation in mediating NSAIDs effects. Subsequently, we developed a pharmacology-informed framework for the environmental risk assessment (ERA) of mixtures, which may pave the way for the future implementation of predictive toxicology approaches into the regulatory decision making process. Successively, we identified a novel putative mechanism of toxicity within the gastrointestinal (GI) tract for the non-selective NSAID diclofenac, implicating the resolution of inflammation in the propagation of NSAIDs effects. We also managed to validate the transendothelial migration of neutrophils into the intestine as a key event driving the pathogenesis of NSAIDs-induced enteropathy. Moreover, we revealed significant differences between traditional non-selective NSAIDs and COX-2 selective inhibitors' phenotypic effects within the GI tract at the cellular and transcriptomic level. Our work supports the notion that NSAIDs effects extend well beyond COX inhibition on a system-wide level, and demonstrates the significance of immunomodulatory processes in mediating these effects within the GI tract.

6.6 References

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Appendix 4.1

The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including:	4.4.6
		a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated.	
		b. The experimental unit (e.g. a single animal, litter, or cage of animals).	4.4.1
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.	4.4.6
		b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	4.4.6
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori*. If no criteria were set, state this explicitly.	4.4.1
		b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.	No exclusions.
		c. For each analysis, report the exact value of *n* in each experimental group.	4.4.6
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.	4.4.6
		b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	4.4.6
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	The sole operator, no blinding was possible.
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).	4.4.8, 4.4.9, and 4.4.13
		b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	4.4.8, 4.4.9, and 4.4.13
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used.	4.4.9, 4.4.13, 4.5.5
		b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	N/a
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.	4.4.1
		b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	4.4.1
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including:	4.4.6
		a. What was done, how it was done and what was used.	4.4.6
		b. When and how often.	4.4.6
		c. Where (including detail of any acclimatisation periods).	4.4.6
		d. Why (provide rationale for procedures).	4.4.6
Results	10	For each experiment conducted, including independent replications, report:	4.5.5
		a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).	4.5.5
		b. If applicable, the effect size with a confidence interval.	

Appendix 4.2

RNA-Seq: Quality control (QC), trimming, mapping, counting, and clustering

After successful library preparation and sequencing run, the number of reads generated for each sample exceeded 23 million (Table S4.1) and was very consistent between samples, suggesting a balanced library pool. The QC process revealed that 'per base sequence quality' for all samples had high quality scores (>30) along the whole length of the reads (Figure S4.1).

Figure S4.1. Per base sequence quality along the read lengths for each sample. *The green shaded area represents a high quality score (>30), which is achieved by every sample.*

Similarly, ‘per sequence quality scores’ confirmed that the overall quality score of the reads for each sample was very high (mean scores >30) (Figure S4.2).

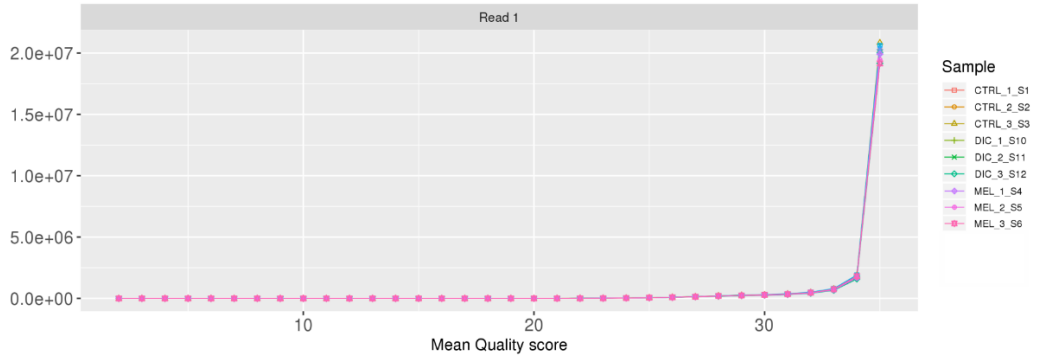

Figure S4.2. Overall per sequence quality scores of the reads for each sample. *Mean quality scores exceeded 30 for all samples, indicating high levels of quality.*

Equally, ‘per base sequence content’ showed little to no difference between the proportions of the four nucleotides across the read’s length. The library preparation caused an anticipated bias at the beginning of the reads, which are represented by the fluctuations seen in Figure S4.3 below.

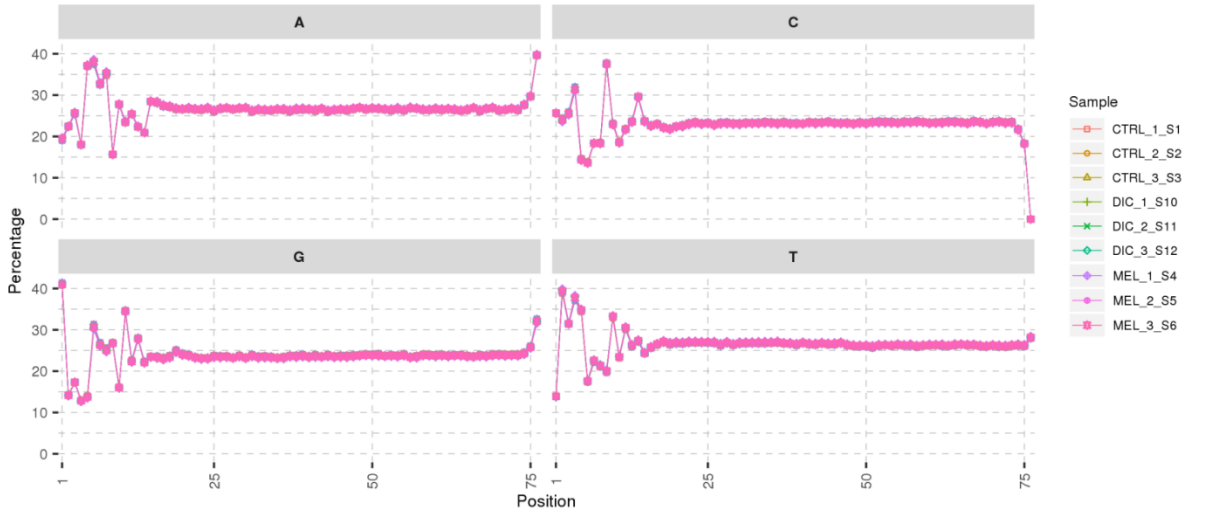

Figure S4.3. Per base sequence content analysis for each sample.

‘Per sequence GC content’ was assessed in order to identify potential contamination or a biased subset in the library. Figure S4.4 shows a normal distribution of GC content across our reads, centred at the overall GC content of the underlying genome. The analysis showed no evidence of contamination, and the peaks are well aligned with each other.

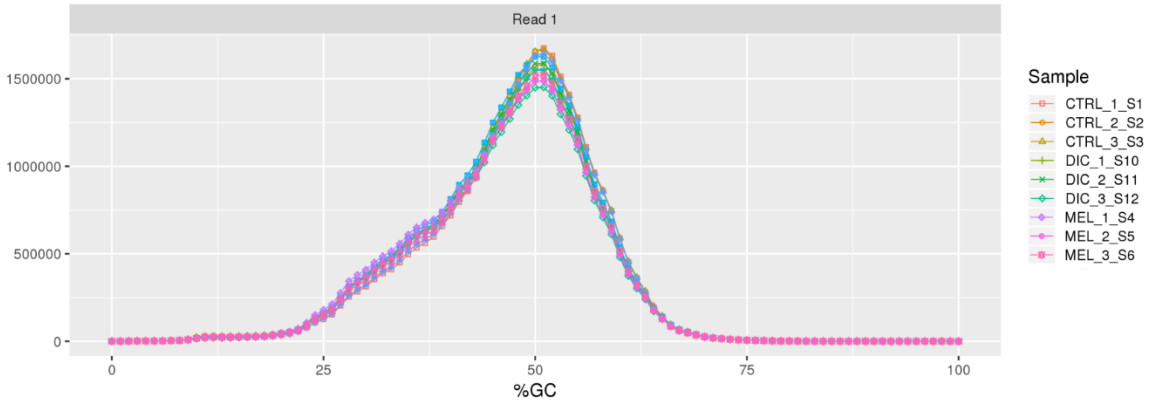

Figure S4.4. Per sequence GC content analysis to identify contamination or biased subset in library. *Normal distribution among all samples, no evidence of contamination or bias.*

‘Sequence length distribution’ of the reads in our libraries was assessed to ensure they match the sequence length used in the protocol, prior to trimming. The sequence length of our reads matched the expected length (~75 bp), which is confirmed by the data visualisation displayed in Figure S4.5.

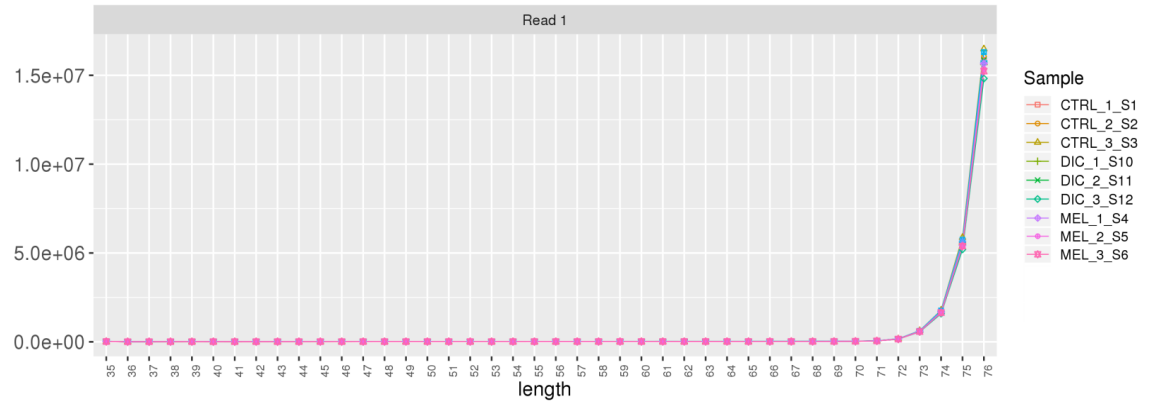

Figure S4.5. Sequence length distribution analysis to assess sequence length of reads in each sample. *All samples contain reads of the expected length (~75 base pairs).*

Sequence duplication levels, displayed in Figure S4.6, were assessed to identify potential read duplications. High levels of duplication may indicate an enrichment bias (e.g., PCR over-amplification) or contamination. However, RNA-Seq libraries will normally show sets of reads at higher duplication levels, which could potentially correspond to highly expressed transcripts. Duplicate reads are not removed from the data, and any peaks after ~>1K copies per read may indicate high polyA content, mitochondrial RNA and/or ribosomal RNA. The profile generated for our data is as expected for this type of experiment.

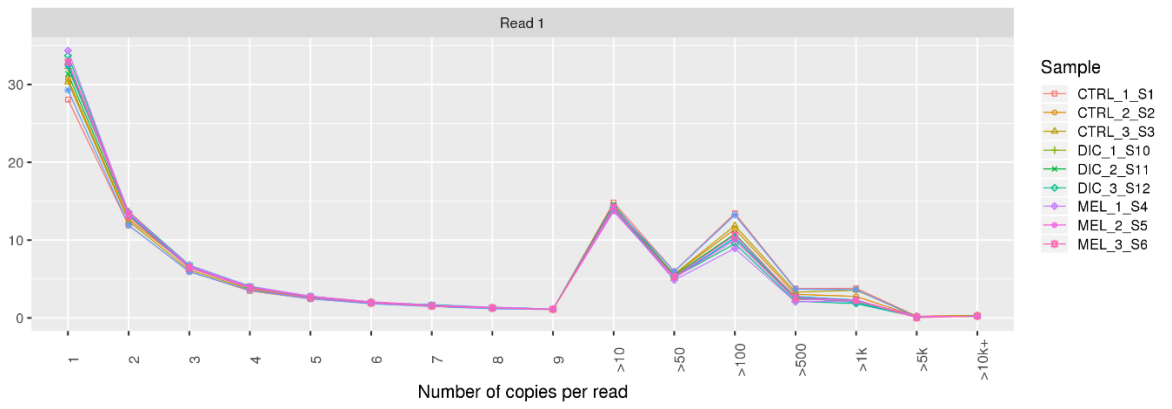

Figure S4.6. Sequence duplication analysis. *Each sample profile is as expected for this type of experiment.*

The number of reads remaining after trimming of low-quality bases and adapter sequences from the 3' end of reads, and the % of reads removed, are displayed in Table S4.1. The number of reads removed due to the trimming process was very low, as expected from good quality reads.

Table S4.1. Number of reads before and after trimming.

Sample	Number of raw reads	Number of reads after trimming	% removed
CTRL_1_S1_R1	24,577,096	24,575,984	0.0045
CTRL_2_S2_R1	24,703,934	24,702,696	0.005
CTRL_3_S3_R1	25,516,305	25,515,169	0.0045
DIC_1_S10_R1	24,172,347	24,171,244	0.0046
DIC_2_S11_R1	24,632,880	24,631,763	0.0045
DIC_3_S12_R1	23,058,241	23,057,198	0.0045
MEL_1_S4_R1	24,164,306	24,163,258	0.0043
MEL_2_S5_R1	23,641,507	23,640,450	0.0045
MEL_3_S6_R1	23,600,565	23,599,469	0.0046

‘Per base sequence content’ was re-assessed to ensure that even proportions of bases across the reads was maintained after the trimming process. Figure S4.7 indicates that the proportions of bases remained uniform after trimming, with an expected drop in the proportion of A nucleotides at the end of the reads due to removal of adapters. Adapters were trimmed due to their interference with downstream analyses, such as mapping of reads to the reference genome.

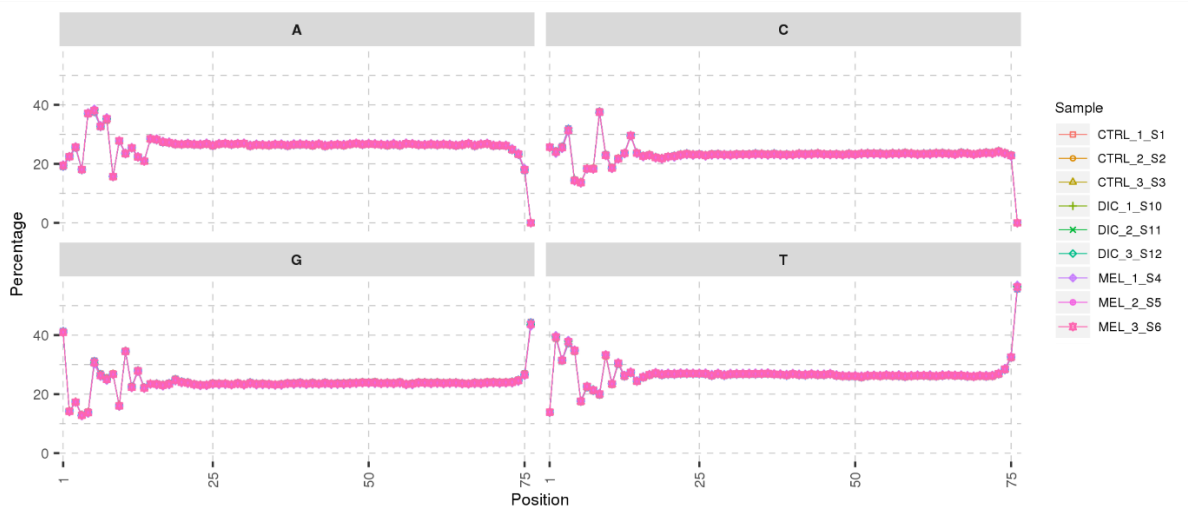

Figure S4.7. Per base sequence content analysis after trimming. *Uniform proportion of bases maintained for each sample, with an expected drop in the proportion of A nucleotides due to trimming of adaptor sequences of reads.*

Table S4.2 summarises the results of the mapping, describing the percentage of uniquely mapped reads, the percentage of reads that mapped to multiple loci, and the percentage of unmapped reads. It is expected that >75% of reads from each sample will uniquely map to the genome. This expectation was confirmed for all the samples analysed in this study as they displayed >90% unique mapping efficiency. Reads which mapped to multiple loci, to overlapping gene regions, or with a mapping quality score <10, were discarded to avoid ambiguity and false positives in the differential expression analysis.

Table S4.2. Summary of the read mapping process.

Sample	% Uniquely mapped	% Multiple loci	% Unmapped	Number of input reads	Number of uniquely mapped reads
CTRL_1_S1	91.34	6.27	2.39	24575984	22448039
CTRL_2_S2	91.15	6.08	2.77	24702696	22517390
CTRL_3_S3	91.04	6.4	2.57	25515169	23228679
DIC_1_S10	90.92	6.51	2.57	24171244	21976817
DIC_2_S11	90.77	6.65	2.58	24631763	22359082
DIC_3_S12	91.19	6.16	2.64	23057198	21026654
MEL_1_S4	91.16	6.06	2.78	24163258	22026624
MEL_2_S5	91.19	6.15	2.65	23640450	21557964
MEL_3_S6	91.24	6.24	2.52	23599469	21532909

Read mapping distribution between different genomic regions is summarised in Figure S4.8, indicating that the largest percentage of reads in each sample is mapped to coding regions (>50%), as expected.

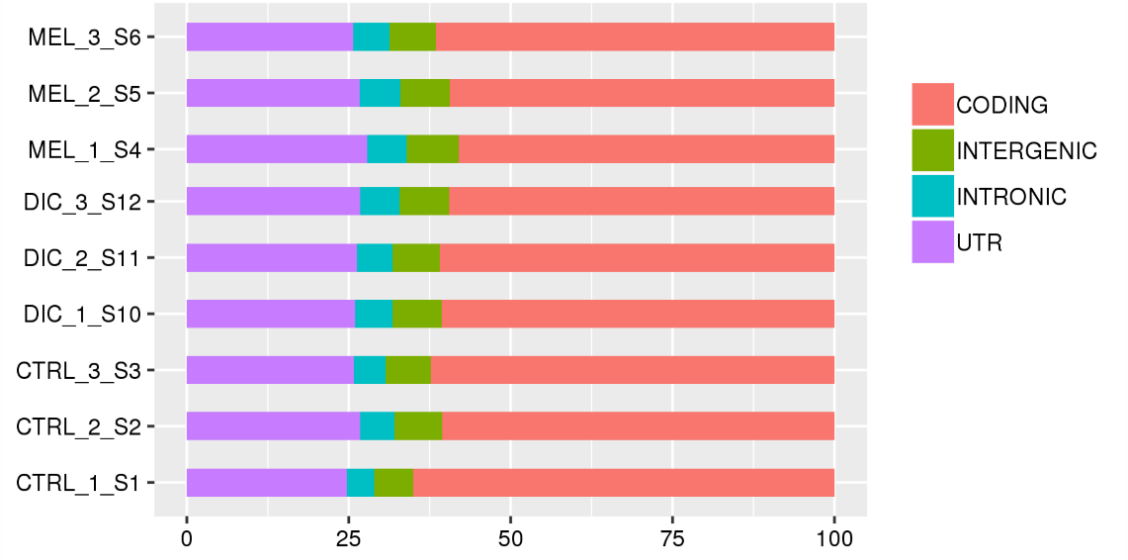

Figure S4.8. Summary of read mapping distribution between genomic regions. *As expected, the majority of reads mapped to coding regions.*

The read coverage (y-axis) along the gene body (x-axis) from 5’ to 3’ was compiled for all the genes and summarised in Figure S4.9. The expected distribution would be a smooth curve with consistently high coverage across the gene body for all samples. The analysis of our data revealed a modest skew in the curve with lower coverage on the 5’ end, which may suggest a small degree of sample degradation. However, the distribution is very consistent for all samples, indicating that sample degradation, if any, was consistent throughout the sample batch, without a negative impact on the data analysis procedure. In addition, this observation is in contrast with the high quality of the samples quantified using both spectrophotometric measurements (Qubit, Nanodrop) and the Agilent 2100 Bioanalyzer system. Hence, the biological and technical relevance of such distribution remains unclear and should be interpreted with caution.

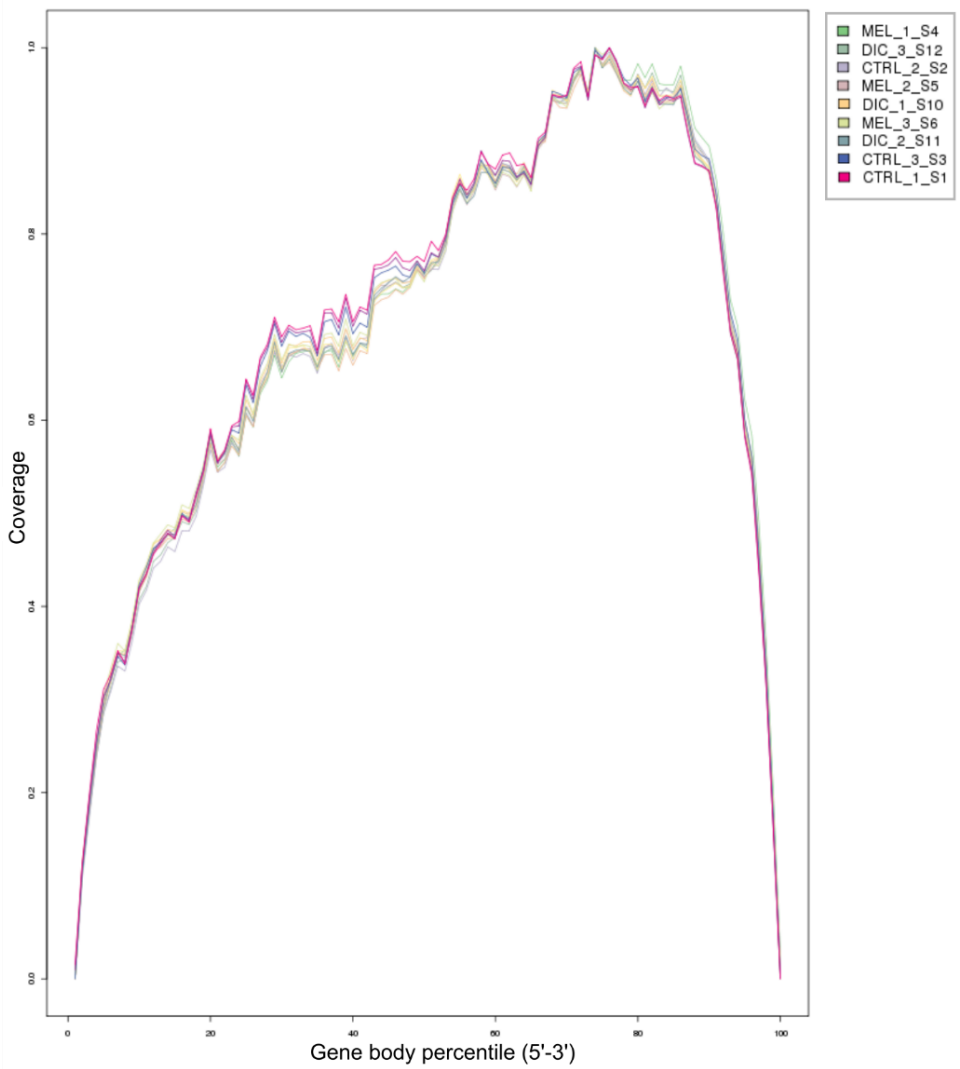

Figure S4.9. Read coverage along the gene body from 5’ – 3’. *Lower coverage on the 5’ end may indicate some small degree of degradation; however, this is consistent between all samples, and other sample quality metrics did not indicate any sample degradation.*

The proportion of reads that map to genomic features, other genomic regions, and to ambiguous locations is summarised in Table S4.3. These proportions are presented for each sample in Figure S4.10 and show that the vast majority of reads map within features, with a similar distribution for each sample.

Table S4.3. Summary of the proportion of reads mapping to features, other genomic regions and to ambiguous loci.

Sample	Uniquely mapped	In features	Not in features	Ambiguous
CTRL_1_S1	22448039	19886205	2065708	496126
CTRL_2_S2	22517390	19290425	2599827	627138
CTRL_3_S3	23228679	20129277	2532333	567069
DIC_1_S10	21976817	18672801	2714041	589975
DIC_2_S11	22359082	19091012	2653789	614281
DIC_3_S12	21026654	17756965	2679253	590436
MEL_1_S4	22026624	18511499	2881556	633569
MEL_2_S5	21557964	18170049	2786481	601434
MEL_3_S6	21532909	18462600	2530444	539865

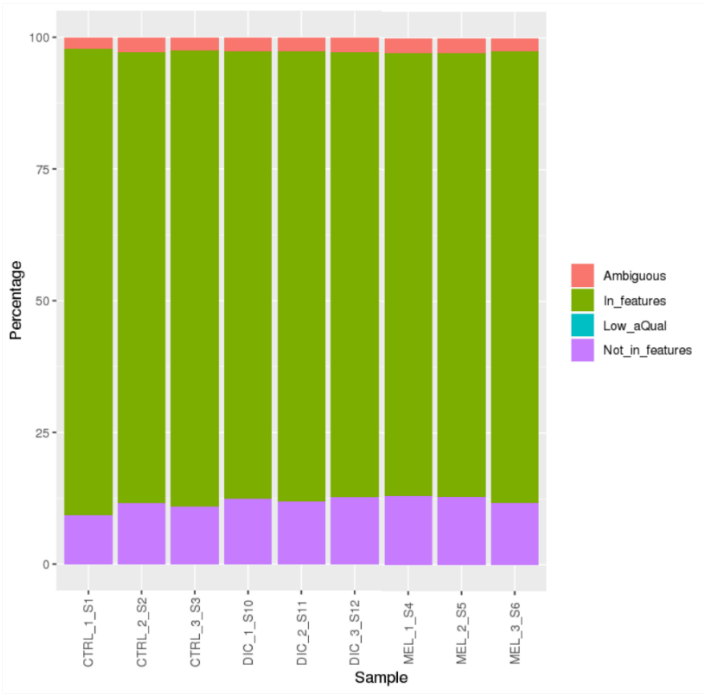

Figure S4.10. Proportions of the reads within each sample mapping to genomic features, other genomic regions (not in features), and ambiguous locations. *Green bars represent the proportion of reads mapping to genomic features; Purple bars represent the proportion of reads mapping to other genomic regions (not in features); pink bars represent the proportion of reads mapping to ambiguous locations.*

The distribution of counts is summarised in Figure S4.11, where the percentage of genes (y-axis) per raw count value (x-axis) is plotted for each sample. All the of samples analysed in this study share a very similar distribution, with the majority of genes showing a raw count value between 1 and 100. The number of genes detected (count ≥ 1) in each sample is expected to be approximately similar between samples, which is confirmed by the data displayed in Table 4.5, resulting in around 25,000 genes per sample.

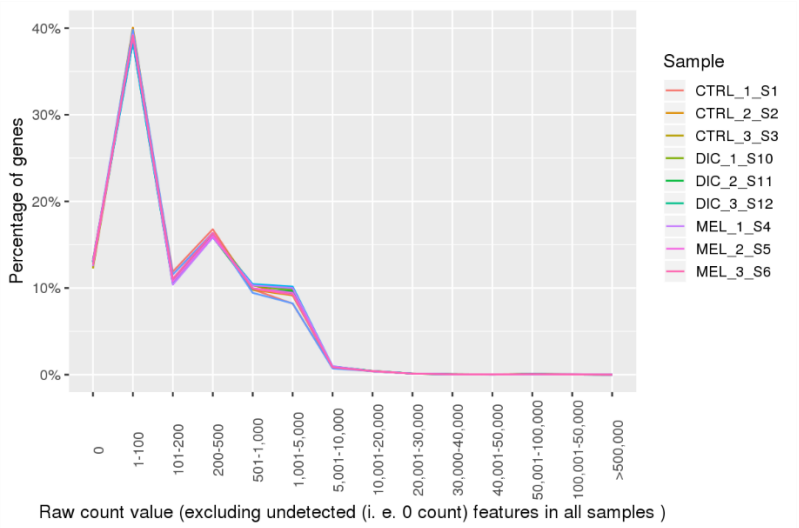

Figure S4.11. Summary of distribution of counts for each sample. *The percentage of genes per count value shows a similar distributions for all samples, with the majority of genes showing a count value between 1 and 100.*

Table S4.4. Number of genes detected per sample. *Each sample should have a similar number of genes.*

Sample	Number of genes detected
CTRL_1_S1	25,220
CTRL_2_S2	25,409
CTRL_3_S3	25,445
DIC_1_S10	25,141
DIC_2_S11	25,226
DIC_3_S12	25,217
MEL_1_S4	25,366
MEL_2_S5	25,183
MEL_3_S6	25,320

The library composition was assessed to confirm that protein coding genes represent the main RNA type in all samples (Figure S4.12).

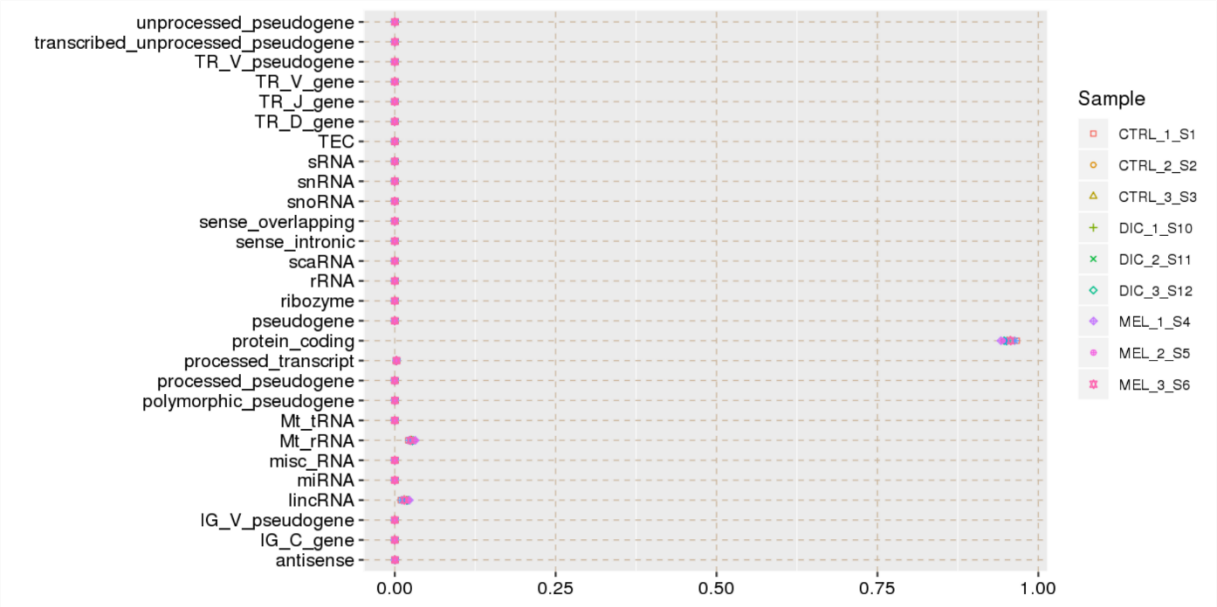

Figure S4.12. Summary of library composition analysis. *Protein coding genes should represent the main type of RNA in all samples.*

Sample clustering was assessed using principal component analysis (PCA) plots and heatmaps, which were derived from normalised and rlog transformed counts, for all groups as a whole and the individual comparisons (control *vs* diclofenac *vs* meloxicam; control *vs* diclofenac; control *vs* meloxicam). Group analyses are presented in Figure S4.13, and the individual analyses are presented in Figure S4.14, and Figure S4.15. The PCA plots revealed a non-optimal clustering of the control samples. This variability can be explained by the observation of the gene expression heatmap, which identified one control sample (CTRL_1_S1) as a potential outlier. However, the biological relevance of this observation remains unclear, as the exclusion of this sample did not improve the overall clustering, indicating that the definition of "outlier" may not be correct. However, it is important to highlight that each sample was formed by four individual larvae. Although our experimental procedure was designed to minimise any potential source of variability, it is possible that the observed inter-sample variability was just a result of biological variability. The heatmaps comparing each sample are presented in Figure S4.16 below.

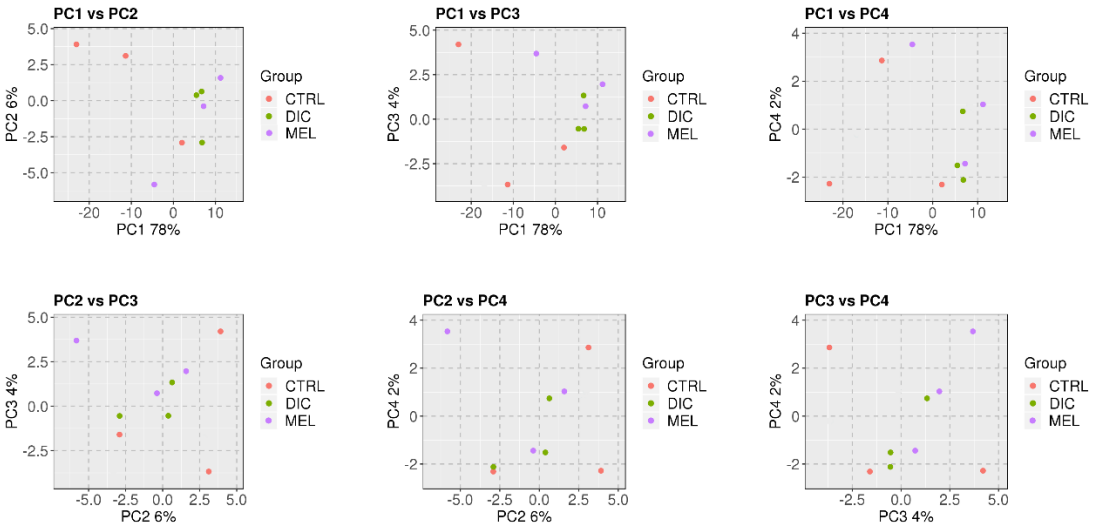

Figure S4.13. Whole group PCA plots assessing sample clustering. *Pink dots represent control samples; green dots represent diclofenac samples; purple dots represent meloxicam samples.*

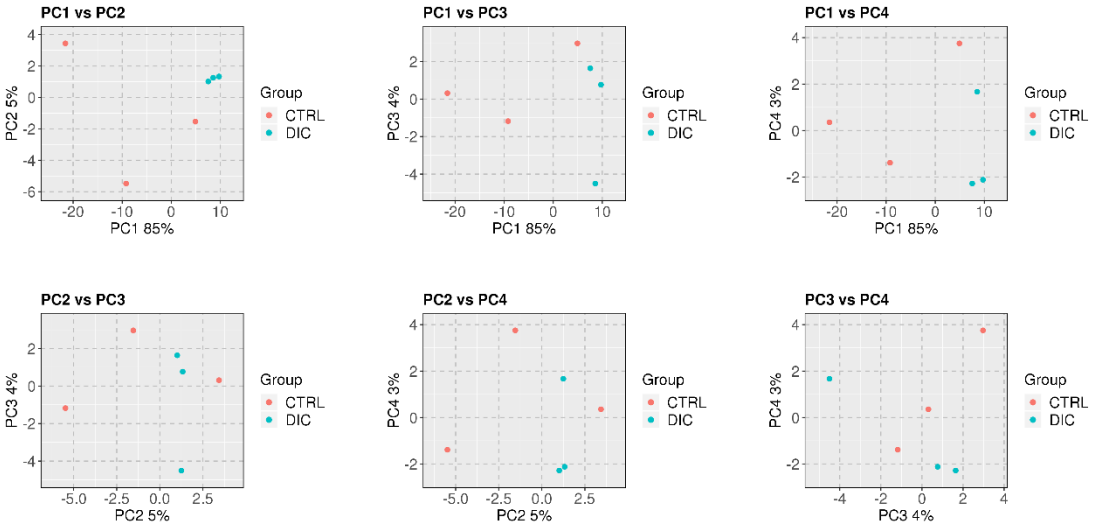

Figure S4.14. PCA plots assessing sample clustering between control and diclofenac samples. *Pink dots represent the control samples; blue dots represent the diclofenac samples.*

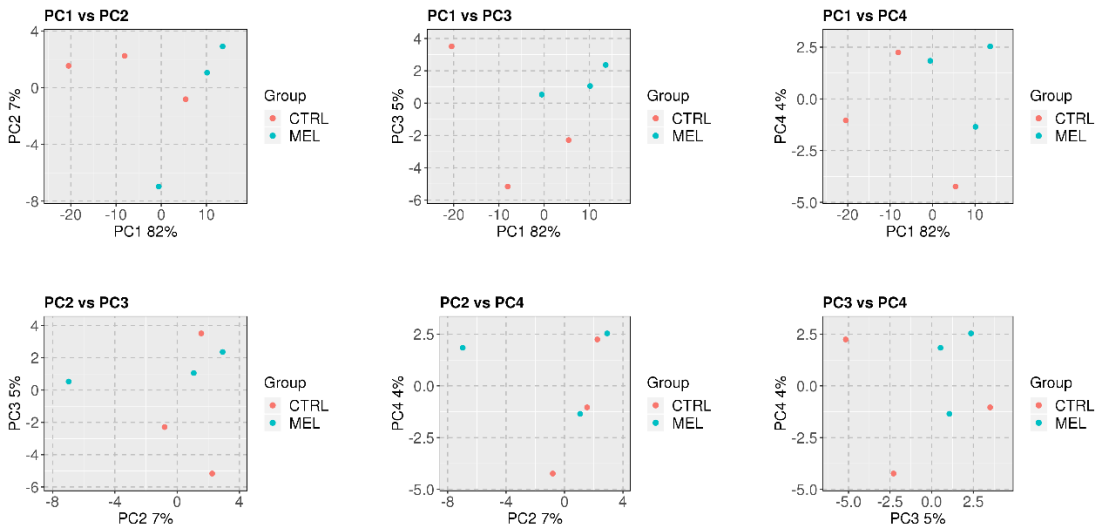

Figure S4.15. PCA plots assessing sample clustering between control and meloxicam samples. *Pink dots represent the control samples; blue dots represent the meloxicam samples.*

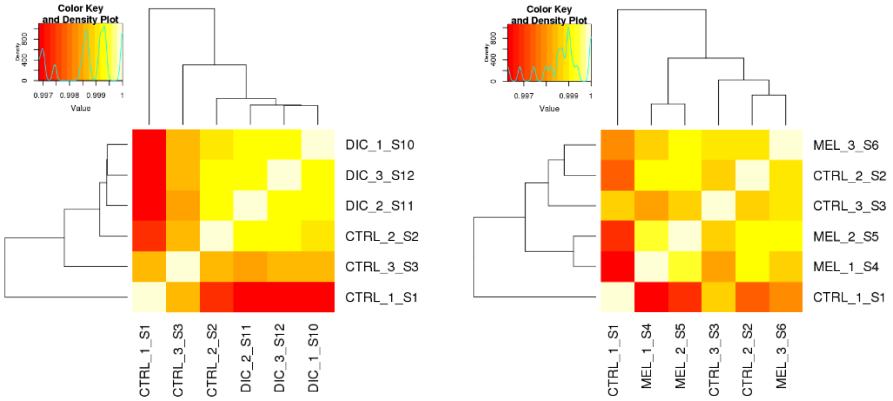

Figure S4.16. Correlation heatmaps displaying the relative similarity between samples. *The heatmaps suggest that sample 'CTRL_1_S1' may be different from the other control samples.*

Multidimensional scaling (MDS) and scatter plots were generated to assess the relationships between the different samples. The MDS and scatter plots for diclofenac (Figure S4.17 and Figure S4.18, respectively) highlight the similarity between the three diclofenac samples and the three control samples. There seems to be a relatively high level of similarity between the treated samples.

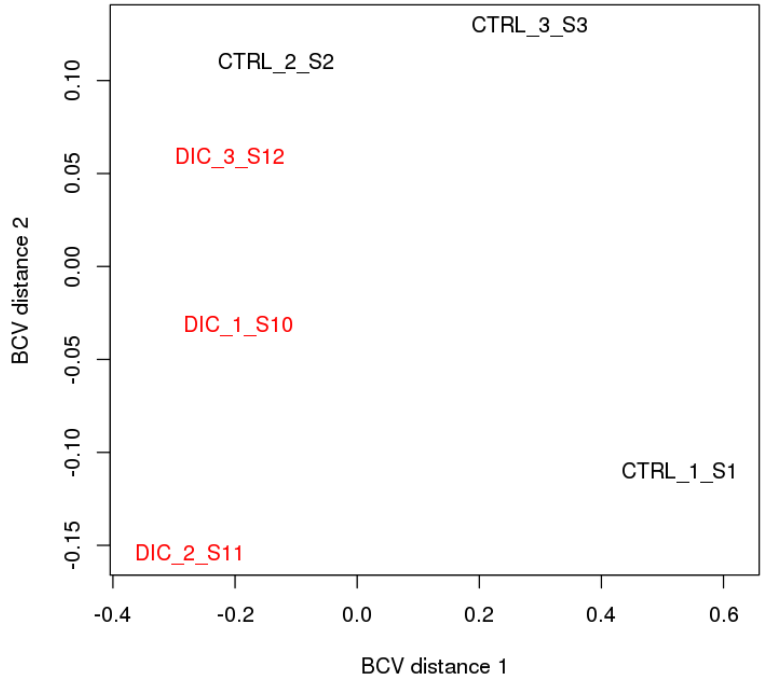

Figure S4.17. MDS plot control and diclofenac samples. *Smaller distance between groups indicates a higher degree of similarity between samples. (i.e. diclofenac samples have a small BCV distance between them, whereas the controls are slightly further apart).*

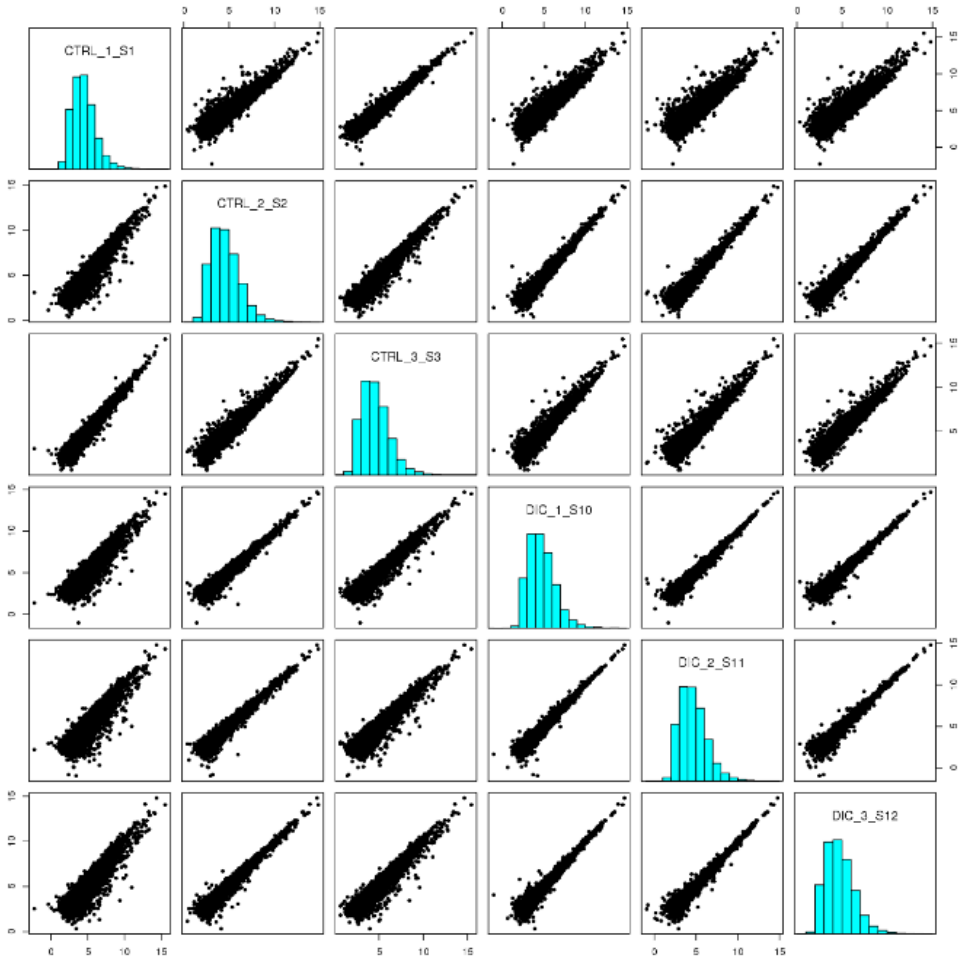

Figure S4.18. Scatter plot comparing control versus diclofenac treated samples. *Black dots closer to the mid-line represent a high level of similarity between samples, whereas data points further from the mid-line represent a higher level of dissimilarity. The scatter plots comparing samples of the same treatment group tend to display a high level of similarity, with the majority of data points lying around the midline. The plots comparing control samples with diclofenac samples tend to have a higher degree of variability, with the data points more distributed either side of the midline.*

The meloxicam MDS and scatter plots (Figure S4.19 and Figure S4.20, respectively) also indicate a relatively close relationship between the treated samples.

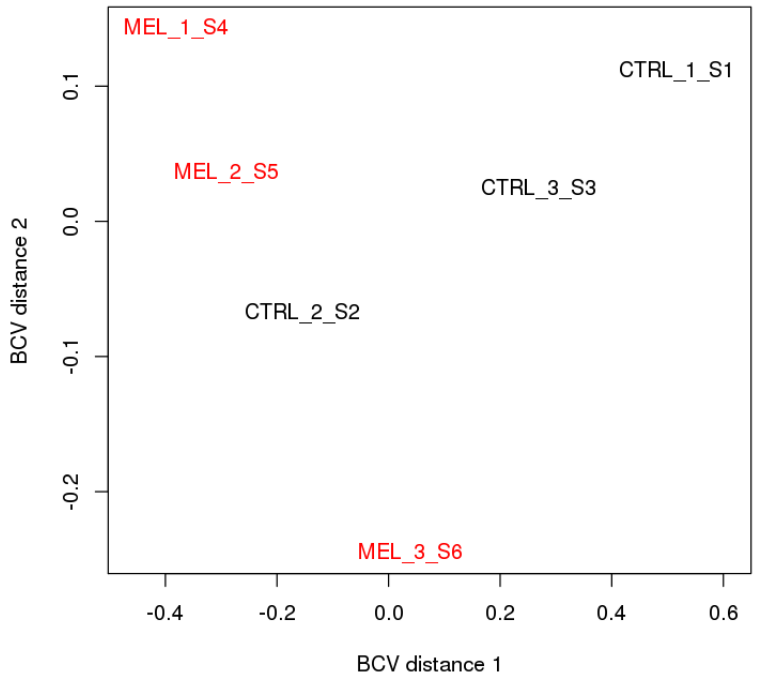

Figure S4.19. MDS plot control and meloxicam samples. *Smaller distance between groups indicates a higher degree of similarity between samples. (i.e. meloxicam samples have a small BCV distance between them, whereas the controls are slightly further apart).*

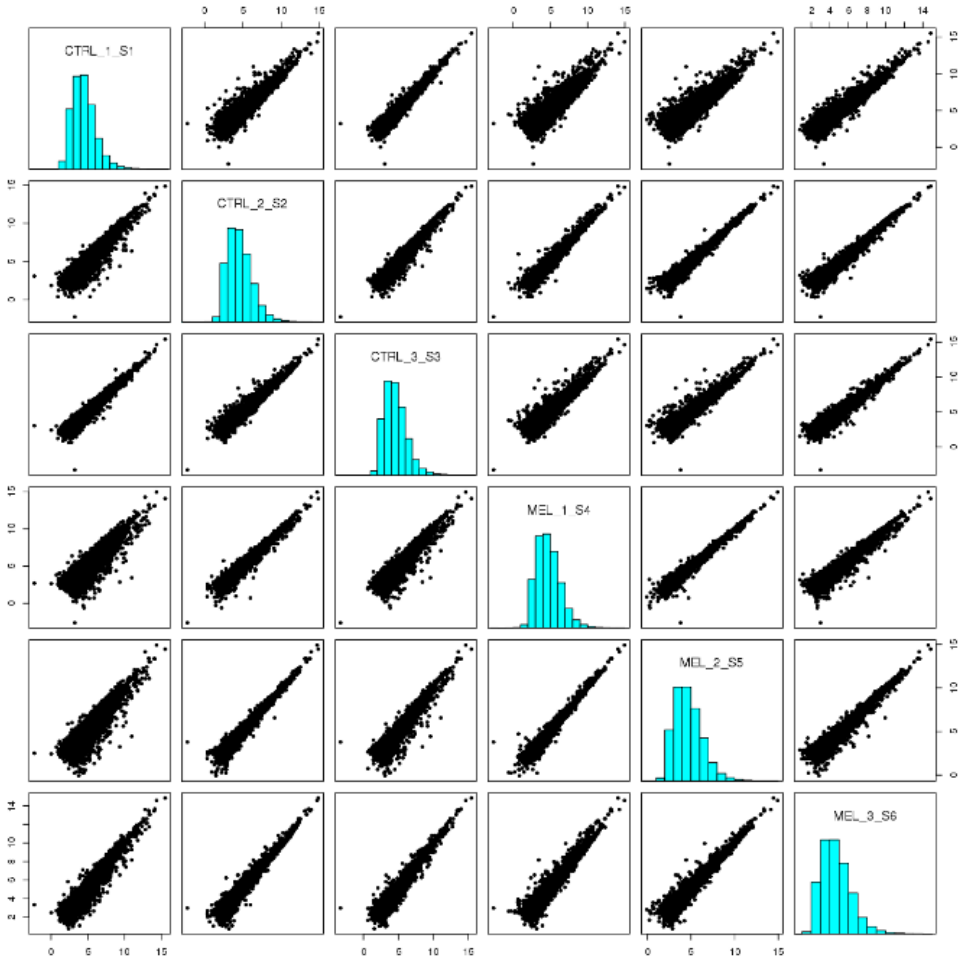

Figure S4.20. Scatter plot comparing control versus meloxicam treated samples. *Black dots closer to the mid-line represent a high level of similarity between samples, whereas data points further from the mid-line represent a higher level of dissimilarity. The scatter plots comparing samples of the same treatment group tend to display a high level of similarity, with the majority of data points lying around the midline. The plots comparing control samples with meloxicam samples tend to have a higher degree of variability, with the data points more distributed either side of the midline.*

On the whole, the scatter plots comparing the variability between the samples of the same treatment group look to have a tight distribution (dots closer to the angled line), which suggests that replicate samples produced similar gene expression profiles (i.e. the three diclofenac samples showed a similar distribution of gene expression).

RNA-Seq: Differential gene expression analyses

A dispersion plot was generated for each comparison displaying the average count per million (CPM) for each gene, and the common dispersion (red line) for the whole dataset (Figure S4.21). The data appeared to be in line with the expectation for both comparisons, as with the increasing average log CPM the dispersions generally decrease in BCV.

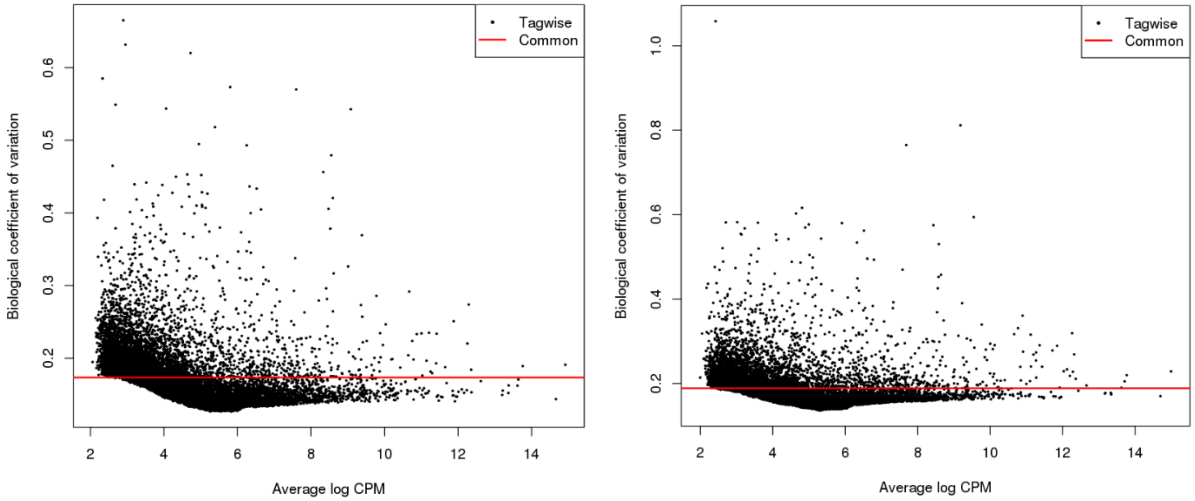

Figure S4.21. Dispersion plots for 'control vs diclofenac' and 'control vs meloxicam', respectively. *The plot on the left-hand side represents the dispersion analysis for the control versus diclofenac comparison, whereas the dispersion plot on the right-hand side represents the control versus meloxicam comparison. Black data points represent genes; the red line represents the common dispersion.*

The detailed results of the differential gene expression analysis are provided in a data file uploaded onto the Brunel Data Repository System (FigShare), which can be retrieved from the following address: https://figshare.com/s/822b6ffcacec179b6e3a. The MA (ratio intensity) plots for each comparison (diclofenac vs control, meloxicam vs control) display a two-dimensional visualisation of the significant DEGs (FDR ≤ 0.05) as a distribution between log_2FC and average log CPM (Figure S4.22). It is clear that there are a larger proportion of DEGs in the left-hand plot comparing 'control *versus* diclofenac' samples.

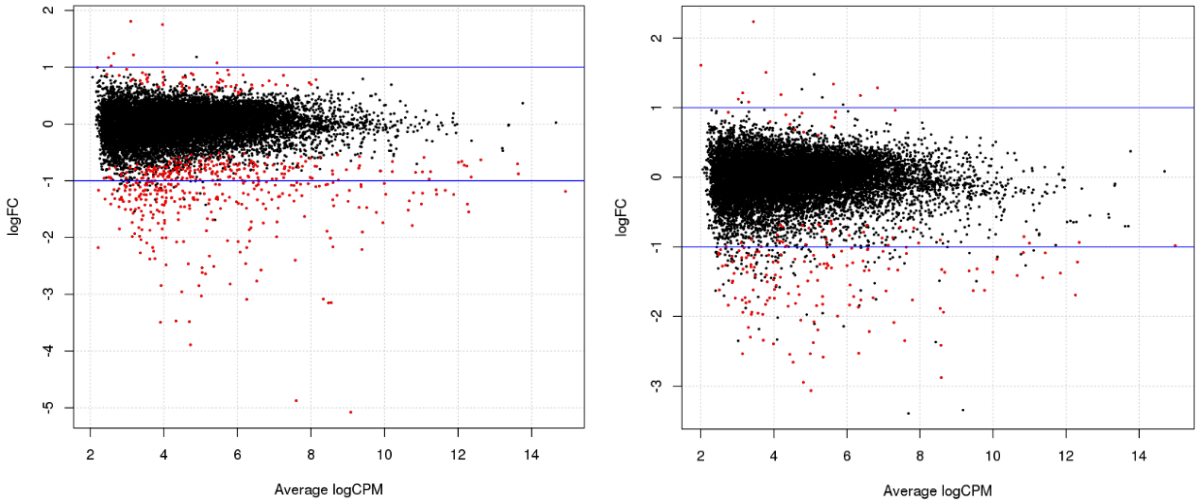

Figure S4.22. MA plots for 'control vs diclofenac' and 'control vs meloxicam', respectively. *Ratio intensity is graphed for each comparison in the MA plots. Each data point represents a gene; red data points indicate statistically significant differentially expressed genes (FDR ≤ 0.05), whereas black data points represent genes which are not differentially expressed. Blue lines represent fold change limits of 1 and -1.*

Appendix 5.1

The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including:	5.4.8
		a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated.	5.4.1
		b. The experimental unit (e.g. a single animal, litter, or cage of animals).	
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.	5.4.8
		b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	5.4.8
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori*. If no criteria were set, state this explicitly.	5.4.1
		b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.	No exclusions
		c. For each analysis, report the exact value of *n* in each experimental group.	5.4.8
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.	5.4.8
		b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	5.4.8
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Sole operator, no blinding possible.
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).	5.4.9
		b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	5.4.9
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used.	5.4.10, 5.5.5
		b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	N/a
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.	5.4.1
		b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	5.4.1
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including:	5.4.8
		a. What was done, how it was done and what was used.	5.4.8
		b. When and how often.	5.4.8
		c. Where (including detail of any acclimatisation periods).	5.4.8
		d. Why (provide rationale for procedures).	
Results	10	For each experiment conducted, including independent replications, report:	5.4.10, 5.5.5
		a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).	5.4.10, 5.5.5
		b. If applicable, the effect size with a confidence interval.	