Pharmacogenomics of non-steroidal anti-inflammatory drug-induced gastrointestinal complications

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Doctor in Philosophy

By

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification

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This research was carried out in the Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool.

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To my beloved family who paid the prize…..thank you, and to all who contributed to this thesis, most especially my supervisors- Professor Sir Munir Pirmohamed and Dr Dan Carr thank you!

Table of contents

Publications

Manuscript in preparation

A loss-of-function polymorphism of *UGT1A6* is not associated with non-steroidal anti-inflammatory drug-induced gastrointestinal complications (2016). C. Esume; D. Carr; M. Pirmohamed; C. Musumba; E. Zhang.

ABSTRACT

Over 30 million people worldwide use aspirin and other non-aspirin non-steroidal anti-inflammatory drugs (NSAID) on a daily basis. It is estimated that over 25% of patients treated for arthritis with NSAIDs have experienced gastrointestinal (GI) complications that required hospital admission and 60% of deaths from adverse drug reactions (ADRs) are attributable to NSAID use.

Significant non-genetic risk factors for the development of NSAID-related gastrointestinal complications include gender, *H. pylori* infection, and concomitant medications. Three genes (*UGT1A6*, *PAI*-1 and *EYA1*) with biological plausibility for roles in NSAID-related ulcers were analysed. Our analysis of genetic risk factors for NSAID-related GI complications in 1197 case-control subjects showed no association between a *UGT1A6* polymorphism and NSAID-induced GI toxicity (p=0.052). Furthermore, a meta-analysis of *UGT1A6* studies confirmed that there was no association between NSAID-related GI complications and *UGT1A6*. The PAI-1 4G/5G polymorphism was also not associated with NSAID-related ulcers and bleeding (n=756), while the *EYA1* rs12678747 single nucleotide polymorphism (SNP) was significantly (p<0.05, OR 1.52; 95% CI 1.21, 1.91) associated with binary ulcer status. In healthy volunteers, *EYA1* gene expression in gastric biopsies was not related to the carriage of this SNP; this contrasts with the difference observed in patients with ulceration suggesting that there may be a SNP-disease interaction, which needs further study. EYA1 was found to be expressed in atypical gastrin secreting (AGS) cells, but the relative expression was significantly (p<0.05) lower than in healthy human gastric epithelial cells and in renal cortical epithelial cells.

Functional assays performed to explore the mechanism of NSAID-related gastric cell death and validate a plausible role for *EYA1* in this process showed that there are multiple cell death pathways occurring concurrently in this cell model depending on the concentration of aspirin. At lower concentrations (10-20mM), PARP cleavage was observed suggestive of an apoptotic process, while at 50mM, necrotic cell death was the predominant mode of cell death. There was a significant (p<0.05) concentration-dependent decrease in the caspase 3 and 7 activity in AGS cells despite a significant (p<0.05) fall in the viability of the cells compared to the controls, suggesting that there is a role for non-caspase dependent mechanisms in the cell death.

In summary, the thesis has focused on the clinical, molecular and functional aspects of peptic ulceration caused by NSAIDs (including aspirin). A novel pathway involving EYA1 has been investigated; this needs further work to define the exact mechanisms by which EYA1 leads to cell death and gastrointestinal injury in patients taking NSAIDs (including aspirin).

Chapter 1

General introduction

Contents

Chapter 1

1.1 Adverse drug reactions

1.1.1 Definition of adverse drug reactions

The World Health Organisation defines adverse drug reaction (ADR) as "noxious and unintended responses to drugs occurring at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function" (WHO, 1972). Due to the vague nature of this definition, because of the use of words like "noxious", Laurence and Carpenter, in 1998 defined ADR as "A harmful or significantly unpleasant effect caused by a drug at doses intended for therapeutic effect (or prophylaxis or diagnosis) which warrants reduction of dose or withdrawal of the drug and/or foretells hazard from future administration" (Laurence & Carpenter, 1998).

These two definitions did not include human errors, a common cause of ADR (Routledge, 2012); effects of contaminants and supposedly inactive excipients as causes of adverse effects. In a bid to include these entities, Edwards and Aronson defined ADR "as an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product" (Edwards & Aronson, 2000).

More recently, the European Parliament and Council of the European Union in 2010, proposed to amend the definition of ADR "to ensure that it covers noxious and unintended effects resulting not only from the authorised use of a medicinal product at normal doses, but also from medication errors and uses outside the terms of the marketing authorisation, including the misuse and abuse of the medicinal product" (EU, 2010).

Some authorities have tried to distinguish ADR from adverse drug events (ADE) which have been defined as harm associated with ADR as well as harmful effects from human error (Edwards & Aronson, 2000). In general, the term ADR infers causality, while ADE can be applied to any event occurring in relation to the drug or intervention irrespective of causality.

1.1.2 The public health burden of adverse drug reactions

ADR are estimated to cause over 100 000 deaths annually in the United States (Lazarou et al., 1998). However, the report was highly criticised since the death rate was extrapolated from admission rates in 1994, yet based on rates of ADR taken from studies done pre 1981. Moreover, it was felt that publication bias could have also contributed to what they regarded as inflated mortality data (Kvasz et al., 2000).

A systematic review of hospital admissions in the United States, noted that between 5% and 7% of all hospitalizations are due to an ADR with a further 10% to 20% of all hospitalized patients experiencing an ADR during the period of hospital admission (Alhawassi et al., 2014). Between 3% and 6% of ADR are fatal or have serious complications, with an estimated 140,000 fatalities due to ADR occurring yearly in the USA (Lazarou et al., 1998; Moore et al., 1998; Wester et al., 2008). The estimated impact on hospital costs exceeds $30 billion, or 5% of total hospital running costs per annum (Patel et al., 2007; White et al., 1999).

An Italian study conducted over a 2-year period reported that of a total of 2,561,400 Emergency Department (ED) visits made, 8,862 were ADR-related, with an overall prevalence rate of 3.5 per 1,000 ED visits. There were 4,111 serious ADR (46.4%), and among these 1,332 (15% of total ADR) led to hospitalization. The fatality rate for all ADR reported in the ED was 1.5%. This study stated further that the most commonly involved drugs were acetylsalicylic acid (34.5% being preventable), amoxicillin/clavulanic acid (32.1% preventable), and warfarin (48.6% preventable). The most frequent suspect drugs in serious ADRs and in inpatients that died within 30 days after discharge were aspirin with 437 deaths, (43.5%) preventable; warfarin with 420 deaths (55.7%) preventable and insulin and analogs with 312 deaths (67%) preventable (Perrone et al., 2014). This makes aspirin a leading cause of mortality from ADR, a significant percentage being preventable.

In one of the largest prospective analysis of ADR in the National Health Service (NHS) Hospitals in the United Kingdom, it was reported that 6.5% of 18,820 admissions were related to ADRs and this figure generally correlates with data pooled from older studies and more recent reports (Davies et al., 2009; Pirmohamed et al., 2004). Over a six-month period, a prospective analysis of 3695 patient-episodes assessed for ADR throughout their admission and each ADR analysed for causality, severity, avoidability and whether it impacted on the length of hospital stay, revealed that of the number assessed, 545 (14.7%) experienced ADR and 50% of these ADR were avoidable (Davies et al., 2009).

A systematic review estimated that about 1.6 million bed days, which is equivalent to 13 to 14 four hundred-bed hospital annually are attributable to in-patient ADR (Wiffen et al., 2002), while another group reported that ADR related bed stay in the two NHS hospital where their study was conducted as 7659 and 9793 bed days respectively. The overall cost

for hospital bed day annually was estimated at £446M for both hospitals (Pirmohamed et al., 2004).

1.1.3 Classification of adverse drug reactions

ADR has been classified as follows:

Type A: Augmented pharmacologic effect, predictable and dose dependent.

Type A reactions are the most common type of ADR and account for 80% of ADR (Pirmohamed & Park, 2001). They are usually a sequel to the known primary pharmacologic effects of the drug, and hence are often predictable. Examples of these include bleeding and thrombosis from use of warfarin (Srinivasan et al., 2004) and renal impairment from use of NSAID amongst others (Patrono & Dunn, 1987). Predictive animal models are often available for type A ADR.

Their severity can vary, ranging from mild to severe and are associated with phase I-III drug metabolism pathways as well as pharmacodynamic variability (Edwards & Aronson, 2000; Pirmohamed et al., 2004; Pirmohamed & Park, 2001). Various relevant factors that could increase the incidence and severity of type A ADR include age, drug-drug interactions, pharmacogenetic variations in pharmacokinetic/pharmacodynamics-related genes and gender (Ajayi et al., 2000; Meyer, 2000).

Type B: These are unpredictable, idiosyncratic, and have no clear dose-response relationship or predictability from the known pharmacological effects of the drug. Type B reactions account for about 20% of ADR, and are usually discovered during phase IV drug development but occasionally in phase III. They occur in susceptible individuals and are characterized by variability in onset of symptoms from a few minutes to sometimes

several months. There are no known widely acceptable animal models of type B ADR. The severity of the response is initially variable but increases when the subject is re-challenged with the same drug (Pirmohamed, 2010; Pirmohamed & Park, 2001). As shown in table 1.1, the classification has further been extended to types C, D, E and F. Type C is dose and time dependent, while type D refers to delayed reactions. Type E and F ADR imply events that occur at the "end of use" and are "attributed to treatment failure", respectively (Perrone et al., 2014).

Table 1.1: Classification of adverse drug reaction (modified from Edwards and Aronson, 2000)

Type of reaction	Mnemonic	Features	Examples	Management
A: Dose related	Augmented	Common Related to the pharmacological action of the drug. Predictable, low mortality	Digoxin toxicity, serotonin syndrome with SSRIs, Anticholinergic effects of tricyclic antidepressants	Reduce dose or withhold Consider effects of concomitant therapy
B: Non-dose-related	Bizarre	Uncommon Not related to a known pharmacological action of the drug, Unpredictable High mortality	Immunological reactions: Penicillin hypersensitivity Idiosyncratic reactions: acute porphyria, malignant hyperthermia, Pseudo allergy (e.g., ampicillin rash)	Withhold and avoid in future
C: Dose and time-related	Chronic	Uncommon May be related to the cumulative dose	Hypothalamic-pituitary-adrenal axis suppression by corticosteroids	Reduce dose or withhold; withdrawal may have to be prolonged
D: Time-related	Delayed	Uncommon Usually dose-related Occurs or becomes apparent sometime after the use of the drug	Teratogenesis (e.g., vaginal adenocarcinoma with diethylstilbestrol) Carcinogenesis Tardive dyskinesia with dopamine agonists	Often intractable
E: Withdrawal	End of use	Uncommon Occurs soon after withdrawal of the drug	Opiate withdrawal syndrome Myocardial ischemia(β-blocker withdrawal)	Reintroduce and withdraw slowly
F: Unexpected failure of therapy	Failure	Common Dose related Often caused by drug interactions	Inadequate dosage of an oral contraceptive, particularly when used with specific enzyme inducers	Increase dosage Consider effect of concomitant therapy

A more recent classification is based on the dosage of the drug, the time course of events, and the patients' susceptibility (DoTS classification). It is thought that this new classification may improve drug development and management of drug adverse effects (Aronson & Ferner, 2003).

1.1.4 Scales for assessment of severity and causality of adverse drug reaction

ADR have been classified based on the severity and seriousness of the adverse reactions. This may be ranked as mild, moderate or severe. The Modified Hartwig Severity Scale (Table 1.2) ranks adverse effects using numbers 1 to 7. Scale 1 refers to an ADR that does not necessitate a change in treatment with the offending drug, while 7b, describes the most severe ADR, directly linked to a patient's death (Davies et al., 2009). This grading, though, may be useful, but does not usually have international acceptance and may lack continuity in its logic.

Table 1.2: The Modified Hartwig Severity Assessment Scale (Hartwig et al., 1992), adapted from (Davies et al., 2009).

Severity level	Description
1	An ADR occurred but no change in treatment with suspected drug required
2	The ADR required that treatment with drug be held, discontinued or otherwise changed. No antidote or other treatment required. No increase in length of hospital stay
3	ADR required that treatment with the suspected drug be held, discontinued, or otherwise changed and/or antidote or other treatment. No increase in length of stay.
4	Level 3 ADR which caused an increase in hospital stay and by at least one day or is the cause of admission to hospital bed
5	Any level 4 ADR that requires intensive medical care
6	The ADR caused permanent harm to patient
7a	The ADR was indirectly linked to death of patient
7b	The ADR was directly linked to death of patient

A widely used and easy to apply scale, developed to standardize assessment of causality for all ADR and for use in the controlled trials and registration of new medication is the Naranjo probability scale (Naranjo et al., 1981). The Naranjo probability scale essentially consists of 10 questions that seek to establish the causality of a drug in ADR; these are presented in table 1.3. More specific ADR causality scales developed for the causality assessment of drug induced liver injury (DILI) is the Council for International Organisations of Medical Sciences or the Roussel Uclaf Causality Assessment Method (CIOMS/RUCAM) (Danan & Benichou, 1993). This scale, though more complex to apply, had been reported to be superior to the Naranjo scale in terms of causality assessment of DILI (GarcÍA-CortÉS et al., 2008).

Table 1.3: The Naranjo adverse drug reaction causality scale

Question	Yes	No	Do Not Know	Score
1. Are there previous conclusive reports on this reaction?	+1	0	0	
2. Did the adverse event appear after the suspected drug was administered?	+2	-1	0	
3. Did the adverse event improve when the drug was discontinued or a specific antagonist was administered?	+1	0	0	
4. Did the adverse event reappear when the drug was re-administered?	+2	-1	0	
5. Are there alternative causes that could on their own have caused the reaction?	-1	+2	0	
6. Did the reaction reappear when a placebo was given?	-1	+1	0	
7. Was the drug detected in blood or other fluids in concentrations known to be toxic?	+1	0	0	
8. Was the reaction more severe when the dose was increased or less severe when the dose was decreased?	+1	0	0	
9. Did the patient have a similar reaction to the same or similar drugs in any previous exposure?	+1	0	0	
10. Was the adverse event confirmed by any objective evidence?	+1	0	0	
	Total Score:			

Significant improvement in the inter-rater agreement in ADR causality when the Naranjo scale was applied compared to when conventional definitions of ADR were used. Definite causality=≥9; probable causality= 5-8; possible causality=1-4; unlikely causality=≤0 (Naranjo et al., 1981).

1.1.5 Risk factors for Adverse drug reactions

The following factors have been identified and their role in the development of ADR explored:

1.1.5.1 Metabolism and drug-drug interaction

Pharmacokinetic drug-drug interaction is a leading factor associated with development of clinically significant ADR (Williams et al., 2004). This could occur via inhibition or induction of the hepatic monoxygenase enzyme system and has been studied extensively over time (Cadieux, 1989; Kamataki et al., 1996). The pharmacologic activity of a drug, in the point of view of efficacy or toxicity, is determined to a large extent, either directly or indirectly, by systemic exposure to the parent drug and/or its metabolites.

Some drugs undergo first pass metabolism with the generation of metabolites which are several fold more potent than the parent compound. Examples include descarboethoxyloratadine, a metabolite of loratadine, a non-sedating selective H_1-histamine antagonist which is 4 times more active than the parent compound (Friedman et al., 1999), and the 5- carboxylic acid derivative of Losartan, whose potency is about 40 times that of Losartan (Sica et al., 2005). Similarly, some observed toxicity arises as a result of the generation of chemically reactive intermediates, exemplified by chloramphenicol-associated aplastic anaemia as a result of the nitro-reduction of the parent compound.

The concept of variability in metabolism is well known and this explains in part some of the unexpected toxicity and or poor efficacy observed following administration of a standard therapeutic dose of the drug. A good example of this is use of flecainide in the elderly, who by virtue of age-related decrease in hepatic metabolism and renal clearance, accumulate the

drug leading to severe ADR, particularly, life-threatening ventricular tachycardia associated with high serum concentrations of this drug (Meyer, 1992).

Inhibition of the metabolism of some drugs can also lead to severe ADR. For instance, metabolism of terfenadine by the isoenzyme CYP450 3A4, is inhibited by commonly used drugs such as itraconazole, ketoconazole and clarithromycin. Concurrent administration of any these drugs with terfenadine leads to accumulation of terfenadine, with consequent QT prolongation and possible development of dangerous arrhythmias and death (Ajayi et al., 2000; Arky et al., 1998). The co-administration of statins with fibrates is associated with the risk of severe myopathy. This interaction is due to metabolic perturbation by the fibrates especially gemfibrozil that interferes with the hepatic glucuronidation of statins (Santos, 2014).

1.1.5.2 Age and gender

The risk of ADR are greatest at the extremes of life, being that at this points in life, organ development/and or function is compromised. In the neonatal period, most enzymes involved in drug metabolism are immature (Court, 2010); hence, drugs which are eliminated by these enzymes can accumulate and cause increased toxicity while a reduction in organ function as seen in the elderly population can cause reduced drug clearance.

Either way, the chances of ADR occurring is significantly increased in these situations (Ajayi et al., 2000). Preliminary work that looked at the glucuronidation of phenolic substrates such as 1-naphthol and 4-nitrophenol by UDP-glucuronosyltransferase 1A (UGT1A) in human fetal liver showed very low to undetectable activities/levels of these isoforms (Rane et al., 1973).

A group examined a wider range of substances now known to be metabolised by UGT1A, using human fetal livers between 16-25 weeks gestation. The activities of these compounds metabolised by various UGT1 isoforms such as bilirubin, androsterone, testosterone, 1-naphthol and 4-nitrophenol were very low, though detectable with the exception of serotonin which was present at activity levels similar to the liver of an adult (Leakey et al., 1987).

Women have been noted to have a higher chance of suffering ADR than men. Possible areas of difference between genders which hopefully could explain this difference are promptness in seeking medical care when complications arise, inappropriate use of drugs, and the insight and importance placed on ill health between genders, as well as gender-dependent differences in expression of some drug metabolising enzymes, for example CYP3A4 (Ajayi et al., 2000).

1.1.5.3 Pharmacogenetics

Polymorphisms in genes involved in drug metabolism are known to predispose to both idiosyncratic and pharmacological toxicity (tables 1.4 and 1.5). So far, most studies have concentrated on genes that code for enzymes involved in the biotransformation of drugs as risk factor for ADR. However, cell repair mechanisms, elaboration of cytokines and the role of immune response needs extensive work to explore genes that increases susceptibility to ADR (Pirmohamed et al., 1998). Success in these research areas will improve predictability of an individual's susceptibility to an ADR. Aside from clinical and demographics factors, genetic factors are likely to play a key role in an individual's predisposition to NSAID induced GI injury.

Table 1.4: Genetic variations affecting drug metabolism and associated therapeutic agents

Genetic variation	Therapeutic agent
TPMT	mercaptopurine, thioguanine, azathioprine
CYP2D6	modeine, tramadol, tricyclic antidepressants, aripiprazole, clomipramine
CYP2C19	tricyclic antidepressants, clopidogrel, voriconazole
VKORC1	warfarin
CYP2C9	warfarin, phenytoin
HLA-B	allopurinol, carbamazepine, abacavir, phenytoin
CFTR	Ivacaftor
DPYD	fluorouracil, capecitabine, tegafur
G6PD	rasburicase, Nalidixic acid, Nitrofurantoin
UGT1A1	irinotecan, atazanavir
SLCO1B1	simvastatin
IFNL3 (IL28B)	interferon
CYP3A5	tacrolimus

These genes are involved in the metabolism of these drugs and pharmacogenetic variations involving these genes could lead to pharmacokinetic variations requiring dose adjustments as appropriate. Some of these may require genetic testing to enable patient stratification and appropriate therapeutic goals (PharmGKB, 2015).

Table 1.5: Some clinically important genetic polymorphism of drug metabolism enzymes that influence drug response

Enzyme	Frequency of polymorphism	Drug	Alleles involved	Drug effect
CYP2C9	14-28% (heterozygotes)	Warfarin (Wypasek et al., 2015)	CYP2C9*2/*3	Hemorrhage
	0.2-1% (homozygotes)	Tolbutamide (Becker et al., 2008)	CYP2C9*3	Hypoglycemia
		Phenytoin (Caudle et al., 2014)	CYP2C9*3	Phenytoin toxicity
		Glipizide (Becker et al., 2013)	CYP2C9*3	Hypoglycemia
		Losartan (Dorado et al., 2014)	CYP2C9 IVS8-109T	Increased hydroxylation capacity
CYP2D6	5-10% (poor metabolizers), 1-10% (ultra-rapid metabolizers)	Antiarrhythmics	*4, *5, *14, *21, *36	Proarrythmic and other toxic effects
		Antidepressants (Hicks et al., 2013)	*4/*4, *3/*4, *5/*5, 5/*6	Toxicity in poor metabolizers, inefficacy in ultra-rapid metabolizers
		Aripiprazole (Hendset et al., 2007; Oosterhuis et al., 2007)	4x *4/*4, *3-6/*3-6	Tardive dyskinesia
		Opiods (Crews et al., 2012)	*4/*4, *4/*5, *5/*5, 4/*6	Inefficacy of codeine as analgesic, narcotic side effects, dependence
		Carvedilol (Baudhuin et al., 2010)	*3/*10,*4/4-*4/*6	Increased β-blockade
CYP2C19	3-6% (whites) 8-23% (Asians)	Omeprazole (Furuta et al., 1998)	CYP2C19∗2	Lower cure rates at lower dosages
		Clopidogrel (Scott et al., 2013)	*2/*2, *2/*3, *3/*3	Poor antiplatelet activity
Dihydropyrimidine dehydrogenase	0.1%	Fluorouracil (Cai et al., 2014)	IVS14+1G>A	Neurotoxicity, myelotoxicity
N-actyltransferase	40-70% Whites) 8-23% (Asians)	Hydralazine, (Sim et al., 2008)	NAT2*5	Hypersensitivity
		Isoniazid (Sim et al., 2008)	NAT2*5	Drug-induced lupus erythematosus
TPMT	0.3%	Mercaptopurine, Thioguanine, azathioprine (Relling et al., 2011)	*2, *3A, *3B, *3C, *4	Myelotoxicity
UGT1A1	10-15%	Irinotecan (Falvella et al., 2015)	homozygous UGT1A1*28	Diarrhea, myelosupression

Frequencies are for the Caucasian population unless otherwise stated. CYP= Cytochrome P 450 isozyme

1.1.5.3.1 Pharmacogenetics of NSAID-induced gastric injury

Several studies have investigated the role of genetics in NSAID-related GI complications and reports from these studies have highlighted important roles of genetic polymorphisms in enzymes involved in the oxidation and conjugation of drugs, genetic mutations of drug receptor and target sites, drug transport proteins and ion channels (see table 1.6). Promising results have been reported in the association of polymorphisms in metabolizing enzyme genes in the pathogenesis of gastroduodenal complications related to the use of NSAIDs and aspirin.

On the contrary, there have been very few studies that have looked at the role of variations in genes that are involved in cellular repair mechanisms. Hence, Pirmohamed and Park's recommendation for a widened search of candidate genes involving more pharmacodynamic targets, that focus on tissue injury underscores the need to explore plausible genetic roles of cell repair genes in NSAID-related GI complications (Pirmohamed et al., 1998; Pirmohamed & Park, 2001).

Recently, an examination of the association of *CYP2C19* genetic polymorphism with peptic ulcer disease in a Caucasian population (n=1239) with or without peptic ulcer disease, some of whom were NSAIDs users was carried out. It reported that a significant association between a gain of function polymorphism in *CYP2C19* 17 and peptic ulcer disease (OR-1.47, 95%CI-1.12-1.92; *P*= 0.005); an association that was independent of NSAID use. However, there were no associations between peptic ulcer disease and the other 7 CYP2C SNPs (CYP2C8*3{rs11572080 and rs10509681}, CYP2C8*4{rs1058930}, CYP2C9*2{rs1799853}, CYP2C9*3{rs1057910}, CYP2C19*2{rs4244285}, CYP2C19*3{rs4986893} (Musumba et al., 2013). Similarly, a lack of association between upper gastrointestinal symptoms and *CYP2C9*

(OR=0.80, 95%CI=0.41-1.56) or *UGT1A6* polymorphisms (OR=0.85, 95%CI=0.44-1.67) in 160 aspirin users in a coronary unit has been reported by an independent group (Van Oijen et al., 2005b). A number of studies, though still very sparse, looking at the genetic predisposition to NSAID-related GI toxicity have been undertaken and a summary of them are presented in the table 1.6.

Table 1.6: Summary of research on genetic association of NSAID-related GI complications

Study	Design	N	Outcome measures	Gene	Report
(Negovan et al., 2015)	Prospective	211	Endoscopic UGIB	*AGT*	*AGT A-20C* variant allele increases risk
(Martínez et al., 2004)	Prospective	218	Endoscopic UGIB	*CYP2C9*	*CYP2C9 *2* alleles ↑ risk
(Hayashi et al., 2012)	Case-control	548	PUD	*IL-17*	*IL-17* rs2275913 A allele increases risk
(Pilotto et al., 2007)	Case-control	78	Endoscopic UGIB	*CYP2C9*	*CYP2C9*1/*3, CYP2C9*1/*2*↑risk. Higher risk among *CYP2C9*3* carriers
(Van Oijen et al., 2005a)	Prospective	160	Symptomatology	*UGT1A6 CYP2C9*	No association
(Musumba et al., 2013)	Case-control	1239	Endoscopic UGIB	*CYP2C* family	*CYP2C19*17* associated with PUD
(Martin et al., 2001)	Retrospective	54	GU or GU scar	*CYP2C9*	No association found
(Arisawa et al., 2007)	Retrospective	480	PUD	*COX-1*	1676T allele increases risk; c.-842A>G/c.50G>T no association seen
(Van Oijen et al., 2005a)	Retrospective	194	PUD	*COX-1*	c.-842A>G/c.50G>T showed inverse association
(Halushka et al., 2003)	Prospective	38	Products of AA pathway ($PGF_{\alpha2}$, TX_{A2})	*COX-1*	c.-842A>G/c.50G>T haplotype had higher inhibition of PG formation by aspirin
(Piazuelo et al., 2008)	Case-control	354	UGIB	eNOS gene	The 'a' allele associated with lower risk
(López-Rodríguez et al., 2008)	Prospective	69	Ibuprofen safety pharmacokinetics pharmacodynamics	*CYP2C9 CYP2C**	*CYP2C9*3* linked to ↓ metabolism and clearance of R –ibuprofen, *CYP2C8*3=* ↑clearance of R-ibuprofen, ↓iNOS expression, *CYP2C8*3* and *4 linked to ↓ adverse effect

N, number of subjects in study; UGIB, upper gastrointestinal bleeding; PUD, peptic ulcer disease; GU, gastric ulcer; CYP, cytochrome P450 enzyme; AA, arachidonic acid ; TXA_2, thromboxane A_2; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthetase; iNOS, inducible nitric oxide synthetase; PG, prostaglandin, modified from.

1.1.5.4 Physiological and pathophysiological states

Comorbid conditions in an individual can influence the susceptibility to ADRs. There has been an exponential increase in the frequency of idiosyncratic ADR as a result of HIV disease. About 50% of HIV patients receiving high dose co-trimoxazole for Pneumocystis carinii (Pneumocystis jiroveci) pneumonia and 30% taking prophylactic doses presented with rashes, contrasting sharply with 3% in non-infected people (van der Ven et al., 1991). This occurred particularly at a time before highly active antiretroviral therapy was introduced.

Among renal failure patients on renal replacement therapies, there is an increased risk of gastrointestinal bleeding with the use of NSAIDs, with as much as 3 times increase among these patients compared to patients not on NSAID (Jankovic et al., 2009). The risk of gastrointestinal bleeding among patients with chronic renal failure has been noted to be increased while it is estimated that about 3-7% of all deaths among end stage renal disease patients is attributable to upper gastrointestinal bleeding (Wasse et al., 2003).

Derangements in thrombocyte function and bleeding time, and chronic gastric mucosal inflammation is the norm in most uremic patients, and the synergistic effect of NSAIDs (as these are similar mechanisms of NSAID-induced gastrointestinal bleeding), in end stage renal disease patients makes them particularly prone to NSAID-related GI complication (Tschugguel et al., 1995). As in the general population, upper gastrointestinal bleeding is much more common than lower gastrointestinal bleeding in end stage renal disease, and NSAID use correlates positively with upper gastrointestinal bleeding due to gastric mucosal erosion or duodenal mucosal inflammation (Gheissari et al., 1990).

More recently, an association between diabetes and increased risk of gastrointestinal bleeding in non-aspirin users and an inverse association among aspirin users has been

reported; however, the relevance and validity of these findings deserves further exploration (De Berardis et al., 2012).

Disease states involving some key enzyme deficiencies are also know to predispose individuals with such deficiency to severe/life threatening ADR to ordinarily safe medications. A typical example is glucose-6-phosphate dehydrogenase (G6PD) deficiency, a sex-linked disorder prevalent among individuals of African, Mediterranean and Asian descent. It is associated with a deficiency of G6PD in red blood cells and generation of haemotoxic metabolites on exposure to some antimalarials such as mepacrine, primaquine and pamaquine; antibiotics such as nitrofurantoin and sulphonamides; fava beans; and anti-mycobacterials such as dapsone, para-aminosalicylic acid and the sulfones. These individuals develop severe haemolytic anaemia and jaundice when offending drugs are administered (Frank, 2005; Manganelli et al., 2010).

Identifying variability among patients in the point of view of age, gender, genetic background, co-administered drugs, coexistence of other diseases and their concurrent effects on drug metabolism and excretion in drug development clinical trial is a major hurdle which when surmounted improves a drug ADR profile (Ajayi et al., 2000).

1.2 Non-steroidal anti-inflammatory drugs

1.2.1 Classification of non-steroidal anti-inflammatory drugs

NSAIDs can be classified based on their chemical structure or their COX selectivity (see table 1.7; modified from (Mourad & Bahna, 2014).

Table 1.7: Classification of non-steroidal anti-inflammatory drugs

COX-selectivity	Chemical classification
Non selective COX-inhibitors • Aspirin • Aceclofenac, diclofenac, indomethacin, ketorol, sulindac, tolmetin • Dexketoprofen, dexibuprofen, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, loxoprofen, naproxen, oxaprozin • Flufemanic acid, meclofemanic acid, mefenamic acid, Tolfenamic acid • Droxicam, isoxicam, lornoxicam, piroxicam, tenoxicam • Phenazone, phenylbutazone, propyphenazone **COX 2 selective inhibitors** • Celecoxib, etoricoxib, parecoxib, parvocoxib, rofecoxib • At low dose: etodolac, meloxicam, nabumetone, nimesulide	**Carboxylic acids** • *Salicylic Acids*: Aspirin, diflunisal, salsalate, trisalicylate • *Acetic Acids*: Aceclofenac, indomethacin, ketorolac, sulindac, tolmetin • *Propionic Acids*: Dexketoprofen, exibuprofen, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, loxoprofen, naproxen, oxaprozin • *Fenamic Acids*: Flufemanic acid, eclofemanic acid, mefenamic acid, tolfenamic acid **Enolic Acids** • *Oxicams:* Droxicam, isoxicam, lornoxicam, piroxicam, tenoxicam • *Pyrazolones*: Phenazone, phenylbutazone, propyphenazone **Sulfonilides** • Nimesulide **COX 2 selective inhibitors** • Celecoxib, etoricoxib, parecoxib, parvocoxib, rofecoxib

Classification of NSAIDs based on chemical class and selectivity for cyclo-oxygenase (COX) enzyme. NSAIDs with similar chemical structure were grouped together based on their COX-selectivity (left column).

1.2.2 Adverse reactions of non-steroidal anti-inflammatory drugs

NSAIDs, though widely used, are known to have a wide range of ADRs affecting various organ systems of the body, including the skin, heart, respiratory system, blood, kidney and the gastrointestinal tract. They are thought to be responsible for a quarter of ADRs (Mourad & Bahna, 2014). The next sections will be highlighting some of these important ADRs.

1.2.2.1 Hypersensitivity reactions to Non-steroidal anti-inflammatory drugs

NSAIDs trigger a wide variety of hypersensitivity reactions in predisposed individuals, varying in the timing of onset, organ/system affected, the severity of the reaction and the underlying mechanistic processes, i.e. whether immunological or non-immunological (Kowalski et al., 2013). A summary of the phenotypic characteristics of the different types of NSAID-related hypersensitivity reactions are presented in table 1.8 (modified from Kowalski *et al*, 2013 and (Ayuso et al., 2013).

In 1902, Hirschberg was the first to describe an aspirin-induced hypersensitivity reaction, and subsequently several other NSAID-induced hypersensitivity reactions have been reported (Hirschberg, 1902). NSAIDs are the second most common cause of drug-induced hypersensitivity reactions, the first being antibiotics (Gomes & Demoly, 2005; Kowalski et al., 2013). Two independent groups of researchers have reported the prevalence of aspirin-induced hypersensitivity reaction in the general population to be 0.5%-1.9% (Gomes et al., 2004; Isik et al., 2009; Settipane et al., 1980)**.**

Other workers have reported prevalence as high as 5.6%, although these figures vary depending on the population studied, method of assessment and the type of reaction (Doña

et al., 2012; Hedman et al., 1999). In asthmatics with nasal polyps, aspirin associated hypersensitivity is projected to reach 25.6%, while skin manifestations of NSAID hypersensitivity is about 0.3% in the general population, (Settipane et al., 1980). Among adult asthmatics assessed with questionnaires, the prevalence of NSAID hypersensitivity ranged from 4.3-11% and when data from a provocation test was added, it increased to 25% (Jenkins et al., 2004; Kasper et al., 2003). Regardless of the NSAID's chemical structure or anti-inflammatory potency, hypersensitivity reactions have been reported for all known NSAID (Kowalski et al., 2011).

1.2.2.2 Mechanistic processes driving NSAID-related hypersensitivity

NSAID-related hypersensitivity reactions could be either immunologically (non-cross reactive) or non-immunologically (cross reactive) mediated. This has been variously classified by different workers; however, for the purpose of this work, we shall be sticking to the Kowalski *et al* classification (Kowalski et al., 2013) as presented in table 1.8. Some experts have suggested that NSAIDs fulfil the criteria of type B ADR (Mourad & Bahna, 2014).

Table 1.8: Phenotypic characteristics of different types of NSAID-related hypersensitivity reactions (Ayuso et al., 2013; Kowalski et al., 2013).

Types of reaction	Clinical features	Timing of reaction	Type of reaction	Underlying disease	Putative mechanism
NERD	Rhinitis/asthma	Immediate	Multiple NSAIDs hypersensitivity	Asthma/rhinosinusitis	COX-1 inhibition
NECD	Wheals/angioedema	Immediate	Multiple NSAIDs hypersensitivity	Chronic urticaria	COX-1 inhibition
NIUA	Wheals/angioedema	Immediate	Multiple NSAIDs hypersensitivity	None	Unknown, possibly COX-1 inhibition
SNIUAA	Wheals/angioedema/anaphylaxis	Immediate	Single NSAID hypersensitivity	Atopy, food and drug allergy	IgE-mediated
SNIDR	Various symptoms and organs involved (e.g. fixed drug eruptions, nephritis, pneumonitis, SJS/TEN, contact and photo-contact dermatitis, bullous skin reactions, aseptic meningitis	Delayed onset (usually more than 24hrs)	Single NSAID hypersensitivity	None	T-cell mediated, cytotoxic T cells, NK cells

NERD: NSAID-exacerbated respiratory disease; NECD: NSAID-exacerbated cutaneous disease; NIUA: NSAID-induced urticaria/angioedema; SNIUAA: Single-NSAID-induced urticarial/angioedema or anaphylaxis; SNIDR: Single-NSAID-induced delayed reactions.

1.2.3 Renal toxicity of Non-steroidal anti-inflammatory drugs

NSAIDs, a widely used class of pain and inflammation control medications, due to their perceived efficacy and safety, are the commonest cause of drug-induced renal injury. It has been reported that the world wide incidence of renal side effects of NSAIDs is 1-5 % (Anders, 1980). Table 1.9 shows a list of renal complications associated with NSAIDs.

Table 1.9: Renal complications arising from use of NSAIDs.

NSAID-related renal complications	Mechanism
• Acute tubular necrosis • Acute kidney injury • Pre-renal azotaemia • Acute papillary necrosis • Hypertension • Chronic tubule-interstitial nephritis • Acute interstitial nephritis • Membranous nephropathy • Hyponatremia • Minimal change disease • Hyperkalaemia and minimal change disease	The mechanism of NSAID-induced renal complications/ acute kidney injury occurs via inhibition of prostaglandin. PGs are essential for the maintenance of normal physiological functions of the kidney. They are known to improve renal perfusion by causing vasodilation of renal glomerular capillaries thereby enhancing hemofiltration and normal milieu of body fluids. Hence, in the presence of NSAIDs, this homeostasis is impaired and renal hemodynamics altered (Pazhayattil & Shirali, 2014).

1.2.4: Cardiovascular adverse effects of non-steroidal anti-inflammatory drugs

NSAIDs can also cause cardiovascular adverse effects; the mechanisms by which these effects occur are listed in table 1.10.

NSAID-related cardiovascular complication	Mechanism
Fluid and electrolyte retention	Interference with the renin-angiotensin system (RAS) via PG inhibition (Camin et al., 2015).
Hypertension	NSAIDs cause fluid and electrolyte retention, and, long term cause an increase in blood pressure (Plouin, 2006).
Increased risk of cardiovascular events	A number of non-clinical studies have reported that inhibition of prostacyclin (PGI_2) cause artherogenesis via plaque destabilization and thrombosis which could be either a platelet dependent or independent mechanism (Egan et al., 2005; Rudic et al., 2005).
Worse prognosis for ischemic reperfusion injury	In ischemic reperfusion injury that could occur following heart attack, COX-2 has been shown to be cardioprotective and is a major source of PGI_2 (Bolli et al., 2002).
Increased thrombotic events	Reduction in the level of antithrombotic PGI_2, an enhanced synthesis of leukotrienes and generation of reactive oxygen species and consumption of antithrombotic nitric oxide (Antman et al., 2005)
Aggravation of doxorubicin cardiotoxicity	An animal study reported that diclofenac sodium, a non-selective COX- inhibiting NSAIDs aggravated the cardiotoxicity of doxorubicin, suggesting that in the presence of diclofenac sodium, the risk of cardiovascular events due to oxidative stress is increased (Kumar Singh et al., 2010; Singh et al., 2014).
Degradation of aortic elastin protein	Blockage of cross linkage formation (Oitate et al., 2007a; Oitate et al., 2007b)
Endothelial dysfunction	Reduction in available nitric oxide due to COX-2 inhibition (Virdis et al., 2005).

NSAID-related cardiovascular complications occurring via various mechanisms such as modulation of the RAS, fluid and electrolyte retention, inhibition of PGI_2 and subsequent increased thrombotic event and plaque destabilization, COX-2 inhibition. NSAIDs also cause endothelial dysfunction by depletion of available nitric oxide.

1.2.5 The non-targeted adverse drug reactions of non-steroidal anti-inflammatory drugs

The adverse effects of NSAIDs on the central nervous system and the hepatobiliary system are mainly non-targeted as they do not involve specific enzyme or receptor targets. These could be related to any moiety of a drug molecule, and can be limited to a single molecule in a class of drugs. Most off-target ADRs are picked up during post marketing surveillance if missed during drug development and this has led to the withdrawal of NSAIDs like bromfenac, ibufenac and benoxaprofen (Goldkind & Laine, 2006; Goldkind & Simon, 2006).

1.2.5.1 Hepatic toxicity of non-steroidal anti-inflammatory drugs

The majority of this class of drugs are eliminated through hepatic metabolism involving the oxidation and/or conjugation of these drugs. The process of oxidation and conjugation during the phase I and phase II reactions respectively, leads to an increase in the solubility of these intermediate metabolites with consequent biliary and or renal excretion. Also, phase I reactions produce highly reactive intermediaries which may form adducts and thereby cause damage to cellular components. This is established for paracetamol at doses greater than 4g/day and most likely may be applicable for all metabolized NSAIDs (Wehling, 2014), but of course, the liver injury with paracetamol has a clear dose-response relationship, while liver injury with NSAIDs is better categorised as a type B reaction.

An increase in transaminases, a marker for hepatocellular injury, of up to 3.5% has been reported for most NSAIDs including diclofenac (Rostom et al., 2005); (Bessone, 2010). Some NSAIDs like ibufenac, bromfenac and benoxaprofen have either been withdrawn or failed to reach the market due to their high rates of hepatotoxicity (Goldkind & Laine, 2006). However, the hepatotoxicity of most NSAIDs has been reported to be low, ranging from

0.29-3.1/100000 exposed individuals in a population based study. Similarly, a large US drug induced liver injury (DILI) registry reported that only 4 of 133 cases caused by single agents were linked to bromfenac while other NSAIDs caused one or two cases at most (Reuben et al., 2010).

In chronic NSAIDs users, it is recommended that transaminase and bilirubin levels are monitored but the utility of this approach is unclear. Nevertheless, a threefold increase in transaminase levels is an absolute indication for stopping the offending NSAID (Wehling, 2014). A rise in serum bilirubin levels should necessitate instant discontinuation of the NSAID regardless of the transaminase level, according to Hy's law (Temple, 2006).

1.3 Gastrointestinal complications of aspirin and other NSAIDs

Low dose aspirin and other NSAIDs are known to commonly cause gastrointestinal adverse drug reactions, and these may range from mild symptoms like dyspepsia to severe complications like peptic ulceration and in the extreme cases, upper gastrointestinal bleeding and perforation which may be associated with significant morbidity and mortality (Lanas et al., 2011).

1.3.1 Peptic ulcer in aspirin and NSAID-users

Low dose aspirin, generally, defined as between the range of 75mg and 325mg daily (Baigent et al., 2009), is commonly prescribed for the primary and secondary prevention of ischemic heart diseases, cerebrovascular diseases and currently being advocated for the primary prevention of colon cancers and other non-colonic cancers (Baron & Sandler, 2000). An audit

of primary care prescription revealed that in England, in 2007 alone, over 30 million aminosalicylic acid (ASA) prescriptions were issued, while in the US; it is thought that over one-third of the adult population, and over 80% of those diagnosed with a cardiovascular disease use aspirin (Ajani et al., 2006); National Health Service information centre, 2007).

Less severe aspirin-related upper gastrointestinal ADR reported by low dose ASA users include gastroesophageal reflux and/or heart burn, bloating, belching, epigastric discomfort, early satiety, and postprandial nausea and these can be found in about 15-20% of low dose ASA users (Cayla et al., 2008; Yeomans et al., 2008).

As with other medications, compliance with low dose aspirin is affected by the frequency of ADR. The UGLA survey reports that about 15.4% of patients having ASA have upper GI symptoms, mainly gastroesophageal reflux and 12% of these patients did not comply with treatment. Presence of prior dyspepsia was predictive of patients who would have low dose aspirin related GI symptoms (Cayla et al., 2010). Other studies have reported discontinuation rates of about 50% in patients who had been on long term low dose aspirin, despite the overwhelming evidence in support of the cardiovascular protective effects of aspirin. This non-compliance rate is quite alarming as various studies have reported increased incidence of cardiovascular and cerebrovascular events following discontinuation of aspirin (Collet & Montalescot, 2004; Collet et al., 2004).

The GI complications associated with the use of low dose aspirin can affect the entire GI tract including the colon, but most commonly, the gastric antrum, especially the pre-pyloric area, which is the most commonly affected. Damage may initially be in the form of petechial lesions, ecchymosis, erosions, and ultimately ulcers. The extent of injury may vary depending on the dose of aspirin used, the concomitant use of other medications, and patient specific

risk profiles (Yeomans, 2011). The mucosal injury associated with low dose aspirin occurs gradually, rather than being an instantaneous event, with loss of superficial cells of the mucosa with each dose of aspirin used.

Similarly, typical NSAIDs (non COX-selective NSAIDs) cause GI toxicity by way of peptic ulceration and as such it has been suggested that typical ulcers in the elderly usually implies NSAID use, and is stronger evidence than history of use volunteered by the patient (Wehling, 2014). Just like aspirin, use of typical NSAIDs causes abdominal discomfort, bloating, acidic reflux, pain and loss of appetite and these may be present without any obvious endoscopic evidence of disruption of mucosal architecture or may herald more serious mucosal damage and bleeding (Bjarnason, 2013).

1.3.2 Lower gastrointestinal injury in low dose aspirin users

It was previously thought that low dose aspirin does not cause any injury to the lower gastrointestinal tract. In support of this thinking is the fact that ASA is almost completely absorbed before reaching the small intestine and as such the topical effect is limited. Emerging evidence now indicates that ASA can cause mucosa damage below the ligament of Trietz (Sostres & Gargallo, 2012).

Protein loss and bleeding in the small bowel in chronic ASA users significant enough to cause hypoalbuminemia and iron deficiency anemia have been described (Fortun & Hawkey, 2005). In an attempt to evaluate the effect of enteric coated low dose ASA on the small bowel, it was shown that half of twenty healthy volunteers who underwent vidoecapsule endoscopy following ingestion of 100mg of enteric coated aspirin showed some level of

mucosal injury and erosions; median baseline permeability was increased following use of ASA while one patient developed two ulcer lesions (Smecuol et al., 2009). The clinical significance of these data is uncertain and further studies are required to validate them.

1.3.3 Upper gastrointestinal bleeding in aspirin and NSAID users

Aspirin/NSAIDs are some of the most commonly prescribed medications, with an estimated annual production of over 45,000 tons and average yearly consumption of 80 tablets per person worldwide (Vane & Booting, 1998). Over 111million NSAID prescriptions were written in the United States in 2004 (Shaheen et al., 2006).

NSAID-related GI ulceration is the most important adverse drug reaction from a public health perspective. The majority of ulcers are asymptomatic and usually heal naturally; however, occasionally, there could be an erosion into a vessel or perforation of the gut wall, both of which, though less common, are life threatening complications (Sostres & Gargallo, 2012). Several factors increase the risk of GI complications in low dose aspirin users and these are discussed in more detail in subsequent chapters.

Most importantly, concomitant use of NSAIDs and other antiplatelet agents amongst others could significantly increase the risk of GI bleeding in low dose aspirin users. In a meta-analysis of 61 randomized controlled trials, it was estimated that there is an increased risk of major GI bleeding with low dose aspirin (OR,1.55; 95% CI, 1.27-1.90). This risk is even further aggravated when low dose aspirin is taken concomitantly with the antiplatelet, clopidogrel or other anticoagulants (OR, 1.86; 95% CI, 1.49-2.31 and OR, 1.93; 95% CI, 1.42-2.61 respectively) (Lanas *et al*, 2011). In a cohort of 903 patients taking aspirin followed up for 45

months, 41 (4.5%) had significant bleeding requiring admission. In terms of site of bleed, 12 (29%) had a bleeding gastric lesion, 10 (24%) a bleeding duodenal ulcer and 19 patients (46%) had gastroduodenal mucosal lesions. This implies that the rate of major GI bleed is 4.54% and an incidence of 1.2/100 patient-years (Serrano et al., 2002).

1.3.4 Epidemiology and burden of nonsteroidal anti-inflammatory drug complications

Endospically confirmed ulcer lesions are estimated to be present in 15-35% of patients within 3 months of commencement of NSAIDs treatment (Bjarnason, 2013). NSAIDs have been estimated to cause 3500 hospital admissions and 400 deaths from ulcer bleeding per annum in the UK in those aged over 60 years (Langman, 2001). In the United States, NSAIDs led to over 100, 000 hospitalisations and 17,000 deaths per annum (Wolfe et al., 1999). In another study conducted on deaths from adverse drug reactions involving 1225 patients in the United Kingdom, over 60% of these were attributable to NSAIDs (Pirmohamed et al., 2004), making NSAIDs ADR a major cause of mortality directly linked to drug use.

In Germany, GI ADRs from NSAIDs use is perceived to be more dangerous than the risk of driving a car, as 1 out of 1220 (in UK, 1 out of 2000 patients) NSAID users will die of GI ADR each year (Bolten, 2001; Cryer, 2005). An independent estimate reported that in 2009, the cost of managing NSAID-related complicated gastrointestinal ulcers in the UK was $6825 per patient (Belsey, 2010).

1.3.5 Risk factors for NSAID-induced upper GI complications

Various risk factors have been implicated in the development of NSAID-related GI complications. As discussed in section 1.1.4, most of the factors noted above also play a role in NSAID-related gastric complications. Particularly, concomitant use of other anti-platelets,

different NSAIDs, steroids and anticoagulants are associated with increased risk (Valkhoff et al., 2012) while comorbid conditions like diabetes, cardiovascular, hepatic and renal disease (Deighton et al., 2009) have been associated with increased bleeding risk with use of NSAIDs. The risk of gastrointestinal bleeding is increased by 2.1% among users of low dose aspirin with previous history of PUD, and in the same cohort, 13.5% of cases with UGIB reported a previous history of UGIB in contrast to only 2.3% without an UGIB (Lanas et al., 2000). Table 1.11 presents the various risk factors for NSAID-related ulcers.

Table 1.11: Risk factors for NSAID-related gastrointestinal complications, modified from (Valkhoff et al., 2012)

Risk factors	Identified as risk factors due to aspirin (definite/probable/controversial/No)	Identified as risk factors due to NSAIDs (Yes/No)*
Patient-specific risk factors		
History of PUD or GI bleeding	Definite (Lanas et al., 2000)	Yes
Older age	Controversial (García Rodríguez et al., 2001)	Yes
Concomitant use of NSAIDs+coxibs	Definite (Lanas et al., 2006)	Yes, + LDA
Concomitant use of anticoagulants including anti-platelets	Probable (Gulløv et al., 1999)	Yes
Presence of comorbidities	Controversial except patients with hypertension and diabetes and chronic debilitating disorders (Deighton et al., 2009; Sirois et al., 2014)	Yes, especially cardiovascular disease
Male gender	No (Ng et al., 2006; Okada et al., 2009)	No
Excessive alcohol use	Probable (Okada et al., 2009; Serrano et al., 2002)	No
Current Smoking	No (Soriano & García Rodríguez, 2010)	No
High Body Mass Index	Probable (Ng et al., 2006)	No
Concurrent corticosteroids	Controversial (de Abajo & García Rodríguez, 2001; Lanas et al., 2002)	Yes No
Concurrent calcium Channel blockers	Probable (Lanas et al., 2002)	No
Concurrent use of SSRI	Definite (Anglin et al., 2014)	No
History of dyspepsia	Probable (Soriano & García Rodríguez, 2010)	No
H. pylori infection	Definite (Lanas et al., 2002)	Yes
Aspirin related factors		
High dose aspirin	Probable (Derry & Loke, 2000; Lanas et al., 2006; Serebruany et al., 2005)	Yes, high dose
Short aspirin duration	Definite (Lanas et al., 2000; Lanas et al., 2006)	No
Plain aspirin preparation	No (de Abajo & García Rodríguez, 2001; Kelly et al., 1996)	No
Regular aspirin duration	Probable (Kelly et al., 1996)	No

*according to American College of Gastroenterology guidelines (Lanza et al., 2009); SSRI: selective serotonin reuptake inhibitors; LDA: low dose aspirin.

1.4 Mechanistic hypothesis of NSAID-induced upper GI injury

Aspirin and NSAIDs cause GI mucosal injury via both topical and systemic effects. However, the systemic effect is believed to be more dominant in the pathogenesis of NSAID-related ulcers. Strong evidence in support of this is the failure of parenteral or rectal administration of NSAIDs to fully prevent the development of ulcers (Sostres et al., 2010). The systemic effects of NSAIDs are mediated mainly by the inhibition of COX I and II enzymes with the attendant blockage of the synthesis of prostaglandins. However, it is worthy of note here that strong evidence points to the existence of several other prostaglandin-independent mechanisms important in ulcerogenesis.

1.4.1 Direct gastric epithelial injury

Aspirin and most other NSAIDs are weak organic acids with a pKa of 3-5 and this acidic nature of NSAIDs initiates and perpetuates the initial GI mucosal injury by disrupting the epithelial cell barrier of the gastric mucosa. Their ability to remain in the non-ionised form in the highly acidic gastric environment (pH2.5) enhances their lipophilicity, and this as well, favours their cell membrane permeability (Schoen & Vender, 1989). There is a correlation between the ability of NSAIDs to cause transient topical damage and their pKa and lipid solubility. The carboxylic acid group of NSAIDs enhances significantly their water solubility and this improves their interaction with cell membrane phospholipids, thereby enhancing their rate of entry into the gastric epithelial cells (Bjarnason et al., 2007).

While in the intracellular location with a pH of about 7, NSAIDs being weak acids, easily dissociate into the ionised form, thereby causing intracellular ion trapping. Various

functional studies have shown that when NSAIDs accumulate in gastric epithelial cells via the mechanism described above, there is an uncoupling of mitochondrial oxidative phosphorylation and inhibition of the respiratory chain, also known as the electron transport chain (figure 1.1). This leads to a significant consumption of intracellular ATP, increased intracellular calcium and attendant toxicity; and increased generation of reactive oxygen species (ROS) (Musumba et al., 2009).

Besides the direct effect of ROS on oxidation of cellular components like proteins, lipids, and nucleic acids, NSAIDs cause genotoxic injury to cells leading to the initiation of apoptosis and/or necrosis. When oxidative phosphorylation is uncoupled, mitochondrial membrane potential is dissipated with subsequent release of cytochrome c and initiation of apoptosis (Nagano et al., 2005). As stated earlier, the properties of NSAIDs confers on them the ability to attenuate the hydrophobic surface barrier of the gastric mucosa by way of its interaction with the phospholipid bilayer present on the surface of the mucous gel in the GI, thereby, disrupting the hydrophobic barrier properties of this layer.

NSAIDs can also alter GI cell membrane permeability by initiating changes in membrane pore formation, fluidity, thickness, bending stiffness and hydrophobicity. These events cause acid 'back diffusion' and initiate cell death via apoptosis and necrosis with formation of the GI ulcer lesion (Darling et al., 2004; Lichtenberger et al., 2006).

The propensity of aspirin and other NSAIDs to cause injury through the topical effect is highlighted by experiments in laboratory animals which have reported gastric mucosa lesions following oral administration of indomethacin in rats (Esume C et al., unpublished data 2016), while other NSAIDs cause GI injury irrespective of route of administration (Mashita et al., 2006). The role of oxidative stress in the pathogenesis of indomethacin-

related ulcers has been reported and it was suggested that irreversible inactivation of gastric peroxidase occurs with indomethacin treatment in rats (Chattopadhyay et al., 2006). Figure 1.2 illustrates the various mechanistic processes of NSAID-induced gastrointestinal injury.
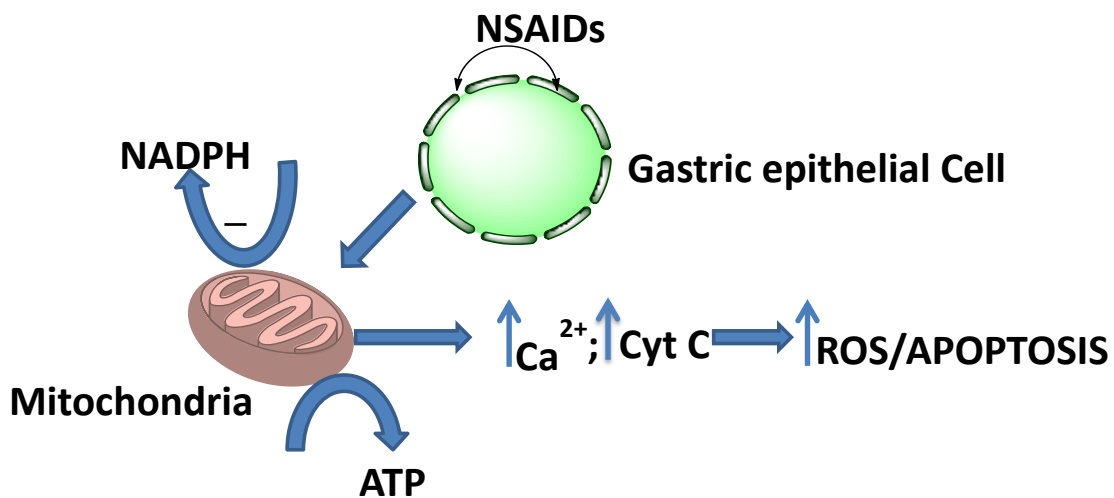
Figure 1.1: A schematic illustration of the topical effect of aspirin leading to gastric cell death via "ion trapping and acid back diffusion" as described by Nagano et al., 2005 and Musumba et al., 2009. Aspirin and other NSAID accumulates in the gastric epithelial cells and this causes uncoupling of the mitochondrial respiration and depletion of ATP, leading to increased intracellular Ca^{2+}, release of cytochrome C and generation of reactive oxygen species culminating in cell death.
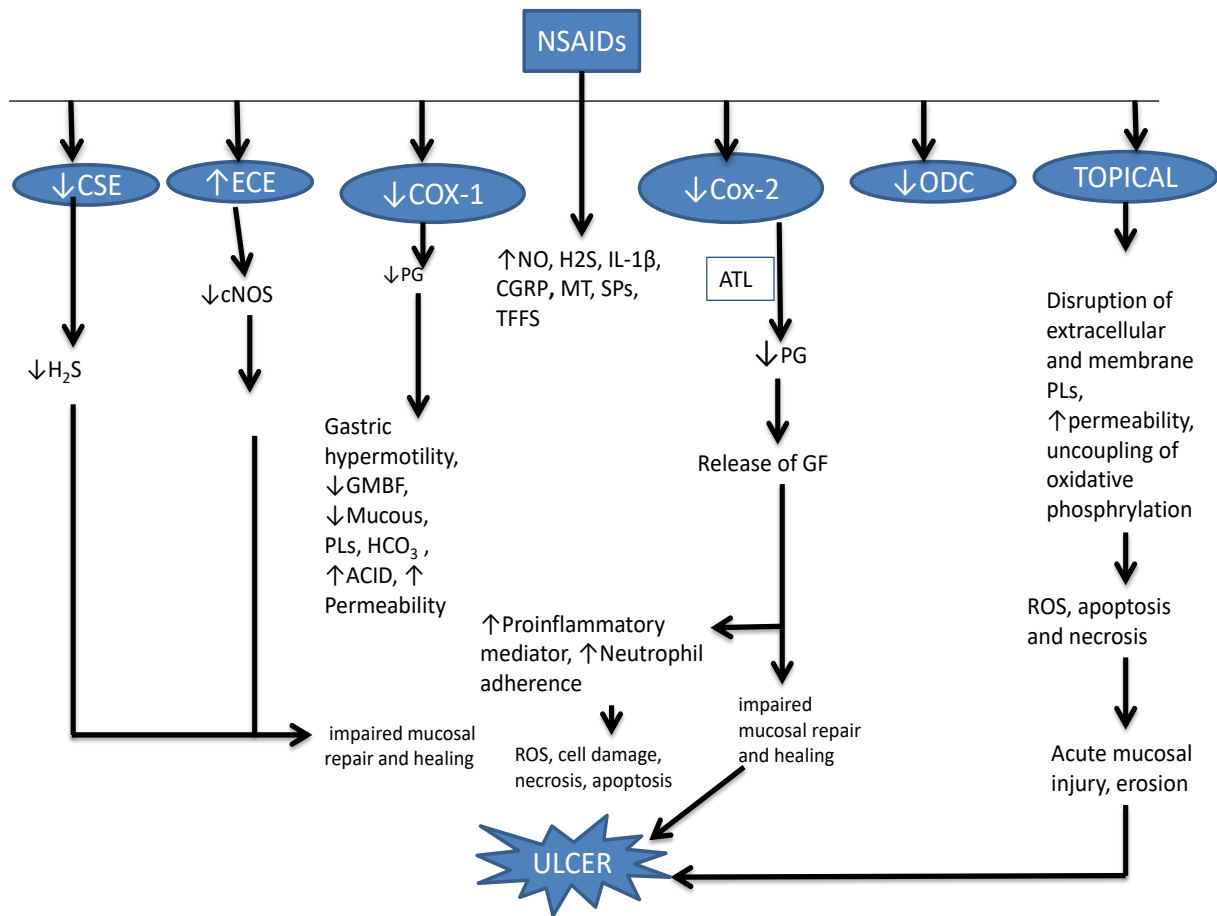
Figure 1.2: Schematic representation of cellular and molecular pathogenesis of NSAID-induced ulcers (adapted from Musumba *et al*, 2009). PLs: Phospholipids; CSE: Cystathione-γ-lyase; ECE-1: endothelin-converting enzyme-1; ATL: Aspirin triggered lipase; cNOS: constitutive nitric oxide; ODC: ornithine decarboxylase; GMBF: gastric mucosal blood flow; CGRP: calcitonin gene related peptide; MT: Melatonin; SPs: stress proteins; TFFs: trefoil factors; ROS: reactive oxygen species.

1.4.2 Inhibition of cyclo-oxygenase 1 and 2 enzymes

There is a strong correlation between NSAID-induced ulcerogenesis and their ability to supress gastric synthesis of prostaglandin and their selective inhibition of COX 1 in preference to COX 2. The COX enzymes are crucial for the conversion of arachidonic acid to prostaglandin endoperoxides and prostanoids, and gastric mucosa integrity is maintained by the continuous generation of prostacyclin (PGI_2) and prostaglandin E_2 (PG E_2). While COX 1 is

constitutively secreted and is in abundance in the gastric epithelium, the expression of COX 2 is inducible, the inhibition of COX 1, following gastric epithelial injury or an ongoing gastric lesion being major inducers of COX 2 expression (Tanaka et al., 2002). So following a perturbation of gastric epithelial cell membrane integrity by various noxious agents, an increase in the expression of the COX enzymes occur leading to an elevated level of gastric cytoprotective prostaglandins.

Aside from the pivotal role that PGI_2 and PGE_2 play in all aspects of gastric cytoprotection and healing, they are both potent vasodilators causing increased mucosal blood flow thereby protecting the mucosa from acid injury. There is a compensatory mechanism that is triggered following acid disruption of the mucosal barrier and this mechanism acts to reduce intracellular hydrogen ions by increasing mucosa blood flow. Failure of these mechanisms cause cell death and ulcer formation (Araki et al., 2000; Takeuchi et al., 2001).

1.4.3 COX and Prostaglandin signalling in NSAID-related ulcers

Four G-protein coupled receptors, EP_1 to EP_4 mediate the various functions of PGE_2 while PGI_2 acts via prostacyclin receptors. Activation of EP_1, EP_2 and EP_4 receptors by PGE_2 triggers an increase in intracellular Ca^{2+} or cyclic adenosine monophosphate activation (cAMP) whereas, coupling of EP_3 causes a decrease in cAMP (Dey et al., 2006).

COX 1 or COX 2 deficient mice do not develop spontaneous gastric lesions and neither does exposure to selective COX inhibitors, even though both conditions attenuate prostaglandin synthesis. It appears that inhibition of both COX enzymes is a prerequisite to the development of NSAID-related ulcers, as mice in which both enzymes were inhibited, or exposed to non-selective COX inhibitors developed severe gastric lesions (Gretzer et al., 2001; Tanaka et al., 2002; Wallace et al., 2000).

The effect of NSAIDs in depleting platelet thromboxane levels following inhibition of platelet COX1 is a driving factor in their tendency to cause GI bleeding. Two groups independently reported up-regulation of COX 1 and 2 at gastric ulcer margins in humans, while in rats; only COX 2 was upregulated (Nguyen et al., 2007; Perini et al., 2003). COX2-dependent prostaglandins are known to play crucial role in ulcer healing and act by promoting growth factor mediated epithelial cell proliferation and angiogenesis, while COX 1 becomes relevant in a COX 2 deficient state (Schmassmann et al., 2006).

Moreover, a delay in onset of repair and restitution was observed in COX 1 knock-out mice with superficial gastric mucosa injury. This could be suggestive of a plausible role of COX 1-derived PGE2 as a mediator of gastric cytoprotection against the initial and early processes that drive the progression of gastric epithelial damage (Starodub et al., 2008).

Meanwhile, inhibition of both COX 1 and COX 2 in knock out mice, greatly attenuated healing ulcer lesions more than selective inhibition of COX 2, with no effect noted with inhibition of COX 1 (Schmassmann et al., 2006). Among most NSAIDs, naproxen has been shown to delay healing of NSAIDs related ulcers to a greater measure than celecoxib, or aspirin and this corroborates reports from whole animal experiments (Dikman et al., 2009).

COX inhibition also leads to gastric hypermotility, which is mediated by COX 1. It is thought that the increased amplitude of smooth muscle contraction results in reduced mucosal perfusion leading to microvascular injury as a result of hypoxia, neutrophil endothelial interaction, and a decrease in the resistance of gastric mucous membrane. A summary of the role of prostaglandins and their mediators of gastric cytoprotection are presented in the table 1.11.

Table 1.12: The role of Prostaglandins and their mediators in gastric mucosal defence, adapted from (Musumba et al., 2009)

Gastroprotective Effect	Receptors (Species)	COX-isoform	References
Inhibit acid secretion	EP_3 (Mouse, rat) IP (mouse)	COX 1	(Kato et al., 2005; Nishio et al., 2007)
Stimulate acid secretion	EP_4 (rat)		(Kato et al., 2005)
Stimulate mucous secretion in stomach	EP_4 (rat)		(Araki et al., 2000)
Stimulate HCO3 secretion in stomach	EP_1 (rat, mouse)	COX 1	(Baumgartner et al., 2004; Takeuchi et al., 2006)
Stimulate HCO3 in duodenum	EP_4 (rat, human)		(Aoi et al., 2004; Larsen et al., 2005)
Maintain mucosal hydrophobicity	? (mouse)	COX 1	(Darling et al., 2004)
Increased GMBF	$EP_2/EP_3/EP_4$ (rat) EP_1 (mouse)	COX 1	(Araki et al., 2000; Komoike et al., 2003)
Facilitate CGRP-mediated hyperemic response	EP_2, EP_4 (mouse)		(Takeuchi et al., 2003)
Supress ↑TNFα suppression	EP_2, EP_4 (mouse)	COX 2	(Taira et al., 2009)
Inhibit neutrophil adherence and activation	EP_2, EP_4	COX 2	(Taira et al., 2009)
Inhibit epithelial cell apoptosis	EP_2, EP_4 (guinea pig)		(Hoshino et al., 2003)
↓ gastric hypermotility	EP_1 (rat)	COX 1	(Araki et al., 2000; Tanaka et al., 2005)
↓epithelial paracellular permeability	? (rat)	COX 1	(Takezono et al., 2004)
Adaptive gastric cytoprotection	EP_1 (rat, mouse)		(Takeuchi et al., 2001)
Accelerate restitution	? (rat, mouse)	COX 1/2	(Starodub et al., 2008; Terano et al., 2001)
Accelerate ulcer healing	EP_4 (mouse)	COX 2	(Hatazawa et al., 2007)
Resist ischemia and reperfusion induced injury	IP	COX 2	(Kotani et al., 2006)

GMBF: gastric mucosal blood flow; CGRP: calcitonin gene related peptide; COX: cyclooxygenase; EP: Prostaglandin E_2 receptor; IP: prostacyclin receptor; ↑: increase; ↓: decrease. Adapted from (Musumba et al., 2009).

1.4.4 Inhibition of ulcer-healing cytokines

1.4.4.1 Concept of adaptive cytoprotection

As stated earlier, secretion of PGs is stimulated by perturbation of the cell membrane of the GI, which can occur following cell trauma from exposure to acid or alkali, in addition to various irritants including hypertonic saline (Nishiyama et al., 1992), thermal injury and ethanol (Katori et al., 1990; Robert et al., 1983). Injury to the GI tract is prevented or at least ameliorated following a challenge with these injurious substances which would ordinarily damage the stomach, a phenomenon known as "adaptive cytoprotection" (Schoen & Vender, 1989).

1.4.4.2 Acid back-diffusion enhances calcitonin gene-related peptide (CGRP) secretion from sensory nerve endings

It is believed that endogenously secreted PGs are mediators of the adaptive cytoprotection and this is thought to be exerted via calcitonin gene-related peptide (CGRP) and substance P-releasing extrinsic sensory primary afferent nerves (Robert et al., 1983). The local protective function of spinal afferent neurons has been reported to occur in virtually all anatomic regions of the GI tract but those involving the gastro-duodenal region have been most extensively studied (Holzer, 1998). There is significant evidence demonstrating that these afferent neurons originating from the dorsal root ganglia have other important roles in GI circulation, secretion, motility, mucosal homeostasis and repair (Holzer, 2007). Acid back-diffusion from the lumen to the mucosal cells disrupts the mucosal barrier and this surge in the intracellular acidity triggers the spinal afferents to release CGRP and nitric oxide (NO), both of which cause immediate hyperaemia of the GI mucosa, and also call into action other

gastric mucosa defence mechanism via secretion of bicarbonate and attenuation of gastric acid secretion (Holzer, 2007; Musumba et al., 2009).

There is an inherent ability of PGs to protect the GI from damage and this occurs even at concentrations that would not interfere with gastric acid secretion. It has been established that there is an early and very crucial role of microvascular damage in NSAID-related ulcerogenesis, which gives credence to the fact that the response from the mucosal vasculature is invariably the most crucial aspect of mucosal defence against injury (Musumba et al., 2009).

Kim and his colleagues assessed gastric blood flow using a laser Doppler flowmeter and reported a significant reduction of blood flow in the gastric antrum of 20 healthy volunteers following administration of ibuprofen (Kim et al., 2007).

1.4.4.3 Biologic effects of CGRP

The release of the CGRP and NO messenger system acts to stimulate the synthesis and release of mucin from the gastric mucosa, and attenuates the myoelectrical activity of the gastric smooth muscles. Interestingly, CGRP itself, by stimulating the release of PGI2 and inhibiting the release of tumour necrosis factor-α (TNF-α) and accumulation of neutrophils in tissues, initiates an anti-inflammatory response (Okajima & Harada, 2006).

On the other hand, following NSAIDs and aspirin use, pro-inflammatory mediators such as TNFα, interleukin-1β (IL-1β), interleukin-8 (IL-8) and platelet activating factor (PAF) are now known to be upregulated and mediate the subsequent event of gastric injury and healing (Watanabe et al., 2004). These mediators (except IL-1β), enhance the ensuing inflammatory response and tissue destruction via mobilization of monocytes and neutrophils to site of

injury, by enhanced expression of adhesion molecules (ICAM-1) on the vascular endothelial walls and CD11b/CD18 from neutrophils (Souza et al., 2004; Watanabe et al., 2004). Physical injury occurs following occlusion of micro vessels by activated neutrophils and the subsequent ischemia causes tissue-destroying ROS and proteases to be released (Musumba et al., 2009). Unlike the other cytokines, IL-1β acts to ameliorate the damaging effects of NSAIDs on the GI by inhibiting PAF and leucocyte adhesion and enhancing elaboration of PG and NO via increased expression of both mediators (Martin & Wallace, 2006). Moreover, a synergistic action of CGRP and COX-derived PGI2 is purported to improve ulcer healing via the an enhanced expression of vascular endothelial growth factor (Ohno et al., 2008).

There is a gender difference in the expression of CGRP in the dorsal root ganglia cells, the reason for this difference being explained by the fact that estrogen enhances the expression and availability of releasable CGRP in the dorsal root ganglion. Hence, induced oestrogen-deficient states attenuate CGRP expression making the mucosa more liable to damage (Shimozawa et al., 2007; Shimozawa et al., 2006).

This could possibly explain the higher frequencies of ulcer in human males than females. A decline in the amount of CGRP elaborated, as reported in ageing rats with reduced number of mucosal CGRP-releasing nerve fibre, has been associated with impaired mucosal mucin synthesis (Holzer, 2007). In humans, there is a perceived association between chronic gastritis and increased expression of gastric CGRP, as well as an association between enhancement of CGRP and substance P expression in the antral mucosal and dyspeptic symptoms in *H. pylori*-infected patients (Dömötör et al., 2007; Mönnikes et al., 2005).

1.4.5 NSAIDs attenuates the gastroprotective effects of nitric oxide and hydrogen sulphide

Two endogenously generated gaseous mediators, nitric oxide and hydrogen sulphide, are important in the maintenance of gastrointestinal mucosal homeostasis and integrity by mimicking most of the biological effects of PGs (Oh et al., 2006). Both enhance the induction of the expression of heme oxygenase 1 (HO-1), a potent antioxidant, anti-inflammatory and anti-apoptotic proteins, and also exert a direct antinecrotic and anti-apoptotic effect on the gastric mucosa (Berndt et al., 2005; Li et al., 2007). The ability of nitric oxide to upregulate the expression of COX 2, with attendant increased elaboration of PGE_2 in rat fibroblasts has been demonstrated (Berg et al., 2005). This leads to increased manifestation of the gastroprotective and other downstream effects of PGE_2 on the GI.

Nitric oxide is generated via the action of nitric oxide synthase on the amino acid L-arginine (Palmer et al., 1988), and this important enzyme occurs in three isoforms, two of which (endothelial NOS and neuronal NOS) are constitutive enzymes, and the third an inducible isoform (iNOS). While the latter is calcium independent, the endothelial and neuronal isotypes are calcium/calmodulin dependent enzymes and synthesize low amounts of NO that perform very essential gastroprotective roles such as promotion of angiogenesis and increased mucosal blood flow thereby leading to improved ulcer healing and repair (Calatayud et al., 2001).

However, the effects of iNOS could be beneficial or detrimental depending on the type of tissue, the duration and level of expression of iNOS, and possibly the oxidation-reduction status of the tissue. Depending on the above factors, iNOS could increase mucosal blood flow leading to enhanced ulcer healing or rejuvenate pro-apoptotic mechanisms and ultimately induce tissue injury and mucosal ulceration (Calatayud et al., 2001).

Non-enzymatic mechanisms of generating NO exists and this includes the use of NO donors and these have been fused to COX inhibitors, to form a compound known as COX-inhibitor nitric oxide donor (CINODs), with the prospect of improving the GI safety of existing COX-inhibitors (Fiorucci S, 2007). The first CINOD to successfully complete phase III trials is naproxcinod (HCT3012) but was declined the United States food and drug agency (FDA) approval for lack of long term data on gastrointestinal and cardiovascular safety and GI benefits over COX inhibitors (Fiorucci & Santucci, 2011) .

There are several pathways through which hydrogen sulphide (H_2S) can be generated, the most crucial being that involving two pyridoxal-5' phosphate-dependent enzymes, that can convert L-cysteine, cysteine and homocystiene to H_2S. These two enzymes, cystathione-β-synthetase (CBS) and cystathione-Y-lyase (CSE), involved in the synthesis of H_2S are expressed in the gastric mucosal cells. H_2S elaborated from the mucosa acts in part, by the activation of ATP-sensitive K^+ channels and also stimulates angiogenesis in a manner independent of VEGF (Wang, 2002).

The effects promote epithelial cell proliferation and increased mucosal blood flow, both of which are essential for ulcer healing and repair (Fiorucci et al., 2007). Furthermore, H_2S has been demonstrated to possess anti-inflammatory properties following the observation that H_2S-donors suppressed leukocyte adherence to the endothelium of blood vessel and caused a reduction in leucocyte migration and extravasation to inflammatory sites and edema formation while inhibition of endogenous H_2S caused an opposing effect from the above (Zanardo et al., 2006). These have been exploited in the search for novel derivatives of mesalamine, used for the management of inflammatory bowel disease (Fiorucci et al., 2007).

Of relevance, there is attenuation of the synthesis of endogenous H_2S after the use of NSAID, demonstrated to be mediated by the suppression of the expression of a key enzyme, cystathione-γ-lyase necessary for the conversion of L-cysteine to H_2S (Fiorucci et al., 2007). Hence, the reduction of mucosal H_2S may well be another mechanism of NSAID-related GI injury, aside the well-known COX inhibition (Fiorucci et al., 2007). Still at an attempt to ameliorate the GI complications of currently used NSAIDs, H_2S-elaborating NSAIDs (ATB-346) with gastroprotective effects similar to that of CINODs have been synthesised (Fiorucci & Santucci, 2011; Li et al., 2007; Wallace et al., 2007).

1.4.6 Inhibition of growth factor production and role of various proteins

Growth factors exert their gastroprotective actions by enhancing mucosa re-epithelisation, granulation tissue formation and stimulation of angiogenesis and are found to be upregulated at ulcer margins following stimulation by cytokines, gastrin and PGE2 (Koike et al., 2007; Szabo & Vincze, 2000). The above effects are mediated essentially by various mechanisms including elaboration of COX-derived PGE2, enhanced expression of NOS and prevention of apoptosis through the modulation of pro-and anti-apoptotic proteins (Kosone et al., 2006; Netzer et al., 2003).

Growth factors such as epidermal growth factors, vascular endothelial growth factor, hepatocyte growth factor, transforming growth factor and trefoil factors are important in the process of restitution while platelet-derived growth factor, fibroblast growth factor-α, insulin-like growth factor-1 and vascular endothelial growth factor facilitate connective tissue restoration and integrity of the vasculature (Hoffmann et al., 2005; Hoffmann et al., 2004). Aside the role of trefoil factors in the promotion of restitution, the peptides acts to

stabilize the mucous gels and exerts some pro-angiogenic and anti-apoptotic effects which are essential for ulcer healing and repair. By suppressing PGE2-mediated growth factor production, NSAID may cause a delay in the repair of GI injury.

The polyamines and other polycations such as spermine, putrescine, and spermidine, adequately expressed in most eukaryotic cells, are important for cell growth and proliferation and are also essential for the various restitutive processes following GI injury from NSAIDs and other mucosal irritants. Because the intracellular levels of polyamines are crucial for determining what effects they exert, with high level promoting rapid cell proliferation and low level inducing apoptosis, their levels are neatly controlled by the biosynthetic enzyme ornithine decarboxylase (ODC) and the catabolic enzyme spermine/spermidine N^1-acetyltransferase (SSAT).

A group demonstrated increased cell death, *in-vitro* in cancer cells, following decrease in the intracellular levels of polyamines caused by the inhibitory effect of NSAIDs on ODC (Saunders, 2008). Available evidences indicate that there could be a role for polyamine depletion in NSAID-induced ulcerogenesis by mechanisms mentioned above. This is consistent with the finding that in rats treated with the NSAID indomethacin, there was elevated gastric acid secretion but significantly reduced gastric ulcer index and elevated mucin content, with attenuation of neutrophil infiltration in those rats administered exogenous spermine (Motawi et al., 2007).

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases found in most peripheral blood cells and gastric epithelial cells, whose secretion is highly regulated at various levels such as inhibition by tissue metalloproteinases (TIMPs), gene expression, and spatial localisation (Egeblad & Werb, 2002). The constitutively expressed MMP-2 plays a role

in the physiological turnover of gastric extracellular matrix, while MMP-9 which appears to be inducible through the action of cytokines, growth factors and hormones has been implicated to play a crucial role during the initial events in development of ulcers.

Indomethacin causes an increase in the activity of MMP-9 while decreasing the activity of MMP-2 in gastric ulcer tissue, and the increase in the MMP-9/MMP-2 ratio correlates with the ulcer severity index (Swarnakar et al., 2005). The generation of ROS regulates MMP expression in a manner that could enhance the transcription of MMP-9 or suppress MMP-2 transcription and this pathways could be PG-dependent or independent (Pan & Hung, 2002).

1.4 Aim of the thesis

A genome-wide association study (GWAS) of NSAID-induced gastrointestinal complications has previously been undertaken at the University of Liverpool, the cohort and recruitment of which was described elsewhere (Musumba et al., 2013). This identified a number of associations between NSAID-related gastrointestinal complications and biologically plausible genes. The "top hit" gene in this study has been identified to be in the *EYA1* gene locus on chromosome 8 ($p<10^{-6}$).

EYA1 is an important transcription factor, and also plays critical role in the apoptosis or repair decision-making of cells following genotoxic injury (Cook et al., 2009; Tadjuidje et al., 2012). The ability of a cell to achieve DNA repair would be dependent on whether the *EYA1* gene is functional or not. As noted earlier, previous studies on the pharmacogenetics or pharmacogenomics of NSAID-induced gastrointestinal complications have concentrated mainly on the polymorphisms of drug metabolising enzymes, genetic mutations in drug transport proteins and ion channels (Pirmohamed, 2011). However, there are currently no

studies which have looked at polymorphisms in genes involved in cell repair in relation to NSAID-induced gastrointestinal injury and particularly, no studies have previously examined the role of *EYA1* in NSAID-related gastrointestinal injuries.

The aim of this study was to elucidate the biological plausibility and functional effects of *EYA1* genetic mutation in the pathogenesis of NSAID-related gastrointestinal injury; we will be looking at it from the molecular and functional points of view using various techniques. We will also examine the role of previously reported polymorphism in *UGT1A6* gene (Shiotani et al., 2009; Van Oijen et al., 2009a; Van Oijen et al., 2009b), and a plausible role for *PAI-1* (Kenny et al., 2013b) polymorphism in the pathogenesis of NSAID and aspirin-related gastrointestinal complications.

Chapter 2

Relationship between a loss-of-function polymorphism of *UGT1A6* and low-dose aspirin related upper gastrointestinal complications in a Caucasian population.

Chapter 2

induced GI complications

Tables

2.1 Introduction

2.1.1 Aspirin and upper gastrointestinal bleeding

Aspirin/NSAIDs are amongst the most commonly prescribed medications, with an estimated annual production of over 45,000 tons and average yearly consumption of 80 tablets per person per year (Vane & Booting, 1998). World-wide, over 30 million people use aspirin and other non-aspirin NSAID on a daily basis, and a retrospective study in the United States reported that approximately 25% of a group of medicaid recipients treated for arthritis with NSAIDs experienced NSAID-related gastrointestinal complications that required further care (Bloom, 1988; Singh & Triadafilopoulos, 1999). In the United States, one-third of the adult population and over 80% of those diagnosed with a cardiovascular disease use aspirin (Ajani et al., 2006). 1n 2007, primary care prescriptions of aspirin were estimated to be over 30 million in England alone (National Health Service information centre, 2007).

NSAIDs are prescribed commonly for their anti-inflammatory, analgesic and antipyretic effects while aspirin is useful for the secondary prevention of ischemic vascular diseases. It is currently being recommended for the primary prevention of cancer of the colon (McGreavey et al., 2005). NSAIDs have been estimated to cause 3500 hospital admissions and 400 deaths from ulcer bleeding per annum in the UK in those aged over 60 years (Langman, 2001). These figures are consistent with a more recent study of adverse drug reaction-related hospital admissions which identified NSAIDs as the most important reason for admission (Pirmohamed et al., 2004). In the United States, NSAIDs led to over 100,000 hospitalisations and 17,000 deaths per annum (Wolfe et al., 1999). In a study conducted on deaths from ADRs involving 1225 patients in the United Kingdom, over 60% of these were attributable to

NSAID (Pirmohamed et al., 2004). Therefore, NSAID-related GI ulceration is the most important ADR from a public health perspective.

The aim of this work was to determine whether *UGT1A6* genetic variability is a risk factor for aspirin/NSAID-related upper gastrointestinal complications. We would also ascertain the allelic and genotype frequencies of *UGT1A6* SNP rs1105879, the prevalence of PUD and upper GI bleeding in a Caucasian population exposed to aspirin and other NSAIDs.

2.1.2 Role of UGT1A6 in aspirin metabolism

Two enzymes are involved in the biotransformation of acetylsalicylic acid via hydroxylation and glucuronidation and both pathways are very important in acetylsalicylic acid metabolism. These enzymes, cytochrome P450 2C9 (CYP2C9) and UDP-glucuronosyltransferase 1A6 (UGT1A6) are highly polymorphic hence, their activity differs in different individuals (Van Oijen et al., 2005a).

Following the absorption of aspirin, the drug is rapidly transformed to salicylic acid via deacetylation. Only about 68% of administered dose reaches the general circulation as nonspecific esterases, mainly in the liver and to a lesser extent in the stomach, hydrolyse the drug (Needs & Brooks, 1985). About 10% of the dose is eliminated unchanged in the urine. Salicyuric acid, a glycine conjugate of salicylic acid is the major metabolite (see figure 2.1), and accounts for about 63% of recovery from the urine, with a large interindividual variability (20-75%). This conjugation with glycine is catalysed by the xenobiotics/medium chain fatty acid:CoA ligase (ACSM2B) (Hutt et al., 1986; Lares-Asseff et al., 2004). However,

variability in *ACSM2B* gene is extremely low compared to *CYP2C9* and *UGT1A6* (Agundez et al., 2009).
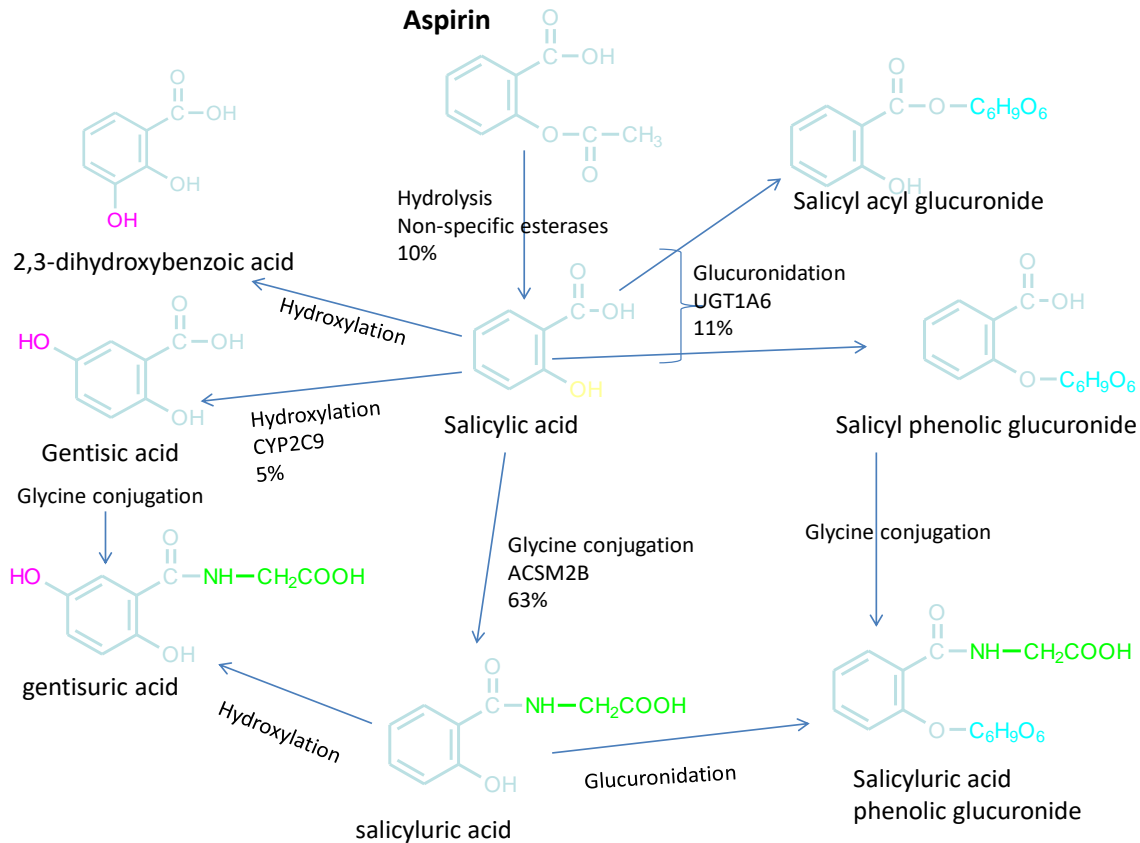
Figure 2.1: Schematic drawing of the pathway for aspirin metabolism, modified from Carr, 2016 (personal communication). The first step is the hydrolysis of aspirin by non-specific esterases in the liver and GI tract to salicylic acid, with only 68% of a dose reaching the systemic circulation. The proportion of each metabolite recovered in urine at therapeutic dose is shown as percentage (%).

Salicylic acid is also glucuronidated to form the acyl and phenolic glucuronides. Although, UGT1A1, 1A3, 1A7-10, 2B4 and 2B7 also catalyse the conjugation of salicylic acid, UGT1A6 is the major enzyme involved in the conjugation of this metabolite *in vivo* (Kuehl et al., 2006). CYP2C9 is also known to oxidize salicylic acid to a minor metabolite, Gentisic acid (2, 5-dihydroxybenzoic acid) (Bigler et al., 2001; Ciotti et al., 1997; Kuehl et al., 2006). UGT family of enzymes are vital in the biotransformation of aspirin/NSAID, morphine, steroids, retinoids

and bile acids to more active and occasionally to more toxic substances (Abbot & Palmour, 1988; Vore et al., 1983; Vore & Slikker, 1985).

2.1.3 Aspirin glucuronidation and UGT1A6 polymorphism

3-UDP-glucoronosyltransferases (UGTs), a family of enzymes, are involved in the glucuronidation of endogenous compounds like bilirubin, drugs and other xenobiotics. Glucuronidation through two UGT families (UGT1A's and UGT2B's) is important for aspirin clearance (Kuehl et al., 2006; Lampe et al., 1999). In aspirin glucuronidation, the most important contributors are *UGT1A1*, *UGT1A6*, *UGT1A7* and *UGT1A9* (Agundez et al., 2009).

UGT1A6 is the founding member of the rat and human *UGT1* family. It is expressed in liver and extrahepatic tissues such as intestine, kidney, testis, and brain and it conjugates planar phenols and arylamines (Bock & Kohle, 2005). *UGT1A6*, like other members of the *UGT1A* family is located on chromosome 2 and encodes a complex gene consisting of 16 exons, whereby alternative splicing of exon 1 to the four common exons (2-5), generates a number of isoforms. About two-thirds of the lumenal domain is encoded by the first exon, while exons 2-5 encodes the remaining one-third of the lumenal domain, as well as the transmembrane domain and the cytosolic tail (Radominska-Pandya et al., 1999).

Two studies have looked at the relationship between loss of function polymorphisms in *UGT1A6* in aspirin users and development of GI complications. None of these studies have confirmed an association between *UGT1A6* polymorphism as a risk factor in the development of ulcer in aspirin and NSAID users (Shiotani et al., 2009; Van Oijen et al., 2009b).

2.1.4 Structure of UGT1A6 enzymes

The UGTs are type 1 transmembrane-based proteins which reside, along with the active site, in the luminal side of the endoplasmic reticulum (ER) (Bock & Kohle, 2005). This is evidenced by the fact that there is a latency period during which UGT activity can be activated following the addition of detergents or pore forming agents such as alamethacin, creating an unrestricted entry of the cofactor UDP-glucuronic acid to the enzyme's active site (Radominska-Pandya et al., 1999). However, lipophilic aglycones, found in high concentrations in the ER plasma membranes, have been reported to have unrestricted access to the active site (Bock & Lilienblum, 1994). There is increasing evidence to suggest that the substrate specificity of the UGTs is affected by their dimeric actions (Ishii et al., 2001).

The UGT1A family of enzymes are encoded by a single large gene spanning about 200kb on chromosome 2q37; being composed of different but homologous first exons. Each is preceded by its own promoter that encodes the N-terminal of the enzyme and a shared set of 5 exons 2-5 that together encode the C-terminal half of the proteins. The N-terminal domain, usually about 260 amino acids long (though variable), is thought to contain the binding site for the aglycones substrate while the 246 amino acid C-terminal domain which is common to all UGT1A is thought to provide the binding site for the common glucuronic acid donor, UDP-glucoronic acid (UDPGA) (Ghemtio et al., 2014).

There is close sequence homology between human *UGT1A6* and other mammalian UGT's for instance, sequence analysis work has determined that the human *UGT1A6* and the cynomolgus monkey *UGT1A6* copy deoxyribonucleic acid (cDNA) have an open reading frame of 1599 bp, with a 96% identity at both nucleotide and amino acid levels (Hanioka et

al., 2006). However, the mammalian *UGT1A6* differs from plant and bacterial UGTs in that the C-terminal region is hydrophobic, folding into a single trans-membrane α helix that traverses the membrane, with a short C-terminal end exposed to the aqueous medium on the other side of the membrane. In plants, the hydrophobic domain is not required for UGT function (Ghemtio et al., 2014). The interaction of compounds with UGT1A6 can be tested by an indirect method, *in vitro,* by measuring the rate of glucuronidation or directly by detecting its effect on the glucuronidation of a probe substrate such as 1-napthtol (Ghemtio et al., 2014).

2.1.5 *UGT1A6* alleles

Several variant alleles have been described for *UGT1A6*, resulting from changes in amino acids resulting in homozygous and heterozygous variant forms; this homo-and heterozygous occurrence of *UGT1A6* markedly affects enzyme activity. The most important of these alleles are two which are defined by changes in the amino acids at p.T181A and p.R184S. The single nucleotide polymorphisms (SNP) under investigation rs1105879, is associated with the amino acid substitution of threonine for alanine at position 181, arginine for serine at position 184 and nucleotide substitution of adenine for cytosine at position 552 (see table 2.1). Carriage of these amino acid substitutions have been shown, *in vitro*, to confer a 30%-50% decrease in enzyme activity compared to the wild type (Ciotti et al., 1997).

UGT1A6*1 and *2 are the most common allozymes accounting for 90% of allozymes of UGT1A6 (Carlini et al., 2005). Several other UGT1A6 allozymes with various combinations of amino acid variants have been described. These variants include *UGT1A6*1, UGT1A6*2, UGT1A6*3, UGT1A6*4, UGT1A6*5, UGT1A6*6, UGT1A6*7, UGT1A6*8 and UGT1A6*9*. A

detailed presentation of polymorphisms affecting the *UGT1A6* gene locus is presented in table 2.1. Some of the NSAID-related pharmacogenomic targets for *UGT1A6* gene seem to be SNP rs1105879 and rs2070959, while the functional effect of the SNP rs6759892 is yet to be fully elucidated (Agundez et al., 2009).

Allelic variations in the coding region of the human *UGT1A6* has been observed to cause over 25- and 85-fold differences in enzyme activity and immunoreactive protein respectively (Nagar et al., 2004). Polymorphisms of the cis-acting response elements and the trans-acting transcription factors are thought to be responsible for the variability in enzyme levels (Bock & Kohle, 2005). Interplay of the above factors culminates in a high interindividual variability in the activity and expression level of *UGT1A6*.

Table 2.1: Major single nucleotide polymorphism affecting the open reading frame for *UGT1A6*, modified from: http://www.pharmacogenomics.pha.ulaval.ca/files/content/sites/pharmacogenomics/files/Nomenclature/UGT1A/UGT1A6.htm

Allele naming	Protein	Nucleotide change	Amino acid change	References
[£]UGT1A6 *1a	UGT1A6.1			
UGT1A6*1b	UGT1A6.1	-1535(G>A)/ -427(G>C)		Krishnaswamy et al., 2005
UGT1A6*1c	UGT1A6.1	-427(G>C)		Krishnaswamy et al., 2005
UGT1A6*1d	UGT1A6.1	-1535(G>A)/ -1377(G>A)/ -427(G>C)		Krishnaswamy et al., 2005
[Δ]UGT1A6 *1e				
	UGT1A6.1	105(C>T)	$D^{35}D$	Saeki M, 2005
[¥]UGT1A6 *1f	UGT1A6.1	IVS1+120(A>G)		Saeki et al., 2005
[α]UGT1A6 *1g	UGT1A6.1	IVS1+142(C>T)		Saeki et al., 2005
[±]UGT1A6*2a	UGT1A6.2	19(T>G)/315(A>G)/541(A>G)/552(A>C)/ IVS1+130(G>T)	$S^7A/L^{105}L/T^{181}A/R^{184}S$	Saeki et al., 2005
UGT1A6*2c	UGT1A6.2	19(T>G)/315(A>G)/541(A>G)/552(A>C)/ IVS1+109(C>T)/IVS1+130(G>T)	$S^7A/L^{105}L/T^{181}A/R^{184}S$	Saeki et al., 2005
UGT1A6*2d	UGT1A6.2	19(T>G)/279(A>G)/315(A>G)/541(A>G)/ 552(A>C)/IVS1+130(G>T)	$S^7A/S^{93}S/L^{105}L/T^{181}A/R^{184}S$	Saeki et al., 2005
UGT1A6*2e	UGT1A6.2	-1710(C>G)/ -1377(G>A)/ -1310 (del AGGAG)/-652(G>A)/19(T>G)/ 315(A>G)/541(A>G)/552(A>C)	$S^7A/L^{105}L/T^{181}A/R^{184}S$	Krishnaswamy et al., 2005

Allele naming	Protein	Nucleotide change	Amino acid change	References
UGT1A6*3a	UGT1A6.3	-1710(C>G)/ -1377(G>A)/ -1310 (del AGGAG)/-652(G>A)/ 19(T>G)	S^7A	Krishnaswamy et al., 2005
UGT1A6*3b	UGT1A6.3	-1710(C>G)/ -1377(G>A)/ -1310 (del AGGAG)/-652(G>A)/ 19(T>G)/315(A>G)	$S^7A/L^{105}L$	Krishnaswamy et al., 2005
UGT1A6 *4a	UGT1A6.4	19(T>G)/315(A>G)/552(A>C)	$S^7A/L^{105}L/R^{184}S$	Nagar et al., 2004
UGT1A6 *4b	UGT1A6.4	19(T>G)/315(A>G)/552(A>C)/627(G>T)	$S^7A/L^{105}L/R^{184}S/V^{209}V$	Saeki et al., 2005
UGT1A6 *4c	UGT1A6.4	-1710(C>G)/ -1377(G>A)/-1310 (del AGGAG)/-652(G>A)/ 19(T>G)/ 315(A>G)/ 552(A>C)	$S^7A/L^{105}L/R^{184}S$	Krishnaswamy et al., 2005
UGT1A6*5	UGT1A6.5	541(A>G)	$T^{181}A$	Krishnaswamy et al., 2005
[€]UGT1A6*6	UGT1A6.6	19(T>G)/308(C>A)/315(A>G)/541(A>G)/552(A>C)/IVS1+130(G>T)	$S^7A/S^{103}X/L^{105}L/T^{181}A/R^{184}S$	Saeki et al., 2005
UGT1A6*7	UGT1A6.7	19(T>G)/269(G>A)/315(A>G)/541(A>G)/552(A>C)/IVS1+130(G>T)	$S^7A/R^{90}H/L^{105}L/T^{181}A/R^{184}S$	Saeki et al., 2005
[β]UGT1A6*8	UGT1A6.8	541(A>G)/552(A>C)	$T^{181}A/R^{184}S$	Ciotti et al., 1997
UGT1A6*9	UGT1A6.9	552(A>C)	$R^{184}S$	Ciotti et al., 1997

[‡]High enzyme activity *in vitro* (Krishnaswamy et al., 2005b; Nagar et al., 2004); [β]low enzyme activity *in vitro* (Ciotti et al., 1997), high activity (Krishnaswamy et al., 2005d); [£]wild type, with normal *in vivo* and *in vitro* enzyme activity; [Δ]silent effect and renamed from UGT1A6*1b; [¥]renamed from UGT1A6*1c; [α]Renamed from UGT1A6*1d; [€]renamed from UGT1A6*5.

2.1.6 Regulation of UGT1A6 expression

Two modes of expression have been proposed; a high constitutive/lower inducible expression and with a low constitutive/high inducible expression. This postulate was based on the finding of high constitutive expression in rat kidney and low basal expression in rat liver with high inducibility by Ah-type inducers (Munzel et al., 1994).

Factors involved in the regulation of the expression of *UGT1A6* include environmental, genetic and tissue-specific factors. *HNF1* and *HNF4* are thought to regulate tissue specific high constitutive expression of *UGT1A6* (Mackenzie et al., 2003). The effect of gender is controversial; one group reported that there is a 50% reduction in hepatic *UGT1A6* expression in females (Court et al., 2001), while another group observed no gender difference in the level of UGT activity (Nagar et al., 2004).

Environmental factors controlling UGT expression include drugs, hormones and other xenobiotics that act as ligands for multiple sensors such as constitutive androstane receptor (CAR), the aryl hydrocarbon (Ah) receptor, pregnane X receptor (PXR), and transcription factor mediating stress responses, Nrf2 (Bock & Kohle, 2005).

2.2 Methods

2.2.1 Study subjects

This study was a multi-centre observational study involving 15 hospitals in the United Kingdom (figure 2.2). Endoscopy databases of participating hospitals were used to identify patients who had undergone endoscopy between July 2005 and June 2011. Following identification of the patients, they were invited to participate in the study either via

telephone calls, letter or were recruited prospectively in hospital as in-patients or day-cases during their attendance at endoscopy from January 2008. Ethical approval was given by the Liverpool Research Ethics Committee.

The patients were assigned to 3 different groups as follows: 1) patients who have endoscopic diagnosis of peptic ulcer disease (PUD) within 2 weeks of use of NSAID and /or aspirin; 2) endoscopic confirmation of PUD without history of use of NSAIDs and/or aspirin within 3 months of diagnosis; and 3) subjects without endoscopic evidence of PUD, whose use or non-use of NSAID was established before endoscopy was performed. Study participants in groups 1 and 2 were recruited pro- and retrospectively, while group 3 were recruited prospectively by a random selection of outpatient attendees scheduled for upper gastrointestinal (UGI) endoscopy at the Royal Liverpool University Hospital.

Eligibility criteria were age over 18 years at the time of recruitment, capacity to give informed consent and clinical indication for undergoing UGI endoscopy. Patients who were unable or refused to give consent, subjects who could not be contacted, patients with malignant peptic ulcer, previous gastrointestinal surgery or Zollinger Ellison syndrome and those with blood borne transmissible diseases were excluded from the study (Musumba et al., 2013).

2.2.2 Patients' demographic characteristic and medication history

All patients participating in this study were interviewed using a structured questionnaire with emphasis on medical and drug history, demographic data, co-morbidities, alcohol intake, smoking habits, and previous *H. pylori* eradication. Pre-endoscopy interview forms and endoscopy reports were carefully checked for details of patients' clinical presentation, ulcer characteristics and use of ulcerogenic drugs like NSAIDs. Further information was

sourced from case notes or when necessary, the patients' general practitioner was contacted. From the drug history, those that had used NSAID as part of a treatment for a continuous period of 1 week or more, for the 2 weeks preceding endoscopy were defined as NSAID users, while non-users were defined as those who had not used any NSAID in the 3-months prior to endoscopy.

2.2.3 Endoscopy and *H. pylori* infection

PUD was defined as a breach in mucous membrane ≥ 3mm or from the endoscopist's description. Upper gastrointestinal bleeding (UGIB) was defined, according to the Forrest criteria as hematemesis, melena or anaemia and/or endoscopic stigmata of a recent bleed (Forrest et al., 1974). According to the standard of practice in the centres, most patients had gastric biopsy done for the rapid urease test (Pronto Dry, Medical Instruments Corporation, Solothurn, Switzerland or CLO test) and/or histology to test for *H. pylori* infection.

A positive histology or urease test was taken as an indication for presence of *H. pylori* infection and when negative or not done, a positive serology test was taken as evidence of past or current infection, while a negative serology test was taken to indicate absence of infection. Once a patient was enrolled, about 20mls of venous blood was collected in 2 specimen bottles and sample (whole blood) was stored frozen at -80°c.

2.2.4 Genomic deoxyribonucleic acid extraction

Genomic DNA was extracted from 5ml whole blood using the Chemagen DNA extraction kit on the Chemagic Magnetic separation Module 1, according to the manufacturer's protocol (AutoQ Biosciences, Germany).

	Key
A	Liverpool (RLUH)
B	Liverpool (UHA)
C	Liverpool (WTH)
D	Blackpool (BVH)
E	Tyneside (STT)
F	Morecambe (RLI)
G	Blackburn (ELH)
H	Cumbria (NCT)
I	Southampton (SGH)
J	Preston (LTH)
K	Devon (NDH)
L	Bradford (BHT)
M	Teeside (NTH)
N	Chester (COC)
O	Cumbria (WCH)

Figure 2.2: Map of England showing the 15 sites from which the patient cohorts where recruited, tagged A-O. 11 of the centres are located in the North West of England, while 2 are in the North East and the other 2 are in the South of England. To the right of the map is the key showing the details of the study centres (adapted from Musumba, unpublished work, 2016).

2.2.5 Allelic discrimination for *UGT1A6* SNP rs1105879

Genotyping of *UGT1A6* SNP rs1105879 A/C (encoding a p.R184S amino acid substitution) was performed with TaqMan® SNP Genotyping assays (Applied Biosystems, USA) using DNA samples from study participants following the manufacturer's instructions. This PCR based assay utilises the 5' nuclease activity of Taq®DNA polymerase to distinguish between alleles. This custom Taqman assay contains a VIC and a FAM labelled probe, which detects one of the SNP-specific alleles by emitting an allele-specific fluorescent signal after degradation of the probe. Fluorescence and allelic discrimination were determined using the SDS software (version 2.2; Applied Biosystems, USA). The primer (VIC/FAM) sequence of this assay is: GTTCCCTGGAGCATACATTCAGCAG [A/C] AGCCCAGACCCTGTGTCCTACATTC.

20ng of DNA was placed in a sterile 384-well Polymerase chain reaction (PCR) plate, skipping alternate rows to avoid sample contamination. The plate was quickly placed in a Vortexer to dry for 5 minutes. The 5µl reaction mix consisted of x1 Taqman Genotyping Mastermix, x20 drug Metabolism Genotyping Assay (both assays supplied by Applied Biosystems) and Nuclease free water (from miliQ machine). For quality assurance, 10% of the test samples were duplicated and 2 negative controls included in each 384-well plate.

Thermal cycling was performed at the following conditions: 10 minutes at 95° C followed by 45 cycles of the denaturing step at 95°C for 15 seconds and an annealing step for 1 minute at 60°C. Amplification and signal detection was then undertaken using the AB 7900 HT Real time PCR system as per manufacturers' instructions (Applied Biosystems). Genotypes were determined using automatic calling on VIC/FAM signal cluster plots, an example of which is shown in Figure 2.3.
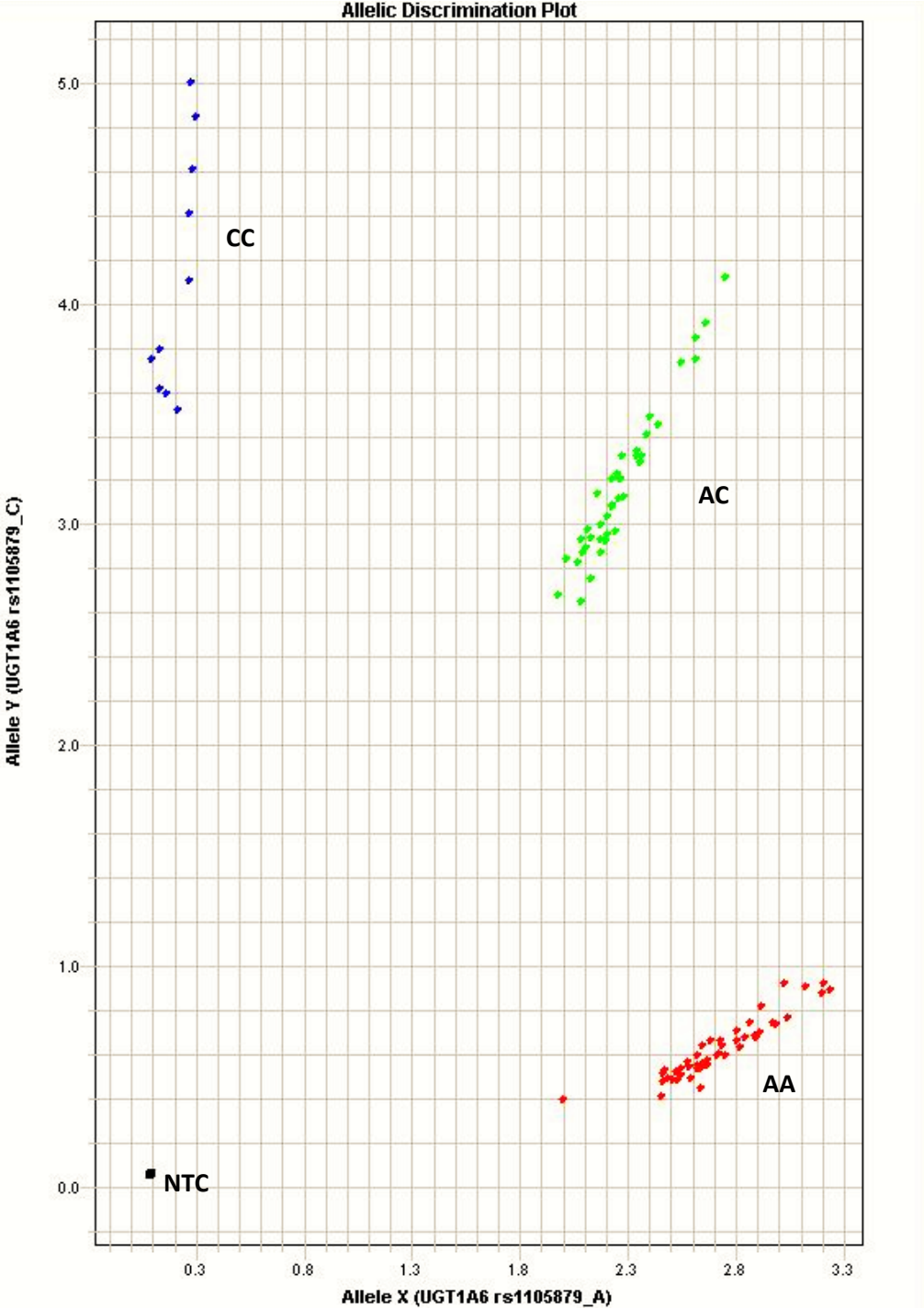
Figure 2.3: Allelic discrimination by TaqMan real-time PCR as displayed by the SDS software. Each cluster represents a genotype call as per whether they are homozygous wild type (AA), homozygous variant (CC) or heterozygous variant (AC). A good calling of SNPs genotype as shown in the graph should have clustering of genotypes into 3 clearly distinguished points from each other and from the normal template control (NTC). The 'A' allele is called closer to the X (horizontal) axis, while the 'C' allele is called towards the Y (vertical) axis in this plot.

2.2.6 Statistical Analysis

Non-Caucasian patients, patients who withdraw from the study and those with missing genotype data were excluded from the analysis. This reduced the sample size to 1197 patients, 800 cases and 397 controls. Genotype frequencies were tested for Hardy-Weinberg equilibrium (HWE) in the control group only and in the sample as a whole, with a *p* value <0.01 assumed to indicate deviation from HWE. Univariate analysis of non-genetic covariates was undertaken, and those showing significance (p<0.1) such as aspirin use, PPI use and gender were included in the multivariate analysis.

In order to test for association between rs1105879 and PUD (ulcer vs. no ulcer), two logistic regression models were fitted. The first (the 'baseline model') included covariates to represent aspirin use, PPI use and gender. The second (the 'genetic model') was the same model, but with the addition of a covariate to represent rs1105879. An additive mode of inheritance was assumed, with wild-type homozygotes coded 0, mutant heterozygotes coded 1 and mutant homozygotes coded 2. The two models were compared using a likelihood ratio test to determine the association between rs1105879 and ulcer status. An odds ratio with 95% confidence intervals was computed for rs1105879. Further analyses were carried out using just gastric ulcer and just duodenal ulcer cases.

The logistic regression analysis was repeated for the additional outcome measure of gastrointestinal bleeding. Tests were conducted to assess the association between rs1105879 and upper gastrointestinal bleeding (UGIB vs. no UGIB), and between rs1105879 and bleeding ulcers (bleeding ulcer vs. non-bleeding ulcer). For all analyses, sensitivity analyses were conducted including only cases without any evidence of *H. pylori* infection.

2.2.7 Meta-analysis

A meta-analysis (see figure 2.4) of closely related studies was done by identifying these publications from the following online sources: Scopus, Pubmed, Medline, Web of Science, EBSCohost, Proquest and Scifinder using "polymorphism", "UGT1A6", "Aspirin", "NSAIDs", "peptic ulcer" and "gastrointestinal bleeding" as search terms.

The inclusion criteria are that the studies were reported in English Language, rs1105879 was genotyped, the study was case-controlled, and have comparable clinical phenotypes and demographic characteristics. However, due to limited number of studies, all ethnicities were included in the meta-analysis. The Forrest plot was constructed using Review Manager Software, version 5.3**.**

2.3 Results

2.3.1 Patients, demographics and data analysis

A total of 1197 patients (46.5% females, n=557) completed the study, of which 800 (66.8%) were cases. In the case group, 466 patients who had an endoscopic diagnosis of ulcer had a history of NSAID use [135 (29%) non-aspirin; 260 (56%) aspirin and 71 (15%) both non-aspirin NSAIDs and aspirin] within 2 weeks prior to presentation with symptoms. 334 patients had ulcer but were not exposed to NSAIDs, while 275 patients neither had ulcer nor were exposed to NSAIDs. 23% (n=107) of PUD patients with history of use of NSAIDs had upper GI bleeding, while 13.8% (n=48) of non-NSAIDs users with evidence of PUD had upper GI bleeding. About 30% of the cases were current smokers while over 37% were previous smokers. Significant alcohol use was also noted in over 15% of both cases and control

groups. Complete detail of patients' distribution and demographics is presented in figure 2.4 and tables 2.2 and 2.3.

Univariate analysis of non-genetic covariates reported a significant association ($p < 0.05$) between gender, *H. pylori* infection, use of PPI, steroid, low aspirin dose, and NSAID and binary ulcer status. A detailed report of the analysis is presented in table 2.3.
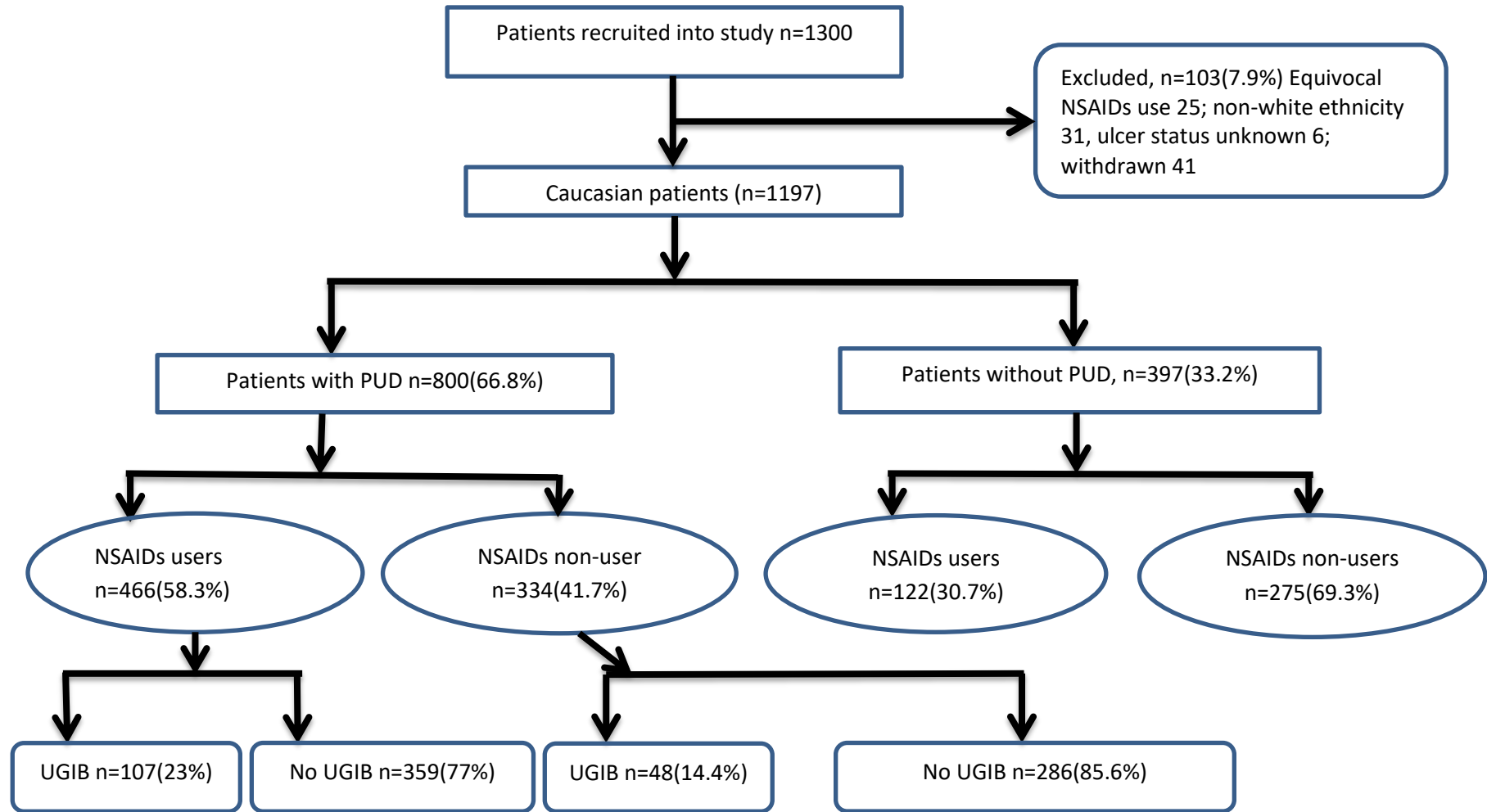
Figure 2.4: Schematic presentation of patients' demographics, cohorts and recruitment process. Of the targeted 1500 patients, 1300 patient were recruited, excluding patients of non-Caucasian background. The case cohort included patients with peptic ulcer disease (PUD) with/without NSAID use while the control cohort is patients without history of PUD with/without history of NSAID use. The case cohort is further stratified based on presence or absence of upper gastrointestinal bleeding among the NSAID users and non-users

Table 2.2: Patient demographics, treatment group and ulcer distribution

Clinical Variable	n (%)
Gender: Male	**640 (53.5)**
Ulcer cases	
NSAID	
Non-aspirin	135 (28.9)
Aspirin	260 (57.8)
Both aspirin and Non-aspirin NSAIDs	71 (15.2)
Total	466 (57.3)
NSAID negative	334 (42.7)
Total	**800**
Ulcer site	
Gastric ulcer	407 (50.9)
Duodenal ulcer	313 (39.1)
Both	62 (7.8)
Pyloric	16 (2.0)
Unknown	2 (0.003)
Total	**800**
Non-ulcer controls	
Non-aspirin NSAIDs	27 (22.1)
Aspirin NSAIDs	85 (69.7)
Both aspirin and non-aspirin NSAIDs	10 (8.2)
No ulcer+ exposure to NSAIDs	122 (30.7)
No ulcer, no exposure to NSAIDs	275 (69.3)
Total	**397**
Total number of participants	**1197**

2.3.2 Genotype and allele frequencies in study patients

The genotype frequencies for rs1105879 for all study subjects were as follows: AA=46%; AC=

43% and 11% for the CC (table 2.4). These frequencies correlate with reports from several

other studies (Agundez et al., 2009; Krishnaswamy et al., 2005a; Krishnaswamy et al., 2005d;

Saeki et al., 2005). The genotype frequencies did not deviate from Hardy-Weinberg equilibrium (p>0.05).

2.3.3 Association between SNP and PUD/UGIB

The data on the logistic regression analysis is summarised in Table 2.5. An analysis between SNP rs1105879 and binary ulcer and bleeding status was done to determine the association between the SNP and NSAID-related ulcers, assuming an additive mode of inheritance with covariates included to represent NSAIDs use, PPI use and gender.

Association was tested by logistic regression using likelihood ratio test (LRT) comparing models with and without a covariate representing the rs1105879 SNP. The analysis suggested a trend towards an association between the SNP and ulcer status, and use of NSAIDs (p=0.052, odds ratio 1.21, 95% CI 1.00, 1.47). A sensitivity analysis was undertaken which included only cases without any evidence of *H.pylori* infection in the case cohort (n=365), which gave a high p value (0.664). This association could be as a result of the confounding effects of *H. Pylori* infection. Further analysis that excluded non-aspirin NSAIDs with covariates to represent use of aspirin, PPI and gender using a LRT as above revealed a lack of association between SNP and aspirin-related ulcers (p=0.106, odds ratio 1.17; 95% CI 0.97, 1.41).

An analysis to determine the association between the SNP and UGIB status was modelled first to include all NSAIDs with case (n=155) and control (n=1042) outcome status, there was no association between NSAIDs use (p=0.725) or aspirin use (p=0.492) and UGIB. A sensitivity analysis excluding cases with *H. pylori* infection in both NSAIDs and aspirin-only users did not change the outcome measure. The above result agrees with previous reports on *UTG1A6* polymorphisms and NSAIDs use; with no association reported in all studies that looked at

this area (Agundez et al., 2009; Oijen et al., 2005; Valkhoff et al., 2012; Van Oijen et al., 2009b).

2.3.4 Influence of PPI use and gender

To further explore the trend towards association between SNP and NSAID-related ulcer, an analysis was modelled to investigate if this trend was due to the interaction between SNP and PPI use and gender. In the entire cohort, 546 PPIs were used by 527 patients with some using more than one PPI. The distribution of PPI use was as follows: omeprazole (242); lansoprazole (211); esomeprazole (53); pantoprazole (32) and rabeprazole (8). Concomitant use of NSAIDs and PPIs was noted in about 250 patients (47.4%).

Introducing PPI use or gender as covariates in the previously fitted genetic model, a LRT was used to compare the new model and the genetic model. In the PUD cohort, 299 (37.5%) patients used PPI while 228 (57.4%) patients in the control used PPI. The analysis revealed no association (p=0.484) between an interaction of PPI status and genotype, and development of ulcers.

In terms of gender, 475 (58.4%) men had ulcer (see table 2.3), revealing a higher incidence of PUD in male patients than females (41.6%). An analysis refitted as above, including an interaction term between SNP and gender did not show any evidence of association (p=0.948) between gender and genotype at SNP rs1105879 and developing an ulcer. This higher incidence of PUD in males supports current available evidence (Wu et al., 2008).

2.3.5 Influence of the location of ulcer

The data on distribution of ulcer based on location is presented in table 2.2. Analysis of the association with SNP was repeated as above but first including only GU (n=407) in the model with those without any ulcers as controls (n=397). Those with both ulcers (62) were excluded from this analysis. There was a statistically significant association (*p*=0.002) between duodenal ulcers and genotype at SNP rs1105879. However, sensitivity analysis including only cases without evidence of *H. pylori* did not show any association (p=0.302). A similar analysis as above reported no association (p=0.383) between gastric ulcers and SNP rs1105879.

Table 2.3: Clinical and demographic characteristics of study participants

	Patients with PUD (n=800)	Patients without PUD (n=397)	Univariate analysis using Chi square		
			Pearson chi-square	*LRT	p
Age, mean (SD)	65.2 (13.6)	57.6 (14.7)	0.159	0.162	0.15
Male sex, n (%)	470 (58.8%)	170 (42.8%)	26.3	26.3	0.001
BMI, mean (SD)	26.9 (7.7)	27.3 (7.8)			0.998
H. pylori Infection	435 (54.4%)	59 (14.9%)	96.8	99.9	0.001
Endoscopy indication, n %)			95%CI	LRT	
Hematemesis/melena	317 (39.6%)	24 (6.0%)	4.396-9.670	6.491	
Dyspepsia/abdominal pain	228 (28.5%)	189 (46.9%)	0.510-0.691	0.593	
Anaemia	109 (13.6%)	68 (16.9%)	0.598-0.691	0.788	
Reflux symptoms	20 (2.5%)	8 (2.0%)	0.559-2.713	1.229	
Other	146 (17.8%)	114 (28.3%)	0.509-0.780	0.629	
NSAID used					
Low dose aspirin	318 (38.1%)	94 (23.3%)	42.6	43.8	0.0001
High dose aspirin	18 (2.2%)	1 (0.2%)	42.6	43.8	0.0001

Diclofenac	104 (12.5%)	18 (4.5%)	26.9	30.9	0.0001
Ibuprofen	77 (9.2%)	15 (3.7%)	15.4	17.3	0.0001
Ketoprofen	4 (0.5%)	1 (0.2%)	7.4	8.4	0.007
Naproxen	30 (3.6%)	6 (1.5%)	7.4	8.4	0.004
Oxicams	15 (1.8%)	0 (0%)	15.4	17.3	0.0001
Coxibs	13 (1.6%)	2 (0.5%)	7.4	8.4	0.007
Ketoprofen	4 (0.5%)	1 (0.2%)	7.4	8.4	0.007
Indomethacin	3 (0.4%)	0 (0%)	26.9	30.8	0.001
Concomitant medications, n (%)					
Proton pump inhibitors	299 (37.5%)	228 (57.4%)	44.6	44.8	0.001
Antisecretory medications	32 (4.0%)	13 (3.3%)	0.493	0.505	0.524
Antiplatelets	44 (5.5%)	19 (4.8%)	0.188	0.190	0.786
Anticoagulants	51 (6.4%)	20 (5.0%)	1.05	1.08	0.365
Steroids	32 (4.0%)	14 (3.5%)	7.8	9.4	0.001
SSRIs	54 (6.8%)	52 (13.1%)	13.2	12.5	0.001
Smoking*, n (%)			**95%CI**	**LRT**	
Never	276 (34.5%)	185 (46.6%)	0.627-0.835	0.723	
Current	247 (30.9%)	106 (26.7%)	0.932-1.374	1.129	
Former	311 (38.9%)	113 (28.5%)	1.118-1.60	1.333	
Alcohol intake*, n (%)			**95%CI**	**LRT**	
Audit score <8	672 (84%)	327 (82.4%)	0.940-1.056	0.994	
Audit score 8-15	126 (15.8%)	57 (14.5%)	0.803-1.430	1.070	
Audit score >15	37 (4.6%)	20 (5.0%)	0.531-1.516	0.531	

Univariate analysis performed by Chi-square shows significant association between NSAIDs use, PPI use, gender, *H. pylori* infection and binary ulcer status, hence these covariates were added to the list of variables used in the logistic regression analysis. *Likelihood ratio test performed using 2 x k table on StatsDirect.

Table 2.4: *UGT1A6* Genotype and allelic frequencies for single nucleotide polymorphism for NSAID users and non-users

		Genotype frequency						Allele frequency			
Status	n	NSAIDs Users (n=588)			Non-users (n=609)			NSAIDs users (n=588)		Non-users (n=609)	
		AA	AC	CC	AA	AC	CC	A	C	A	C
Ulcer	800	0.48	0.41	0.10	0.41	0.46	0.14	0.69	0.31	0.64	0.36
No ulcer	397	0.52	0.39	0.08	0.46	0.45	0.09	0.73	0.27	0.68	0.32
*UGIB	155	0.50	0.36	0.13	0.46	0.40	0.15	0.69	0.31	0.66	0.34
*No UGIB	1042	0.49	0.42	0.09	0.43	0.45	0.11	0.70	0.30	0.66	0.43
Ulcer with UGIB	155	0.50	0.36	0.13	0.46	0.40	0.15	0.69	0.31	0.66	0.34
Ulcer without UGIB	645	0.48	0.42	0.09	0.40	0.46	0.13	0.69	0.31	0.63	0.37
		Aspirin Users (n=426)			Non-users (n=771)			Aspirin Users (n=426)		Non-users (n=771)	
		AA	AC	CC	AA	AC	CC	A	C	A	C
Ulcer	800	0.47	0.42	0.11	0.44	0.44	0.12	0.68	0.32	0.66	0.34
No ulcer	397	0.51	0.42	0.07	0.47	0.43	0.09	0.72	0.28	0.69	0.31
*UGIB	155	0.49	0.37	0.14	0.49	0.38	0.13	0.67	0.33	0.68	0.32
*No UGIB	1042	0.48	0.43	0.09	0.45	0.44	0.11	0.69	0.31	0.67	0.33
Ulcer with UGIB	155	0.49	0.37	0.14	0.49	0.38	0.13	067	0.33	0.68	0.32
Ulcer without UGIB	645	0.47	0.43	0.10	0.43	0.45	0.12	0.69	0.31	0.65	0.35

The genotype and allelic frequencies correlates with reported frequencies for the European population and there was no significant difference (p>0.05) between these frequencies in the case and control groups. UGIB= upper gastrointestinal bleeding; AA=homozygote wild type; AC= heterozygote variant; CC= homozygote variant; *the UGIB status of 2 patients was missing.

Table 2.5: NSAID covariates tested against SNP rs1105879

Covariates tested against *UGT1A6* SNP		n		Test of association		
		Cases	Control	*p*	Odds ratio	Association
$Binary ulcer status		800	397	0.052	1.21 (95% CI 1.00, 1.47)	Indicative
^Binary ulcer status		800	397	0.106	1.17 (95% CI 0.97, 1.41)	No
$Upper GI bleed status		155	1042	0.492		No
$Ulcer and GI bleed status*		155	645	0.131		No
$Ulcer location	GU	407	397	0.383		No
	DU	313	397	0.022		Yes
Gender	female	333	224	0.948		No
	male	470	170			
Use of NSAIDs		588	609	0.05	1.63 95% CI 1.24, 2.13	Yes
Use of PPI		515	670	0.484		No

^excluding non-aspirin NSAIDs, $including non-aspirin NSAIDs; GU= gastric ulcers; DU= duodenal ulcers; PPI= proton pump inhibitors; *the upper GI bleeding status of 2 patients were missing.

2.3.6 Meta-analysis

The following databases: Scopus, Pubmed, Medline, Web of Science, EBSCohost, Proquest and Scifinder were assessed on the 20th of June, 2012 using the search terms "polymorphism", "UGT1A6", "Aspirin", "NSAID", "peptic ulcer" and "gastrointestinal bleeding" identified 4 similar studies that sought to answer the same research question, used case-control study design and similar patient demographics and clinical phenotypes.

Two studies (van Oijen *et. al*., 2005; and Shiotani *et. al*., 2008) were excluded. The first was excluded because it was not a case-control study, while the second paper published in 2008 by the Japanese group was also excluded because the patient's phenotype and

characteristics were the same as the 2009 paper. Hence, a total of 3 studies were used for

the meta-analysis and all studies included were free of biases due to inclusion and exclusion

criteria.

The result of meta-analysis indicates that there was no association between polymorphisms

of *UGT1A6* and aspirin-induced upper GI complications. From the meta-analysis (Figure 2.5),

our study has the largest Mantel-Haenszel weight, accounting for over 70%, while Shiotani *et*

al and van Oijen *et al* weighted 7.1 and 22.3% respectively. There was very minimal

heterogeneity (I2 = 0) between the studies analysed, suggesting that the patient

characteristics and study designs were very similar. However, when all 3 studies were pooled

together, there was no association (P=0.31, OR= 1.11, 0.91, 0.36) between the *UGT1A6*

polymorphism and GI complications in aspirin/NSAID users.

Study or Subgroup	Cases Events	Cases Total	Control Events	Control Total	Weight	Odds Ratio M-H, Fixed, 95% CI
Esume et al 2014	445	813	209	401	70.6%	1.11 [0.87, 1.41]
Shotani, 2009	86	192	14	40	7.1%	1.51 [0.74, 3.06]
van Oijen et al 2009	68	114	179	298	22.3%	0.98 [0.63, 1.53]
Total (95% CI)		1119		739	100.0%	1.11 [0.91, 1.36]
Total events	599		402			

Heterogeneity: Chi² = 1.01, df = 2 (P = 0.60); I² = 0%
Test for overall effect: Z = 1.02 (P = 0.31)
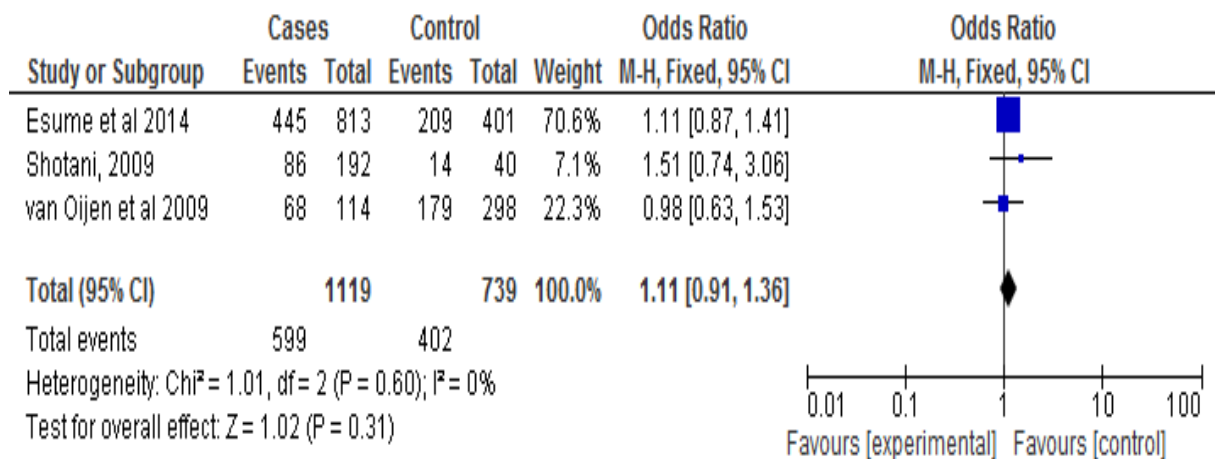
Favours [experimental]   Favours [control]

Figure 2.5: Meta-analysis of available studies on the *UGT1A6* polymorphism and NSAID-induced GI complications. Overall effect size was improved on adding the individual studies but did not change the lack of association between *UGT1A6* polymorphism and aspirin-related gastrointestinal bleeding reported in the individual studies.

2.4 Discussion

The frequency for the variant allele for rs1105879 was 0.31 in the case cohorts that used NSAIDs. There was no statistically significant (p>0.05) difference between this and the allelic frequencies in the non-ulcer group. The allelic frequencies for all groups were not different from those reported for the general population among patients of European descent. Previous reports of the allelic frequency of the variant allele of this SNP in other ethnic groups were 43.5 and 32.5% for Japanese and African populations, respectively (Agundez et al., 2009). This suggests that there is no significant interethnic difference in the allelic frequency of variant *UGT1A6* between African and European populations, while that of patients of Japanese descent was slightly higher (see table 2.4).

The above also holds true for the aspirin-only users, as their allelic frequencies did not vary from that of the general NSAIDs group. Our data reports that the genotype frequencies of the variant alleles are commoner than the wild type; 54 and 46% respectively as represented in figure 2.2.

Similar to previous reports, there was no association between rs1105879 C-allele and binary ulcer status (p>0.05; OR, 1.2; 95% CI, 1.00-1.47); SNP and binary ulcer and bleeding status (p>0.05) for both NSAIDs and the aspirin-only group (Shiotani et al., 2009; Van Oijen et al., 2005b; Van Oijen et al., 2009b). Due to the small sample sizes of the two previous studies, the results could be adjudged inconclusive, but going by the result of our study, with a sample size that is 4-7 fold larger, further weight is added to the argument that, there may be no association between polymorphisms of *UGT1A6* enzymes and UGI complications in patients exposed to NSAIDs and/or aspirin.

The UGT family of enzymes are involved in the phase II detoxification of a variety of endogenous and exogenous compounds and toxins including tamoxifen, estrogen and bilirubin making them less toxic (Mackenzie et al., 1996; Tukey & Strassburg, 2000). UGT1A6 is involved in the glucuronidation of aspirin and other NSAIDs and is the major enzyme involved in the conjugation of salicylic acid *in vivo,* though other closely-related enzymes such as UGT1A1, UGT1A3, UGT1A7, UGT1A8, UGT1A9, UGT1A10 UGT2B4 and UGT2B7 play some role in the formation of conjugates of salicylic acid (Bigler et al., 2001; Kuehl et al., 2006).

Glucuronidation of salicylic acid by UGT1A6 leads to the formation of the phenolic and acyl glucuronide and each of these accounts for about 7% of the recovery from urine, though it varies widely from 1-40%. Furthermore, a minor metabolite, gentisic acid arises from the hydroxylation of salicylic acid by CYP2C9 (see figure 2.1) and accounts for about 4% (1-4%). Gentisic acid, though an aspirin metabolite, is only detectable at very high doses in man (Bigler et al., 2001; Lares-Asseff et al., 2004; MacDonald et al., 2004). Overall, UGTIA6 accounts for about 25-30% of salicylic acid metabolism and it's worth noting that there is significant interindividual, interethnic and gender-related variability in the metabolism of aspirin.

The interethnic differences in aspirin metabolism suggest that genetics play a role in this variability of aspirin metabolism (Carlini et al., 2005; García-Martín, 2008); however, this variability does not seem to play significant role in the pathogenesis of aspirin-related ulcers, as revealed by our reports. *UGT1A6* polymorphisms that involves amino acid changes at position 181 and 184 (rs2070959 and rs1105879 respectively) is associated with a 30-50% reduction in enzyme activity compared to the wild type (Lampe et al., 1999).

From a functional point of view, these two SNPs are thought to be the most relevant targets for the *UGT1A6* gene (Agundez et al., 2009). With the established loss-of-function associated with the polymorphism of SNP rs1105879, it is expected that the level of salicylic acid, the aspirin metabolite degraded by UGT1A6 would be relatively higher in individuals who carry this variant allele due to lower clearance rates and higher drug plasma concentration, and may lead to an increase in the GI complications and other ADRs associated with aspirin.

The lack of significant association between this loss-of-function polymorphism and NSAID ulcers as reported in our study could be as a result of compensation by the alternative pathways involved in the metabolism of salicylic acid- via the salicyluric acid and the gentisic acid pathways catalysed by ACSM2B and CYP2C9 enzymes respectively (Agundez et al., 2009).

This increased elimination of salicylic acid via the gentisic acid and salicyluric acid pathways could compensate for the slow glucuronidation of salicylic acid in carriers of the variant allele, leading to a lack of effect of *UGT1A6* polymorphisms in aspirin-related GI complications. Secondly, the intermediate products salicyluric acid, and gentisic acid are both inhibitors of PG synthesis (Hinz et al., 2000). Attenuation of PG synthesis further drives gastric mucosal injury through loss of mucosa protective-mucin secretion and other gastro-protective effects of PG. Furthermore, carriers of the variant *CYP2C9*3* alleles, as expected, would have a decreased formation of gentisic acid with attendant reduction in the inhibitory effect of this metabolite on PG synthesis and better mucosa protection.

Some, if not most of the ulcerogenic effects of aspirin are known to occur due to the direct/topical effect which occur independent of COX-1 inhibition and prostaglandin depletion or changes in activity of phase I and II drug metabolising enzymes. Aspirin and

other NSAIDs are concentrated in gastric epithelial cells via "ion trapping" and it is through this mechanism that the topical effects of NSAID occur (Bjarnason et al., 2007; Schoen & Vender, 1989). Other NSAIDs that are weak organic acids such as indomethacin (pKa 5.2), phenylbutazone (pKa 4.8) are also concentrated in gastric mucosal cells via a similar process as aspirin (Bjarnason et al., 2007). These events activate the uncoupling of the mitochondrial oxidative phosphorylation and inhibition of the electron transport chain resulting in intracellular ATP depletion and cellular calcium ion toxicity and generation of reactive oxygen species (ROS), as shown in figure 1.1. In contrast to other NSAID that cause GI injury irrespective of the route of administration, aspirin has been shown to cause GI toxicity in mice only when given orally (Mashita et al., 2006).

The direct/topical effects of aspirin are independent of aspirin metabolism by UGT1A6 in the liver and as such may attenuate any likely effect due to polymorphism of drug metabolism enzymes. It is highly possible that the cases of ulcers seen in these patients are as a result of the topical effect of NSAIDs.

There is a gender difference in the expression of UGT1A6 in the liver, with 50% lower level reported in females. This is in accordance with the lower rate of metabolism of paracetamol in females (Bock & Lilienblum, 1994; Court et al., 2001). This lower expression of UGT1A6 would be expected to be associated with a higher tendency for the females to develop ulcers, due to drug and active metabolite accumulation. However, contrary to that expectation, we observed there were a lower proportion of women with ulcers than men. This trend of higher prevalence of PUD in men (58.8%) than women (41.2%) observed in our study has been reported elsewhere (Wu et al., 2008).

However, there are other mechanisms known to protect the female population from developing ulcers, such as the role of CGRP, where expression in the dorsal root ganglia, is gender dependent and the stimulatory role for estrogen enhancing its expression has also been described (Shimozawa et al., 2007; Shimozawa et al., 2006).

Interestingly, alcohol consumption is an established factor that induces UGT1A6 expression, via the CAR receptor (Bock et al., 1987), reflected by significantly higher mRNA, protein and glucuronidation activities of UGT1A6 in the livers of donors with history of more than 14 drinks per week compared with those donors with lower or no history of alcohol consumption (Krishnaswamy et al., 2005c).

Over 25% of the cases and controls in our study were smokers while another significant proportion were former smokers. Alcohol consumption is also noted to be prevalent among participants of the study, with about 20% of both cases and controls having an alcohol audit score greater than 15, implying high alcohol consumption. It is expected that the use of substances which could induce the expression of UGT1A6 would increase the activity of *UGT1A6* even in individuals with the variant allele to higher than expected levels, leading to aspirin metabolite levels similar to that seen in individuals with the wild type allele. Whether this augmented activity of UGT1A6 from the use of alcohol and cigarette smoking blunted the trend towards association between *UGT1A6* SNP and NSAID-related GI complications in our study needs to be further explored.

There are a few studies conducted to determine the association between *UGT1A6* SNP and GI complications in NSAID users, and none of these studies have reported an association (Shiotani et al., 2009; Van Oijen et al., 2005a; Van Oijen et al., 2009b). Of the 4 studies identified (association between *UGT1A6* SNP and aspirin/NSAID-related GI complications),

only 2 met our inclusion criteria and were included in the meta-analysis. Furthermore, the 3 studies used in this meta-analysis had many similarities such as answering the same research question, use of case-control study design and similar patient demographics. This is reflected in the heterogeneity score of zero recorded from our meta-analysis. The pooling of the data in the 3 studies to increase the effect size did not yield any significant association between the SNP and GI complications in aspirin and NSAIDs users (P=0.31, OR= 1.11, 0.91, 0.36). This validates the report of the individual study and one can report a lack of association between *UGT1A6* SNP and GI complications related to NSAID.

The limitations of this meta-analysis include the sample size of the other two studies, very few published data on the study and also inclusion of a genetically different population (Japanese) whose allelic frequency for the variant SNP under study is significantly different than that of the Caucasian population we examined (Shiotani et al., 2009). These limitations probably diminished the power of association in this meta-analysis.

Our study to date is the largest (1200 participants) and hence had the best chance to elucidate the relationship between NSAID use and a loss-of-function polymorphism in the *UGT1A6* gene locus. Prior studies included 192 and 114 patients as reported by Shiotani *et al* and van Oijen *et al,* respectively (Shiotani et al., 2009; Van Oijen et al., 2009b). Secondly, in recruiting our study patients, endoscopy, which is the gold standard for the diagnosis of peptic ulcer disease, was performed to ascertain the presence or absence of ulcers; hence we did not rely on subjective evidence like patients' self-reporting (which could be very deceptive) but on more objective evidence. Our study also took into account the role of *H. pylori* in the pathogenesis of peptic ulcer disease, making it possible to perform a sensitivity analysis, including only cases without any evidence of *H. pylori* infection as cases, which gave

a high p value (p=0.106) suggesting that the trend towards some association earlier seen when *H. pylori* positive patients were included in the analysis could be as a result of the confounding effect of *H. pylori* infection. The limitations of our study are that we had an incomplete data on smoking, and as such we, were unable to stratify patients based on the number of pack-years. This limited our ability to analyse any effect cigarette smoking would have on the role of *UGT1A6* gene in NSAID-related ulcer. Secondly, we did not genotype other *UGT1A6* SNP which are in close LD with rs1105879, particularly, rs2070959 and rs6759892 which are thought to be the most relevant as far as *UGT1A6* polymorphism and aspirin bio-disposition are concerned (Agundez et al., 2009).

In conclusion, we have reported a lack of association between a loss-of-function polymorphism of *UGT1A6* and GI complications associated with the use of NSAID and aspirin. Furthermore, we conducted a meta-analysis of all available publications on this topic with a view to increasing the effect size and improve the chance of finding an association, but the meta-analysis also revealed a lack of association between the *UGT1A6* polymorphism and aspirin/NSAID-related GI complications.

It is important to investigate the role of other mechanisms such as the role of other SNP, the role of pro-apoptotic and anti-apoptotic molecules. Other relevant pharmacogenomic targets as regards aspirin intolerance are ACSM2B, CYP2C9, COX-1 and COX-2 (Agundez et al., 2009). There is increasing evidence of both caspase dependent and caspase independent mechanisms in the pathogenesis of NSAID-induced ulcer (Jana, 2008). Polymorphisms in these pro-and anti-apoptotic genes may explain the inter-individual variability in the development of PUD and this requires further investigation.

Chapter 3

Association of a functional promoter polymorphism (rs1799889) in the Plasminogen activator inhibitor gene (*PAI-1*) and NSAID-related upper gastrointestinal toxicity

Chapter 3

Chapter 3

3.1 Introduction

3.1.1 The role of Plasminogen activator inhibitor (PAI-1) in gastric mucosal protection

PAI-1 is a member of the serine protease inhibitor superfamily that plays crucial role as a primary regulator of plasminogen activator and hence important in the control of the thrombotic and the fibrinolytic systems (Bayramoglu et al., 2014).

PAI-1 has a role in the protection of the gastric mucosa, a concept that was developed following earlier observations of increased expression of plasminogen activator and increased fibrinolytic activity at the edge of gastric ulcers. Specifically, there was a high level of expression and activity of tissue type plasminogen activator (tPA) from biopsy specimens of ulcer lesions. However, the amount and activity of tPA in biopsy samples from non-ulcer lesions of active ulcers or from both ulcer lesions and non-ulcer lesions of inactive ulcers showed no significant difference (Kurose et al., 1990). This group also reported significantly elevated PAI-1 levels in the plasma of gastric ulcer patients with active ulcers compared to patients with inactive ulcers (Kurose et al., 1990). This suggests that there is a role for tPA in the pathogenesis of gastric ulcers as the increased fibrinolytic activity would cause increased local fibrinolysis which may be crucial for ulcer formation.

It has also been reported that there is increased urokinase plasminogen activator (uPA) and PAI-1 activity at the edge of gastric and duodenal ulcers, accompanied by a remarkable reduction in the tissue plasminogen activity in gastric and duodenal ulcer lesions (Kurose et al., 1990). They also observed that there was a partial reversal of tPA towards the normal pattern in healed duodenal ulcers but uPA activity remained persistently high. This led them to speculate that the fibrinolytic system may have a role in the reoccurrence of ulcers at or near previous scars (Wodzinski et al., 1993). Impaired fibrinolysis at ulcer lesions would

compromise the microcirculation in the gastroduodenal mucosal via thrombosis leading to ischemic injuries to the affected mucosa, and further exposure to other noxious factors would then lead to formation of peptic ulcers. Repair of ulcers requires uPA for tissue remodelling (Wodzinski et al., 1993). While early activation of tPA and uPA inhibits haemostasis via fibrin degradation, their later activation is associated with enhanced tissue remodelling with their activity being inhibited by several inhibitors, the most important being PAI-1 (Dupont et al., 2009).

More recently, it was reported that following exposure to aspirin and NSAIDs in humans and mice, there was a 2-6 fold increase in the PAI-1 mRNA expression compared to control, a response which was not observed in PAI-1 null mice. Furthermore, unlike PAI-1-H/K mice (mouse in which PAI-1 is over-expressed in the gastric parietal cells), PAI-1 null mice are susceptible to ethanol and indomethacin-induced gastric lesions, and because of the above findings it is highly likely that these effects are pathways related to PAI-1 (Kenny et al., 2013b). In the presence of *H. pylori* infection, PAI-1 expression, uPA and its receptor uPAR expression are increased and this is associated with inhibition of gastric epithelial proliferation through the suppression of the uPA-mediated release of heparin-binding epithelial growth factor, and inhibition of epithelial cell migration (Kenny et al., 2008).

In the light of the above, it appears that PAI-1 acts to halt fibrinolysis in an autoregulatory manner activated by mucosal injury and a strategy to selectively modulate PAI-1 might be therapeutically useful in preventing ulcerogenesis in selected patients (Kenny et al., 2013b). The biology of PAI-1 is however very complex and there is evidence for actions that are independent of tPA or uPA (Lijnen, 2005).

Fibrin degradation (fibrinolysis) is an important physiological process occurring in the circulatory system but over-activation of the fibrinolytic system may overwhelm the mechanisms which inhibit fibrinolysis, particularly the plasminogen activator inhibitor and the $\alpha2$-plasmin inhibitor ($\alpha2$-PI). When this occurs, bleeding and inflammation may be produced by the active enzyme plasmin followed by the activation of the complement system (Dellas & Loskutoff, 2005; Kurose et al., 1990).

Using various models, it has been demonstrated that plasminogen activators increase at the site of mucosal injury and because of this finding it was thought that the increased fibrinolytic activity in the areas of injury might be important for mucosal bleeding (Kenny et al., 2013b; Wodzinski et al., 1993). The mucosal haemorrhagic changes which occurred in an experimental rat model had been attributed to the microvascular endothelium derived t-PA which is speculated to play a crucial role in this event (Kurose et al., 1988).

An examination of the disturbances in the fibrinolytic system in gastric ulcer patients showed that plasma PAI-1 levels in both active and inactive ulcers was significantly increased compared with the control group while the tPA levels did not differ between cases and controls.

Furthermore, other coagulation factors such as the activated partial thromboplastin time, fibrinogen, prothrombin time, fibrinogen degradation products, plasminogen, antithrombin-111, and $\alpha2$-PI showed no significant difference among the controls and patient with active and inactive ulcers (Kurose et al., 1990). A tight control of the fibrinolytic system is crucial for the much desired homeostasis in the coagulation pathway, as an imbalance in the system could lead to a thromboembolic state while excessive activation of the system would also

trigger increased fibrinolysis and consequently a bleeding disorder (Gils & Declerck, 2004a; Gils & Declerck, 2004b).

PAI-1 plays an important regulatory role in the fibrinolytic system by reducing the generation of plasmin from plasminogen, and it has been observed that a deficiency of PAI-1 by way of a functionally abnormal protein or a lack of plasma PAI-1 antigen results in a hyperfibrinolytic state (Dieval et al., 1991; Schleef et al., 1989).

3.1.2 The biology of plasminogen activator inhibitor (PAI-1)

PAI-1 was first isolated from culture media of human endothelial cells and then later identified in plasma, platelets, placenta and media of fibrosarcoma cells and hepatocytes (Kruithof, 1988a). Other tissues from which PAI-1 has been isolated include vascular smooth muscle cells, mesangial cells, fibroblasts, monocytes/macrophages and stromal cells of adipose tissues (Lijnen, 2005).

Except in platelets where PAI-1 can be stored in an inactive form, in other tissues, it is rapidly and constitutively secreted after synthesis. Its secretion is known to exhibit circadian variation, attaining the highest plasma concentration in the morning and lowest in the evening, while the exact opposite circadian variation has been described with tPA (Wiman & Hamsten, 1990). The synthesis and secretion of PAI-1 is modulated by various agonists such as cytokines, growth factors, endotoxin, hormones, with some evidence of transcriptional regulation of PAI-1 synthesis (Dawson et al., 1991; Lijnen & Collen, 1995b).

PAI-1 is a member of the serpine family whose reactive site peptide bond is located at Arg345-Met346 and is composed of a single chain glycoprotein of about 45kDa with 379

amino acids, but can be up to 381 amino acids due to NH_2 terminal heterogeneity (Stefansson et al., 2003). The *PAI-1* gene is located on chromosome 7, bands q21.3-q22, spanning approximately 12.2kb and consists of nine exons (Lijnen, 2005). The enzyme is stabilized by binding to a PAI-1 binding protein, vitronectin at residues 12-30 of the somatomedin B domain of vitronectin and the active inhibitory form of this protein rapidly transforms to a latent conformation that can be partially reactivated by denaturing agents (Declerck et al., 1988; Deng et al., 1996).

PAI-1 is known to react very rapidly with the single chain and the two-chain tPA and with the two-chain u-PA, but not with the single-chain uPA (Kruithof, 1988b). Plasminogen activators are serine proteases that convert the proenzyme plasminogen in blood into the fibrinolytically and thrombolytically active protease, plasmin. There are several functionally and immunologically active distinct forms of plasminogen activators; however, tPA is thought to be the most important in the initiation of fibrinolysis and thrombolysis (Lijnen, 2005).

3.1.3 Genetic variation of *PAI-1*

The *PAI-1* rs1799889 (accession no. P05121) genetic polymorphism encoded on chromosome 7 (Bayramoglu et al., 2014; Cosan et al., 2009) is associated with variation in PAI-1 plasma levels, with the 4G homozygous individuals having high PAI-1 levels, intermediate levels in heterozygous participants and low PAI-1 levels in 5G homozygous subjects (De Lange et al., 2013).

The 4G/5G polymorphism is perceived as a "response polymorphism" in that the difference between the PAI-1 levels in carriers of 4G and 5G alleles becomes more pronounced in the presence of disease and environmental factors which enhance PAI-1 expression (Hoekstra et al., 2004). This is thought to play an essential role in the regulation of *PAI-1* gene expression, with the 4G allele which occurs at frequency of 0.56 in Caucasians, being associated with higher plasma PAI-1 levels (Dawson et al., 1991). The frequency of the 5G allele of the 4G/5G polymorphism in Black subjects has been reported to be 0.85 (De Lange et al., 2013). Other *PAI-1* genetic polymorphisms which are in close linkage disequilibrium to the 4G/5G polymorphism have been described (Lijnen, 2005).

The role of PAI-1 in various diseases has been demonstrated. For instance, there are reports of increased PAI-1 expression in gastric cancer, a state that is associated with poor disease outcome; and in radiation damage, in experimental colitis, and in Crohn's disease (Lijnen, 2005).

In insulin resistance, high PAI-1 levels were observed with a significant association with body mass index (BMI), triglycerides levels, insulin levels and systolic blood pressure (De Lange et al., 2013; Juhan-Vague et al., 1991). There is also an association between the 4G/5G *PAI-1* polymorphism and waist circumference, triglyceride concentrations and cardiovascular disease risk (De Lange et al., 2013). Altered PAI-1 expression may also predispose to asthma in human subjects, as evidenced by the observation of massive infiltration of PAI-1-producing mast cells in the airways of these individuals and a higher 4G allele frequency in asthmatic patients than in controls (Cho et al., 2004).

3.1.4 The role of Plasminogen activator inhibitor (PAI-1) in the fibrinolytic system

Following trauma and surgery, patients with deficiency of PAI-1 as a result of a homozygous frameshift developed abnormal bleeding (Fay et al., 1997). A study involving patients who were undergoing coronary angiography reported higher plasma PAI-1 levels and lower fibrinolytic activity in patients with severe atheromatosis (Fernandes et al., 2015). Furthermore, an analysis of the gene expression profile of aspirated coronary thrombi in acute myocardial infarction showed that long ischaemic time is associated with a relative increase in the expression of tPA, uPA, and PAI-1 (Helseth et al., 2015).

Increased fibrinolytic activity has been reported in patients with bleeding peptic ulcers and this is decreased by acid suppression and at least in part, by increased plasma PAI-1 levels (Vreeburg et al., 2001). A meta-analysis reported an association between *PAI-1* 4G/5G polymorphism and recurrent pregnancy loss as a result of microthrombosis in Caucasians (Li et al., 2015).

The aim of the work within this chapter was to assess whether the functional polymorphism, *PAI-1* rs1799889 (4G/5G), increases the risk of development of peptic ulcer and its tendency to bleed among NSAID users in a Caucasian population.

3.2 Methods

3.2.1 Patients recruitment, demographics and sample collection

Patient cohort, recruitment and sample collection are as described in section 2.2.1 and 2.2.2.

3.2.2 Genomic Deoxyribonucleic acid extraction

This is as described in section 2.2.4.

3.2.3 Allelic discrimination of *PAI-1* SNP rs1799889

Following genomic DNA extraction, genotyping of *PAI-1* SNP rs1799889 was performed using TaqMan® SNP Genotyping assays according to manufacturer's instructions. Fluorescence and allelic discrimination were determined using the SDS software (version 2.2; Applied Biosystems, USA). The forward primer sequence of this assay was 5'AGACAAGGTTGTTGACAAGAGA3', while the reverse primer sequence was 5'GGCCGCCTCCGATGATAC3'. Details of the thermocycling conditions are as described in section 2.2.5.

3.2.4 Statistical analysis

The statistical analysis was modelled as described in section 2.2.5, but in addition to this, we performed a logistic regression model to determine the association of *H.pylori*, gender and age and NSAID-ulcer status irrespective of *PAI-1* genotype.

3.3 Results

3.3.1 Patients, demographics and data analysis

Figure 3.1 is a flow diagram of the demographic characteristics and patient distribution of the case and control cohorts. Of the 1300 recruited patients, 544 participants were excluded for various reasons as follows (figure 3.1): unknown NSAID status (n=25), non-white ethnicity (n=31), unknown ulcer status (n=6), withdrawal (n=41) and lack of DNA samples to complete SNP genotyping (n=441). Peptic ulcer disease was confirmed by endoscopy in 68% (514) of patients recruited (case cohort) while the remaining 32% did not have any ulcers on

endoscopy. Among the case cohort, the incidence of UGIB was 22.4% and 14.1% among

NSAID users and non-users, respectively.

Table 3.1 presents further details on the socio-demographic characteristics of this study

population. The result of the univariate analysis of the non-genetic covariates relevant to

this population is presented in table 3.2.

Table 3.1: Patient demographics, treatment group and ulcer distribution

| Clinical Variable	n (%)
Gender: Male	**425 (56.2)**
Ulcer Cases	
NSAID	
Non-aspirin NSAIDs	80 (30.8)
Aspirin NSAIDs	148 (57.1)
Both aspirin and Non-aspirin NSAIDs	31 (11.9)
Number of cases NSAID positive	259 (50.4)
Number of cases NSAID negative	255 (49.6)
Total number of cases	**514**
Ulcer site	
Gastric ulcer	237 (46.1)
Duodenal ulcer	228 (44.4)
Both	35 (6.8)
Pyloric	12 (2.3)
Missing	2 (0.4)
Total	**514**
Non-ulcer controls	
Non-aspirin NSAID exposed	11 (15.7)
Aspirin-NSAID exposed	59 (84.3)
Both aspirin and non-aspirin NSAID exposed	0 (0)
No ulcer, with exposure to NSAID	70 (28.9)
No ulcer, and no exposure to NSAID	172 (71.1)
Total number of controls	**242**
Total number of participants	**756**
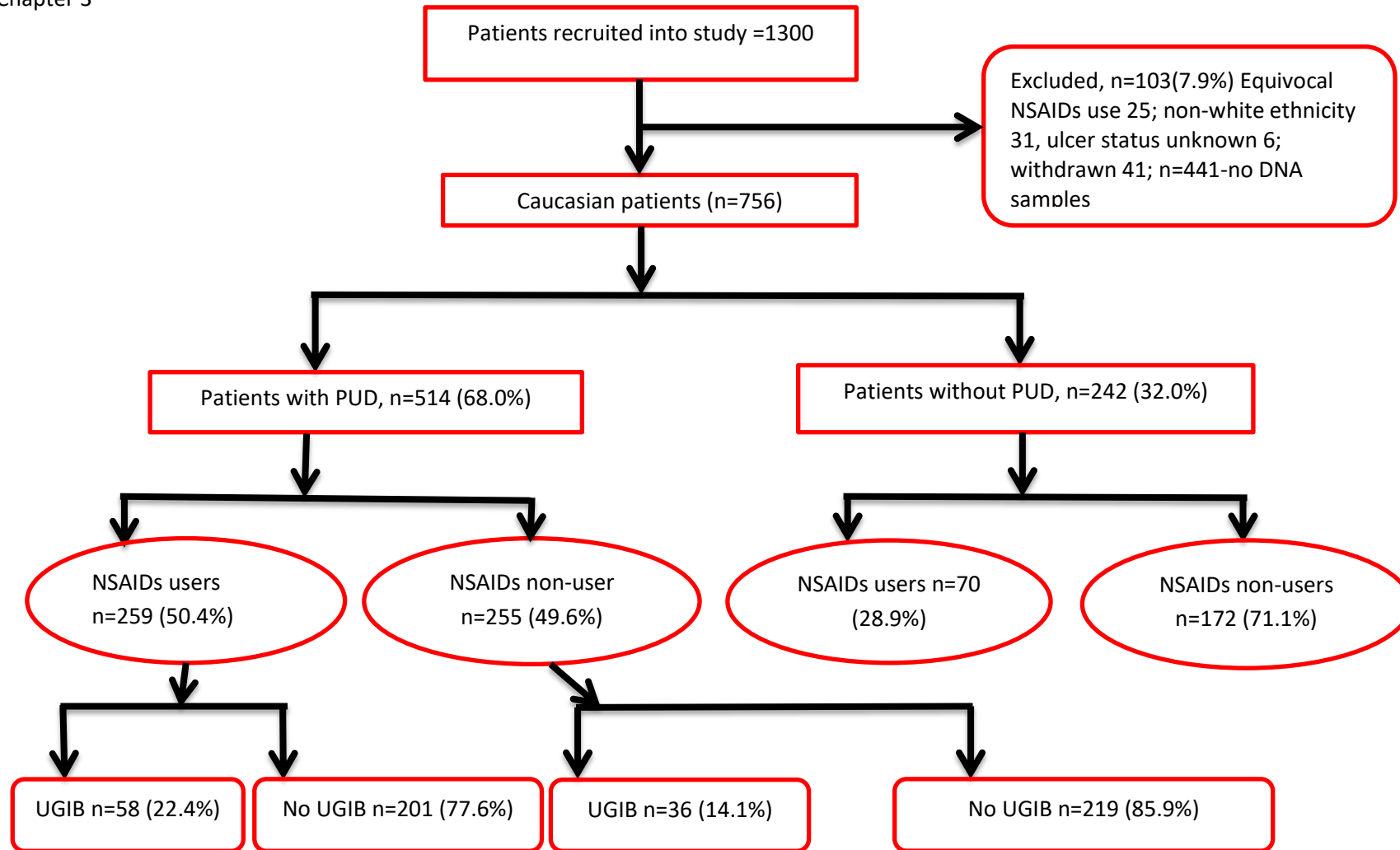
Figure 3.1: Schematic presentation of patients' demographics, cohorts and recruitment process. 1300 patient were recruited, excluding patients of non-Caucasian background. The case cohort included patients with peptic ulcer disease (PUD) with/without NSAID use while the control cohort is patients without history of PUD with/without history of NSAID use. The case cohort is further stratified based on the presence or absence of upper gastrointestinal bleeding among the NSAID users and non-users.

Table 3.2: Clinical and demographic characteristics of study participants

Covariates	Patients with PUD (n=514)	Patients without PUD (n=242)	Univariate analysis using Chi square		
			Pearson chi-square	*LRT	p
Age, mean (SD)	65.2 (13.6)	57.6 (14.7)	29.8	30.3	<0.05
Male sex, n (%)	312 (60.7%)	113(46.7%)	13.1	13.1	<0.05
BMI, mean (SD)	26.8 (7.7)	27.1 (7.8)			>0.05
H. pylori Infection	121 (23.5%)	41 (16.9%)	100.3	108.0	<0.05
Endoscopy indication, n (%)			95%CI	LRT	
Hematemesis/melena	217 (42.2%)	24 (10.0%)	3.496-6.570	4.429	
Dyspepsia/abdominal pain	198 (38.5%)	189 (78.1%)	0.325-0.489	0.487	
Anaemia	92 (17.9%)	58 (23.9%)	0.398-0.441	0.358	
Reflux symptoms	20 (3.89%)	8 (3.3%)	0.359-1.913	0.929	
Other	76 (14.8%)	34 (14.0%)	0.205-0.605	0.429	
NSAID used					
Low dose aspirin	179 (34.8%)	59 (24.4%)	23.7	24.8	<0.05
Non-aspirin NSAID	111 (21.6%)	11 (4.5%)	19.8	20.4	<0.05
Concomitant medications					
Antiplatelets	22 (4.2%)	11 (4.5%)	25.7	26.5	<0.05
Anticoagulants	23 (4.5%)	14 (5.7%)	0.372	0.459	>0.05
Steroids	20 (3.8%)	9 (3.7%)	11.2	12.4	>0.05
SSRI	25 (4.9%)	36 (14.9%)	0.590	0.720	>0.05
Proton pump inhibitors	22 (4.3%)	11 (4.5%)	44.6	44.8	<0.05
Antisecretory medications	32 (6.2%)	13 (5.4%)	0.493	0.505	>0.05

Univariate analysis performed by Chi-square shows significant association between NSAID use, PPI use, gender, age, *H. pylori* infection and binary ulcer status, hence these covariates were added to the list of variables used in the logistic regression analysis. *Likelihood ratio test performed using 2 x k table on StatsDirect.

3.3.2 Genotype and allele frequency of study patients

The genotype frequencies for the *PAI-1* 4G/5G polymorphism in the case cohort were 0.22 for 5G/5G; 0.52 for 4G/5G and 0.26 for the 4G/4G SNP. These frequencies are similar to the reports of the genotype frequency of this SNP in a Caucasian population (Emonts et al., 2007). We also observed a statistically significant ($p<0.05$) higher frequency of the 4G allele (0.54) compared to the 5G (0.46) in our study population; a finding that is similar to previous reports on the allele frequencies of this SNP in Caucasians (Dawson et al., 1991). The *PAI-1* SNP 4G/5G SNP was in Hardy-Weinberg equilibrium ($p > 0.05$).

Table 3.3: Genotype and allele frequencies for *PAI-1* 4G/5G SNP in NSAIDs users and non-users

		Genotype frequency						Allele frequency			
Status	**n**	**NSAIDs Users (n=332)**			**Non-users (n=424)**			**NSAIDs Users (n=332)**		**Non-users (n=424)**	
		5G/5G	4G/5G	4G/4G	5G/5G	4G/5G	4G/4G	5G	4G	5G	4G
Ulcer	514	0.22	0.52	0.26	0.24	0.46	0.30	0.48	0.52	0.49	0.51
No ulcer	242	0.25	0.55	0.20	0.21	0.57	0.22	0.46	0.54	0.48	0.52
UGIB	94	0.18	0.50	0.32	0.17	0.51	0.32	0.45	0.55	0.47	0.53
No UGIB	662	0.24	0.49	0.28	0.20	0.44	0.36	0.49	0.51	0.49	0.51
Ulcer with UGIB	94	0.18	0.50	0.32	0.17	0.51	0.32	0.47	0.53	0.48	0.52
Ulcer no UGIB	420	0.24	0.52	0.24	0.25	0.52	0.23	0.48	0.52	0.46	0.54
Status	**n**	**Aspirin Users (n=238)**			**Non-aspirin users (n=518)**			**Aspirin-users (n=238)**		**Non users (n=518)**	
		5G/5G	4G/5G	4G/4G	5G/5G	4G/5G	4G/4G	5G	4G	5G	4G
Ulcer	514	0.21	0.52	0.27	0.24	0.47	0.29	0.47	0.53	0.48	0.52
No ulcer	242	0.27	0.42	0.31	0.21	0.51	0.28	0.45	0.55	0.47	0.53
UGI bleed	94	0.18	0.50	0.32	0.20	0.50	0.30	0.44	0.56	0.48	0.52
No UGIB	662	0.24	0.48	0.28	0.23	0.49	0.28	0.48	0.52	0.48	0.52
Ulcer with UGIB	94	0.17	0.49	0.34	0.20	0.50	0.30	0.47	0.53	0.48	0.52
Ulcer without UGIB	420	0.22	0.50	0.28	0.22	0.51	0.27	0.48	0.52	0.49	0.51

The genotype and allelic frequencies correlate with reported frequencies for the European population and there was no significant difference (p>0.05) between these frequencies in the case and control groups. However, we observed a significant (p<0.05) difference in the 4G and 5G allelic frequencies. UGIB= upper gastrointestinal bleeding; 5G/5G=homozygote wild type; 4G/5G= heterozygote variant; 4G/4G= homozygote variant.

3.3.3 Influence of PPI use and gender

In terms of gender, 312 (60.7%) men had ulcers (see table 3.2), revealing a higher incidence of PUD in male patients than in females (39.3%). An analysis refitted, including an interaction term between SNP and gender did not show any evidence of association (*p*=0.710) between gender and genotype at SNP rs1799889 and developing an ulcer.

However, a further analysis of the association between gender and binary ulcer status showed a significant association (p=0.001, OR 1.76; 95% CI 1.27, 2.44) between gender and ulcer status (figures 3.4 and 3.5). The higher incidence of PUD in males supports current available evidence (Wu et al., 2008). Similarly, PPI use was significantly associated (p=0.001) with binary ulcer status.

3.3.4 Influence of location of ulcer

In terms of the location of the ulcers, the distribution in this cohort is shown in table 3.1. Test of association between SNP and site of ulcer was performed including only gastric ulcers as cases in the first analysis and then repeating the analysis with only duodenal ulcers as cases and in both cases, no ulcers as control (n=242). Those with both gastric and duodenal ulcers were excluded from the analysis. There was no association between *PAI-1* SNP and gastric or duodenal ulcers (p=0.144 and p=0.170, respectively). Furthermore, gastric and duodenal ulcer sensitivity analysis which included only cases without any evidence of *H. pylori* infection as cases (n=211 for gastric ulcers and n=123 for duodenal ulcers) reported p>0.05 for both (table 3.5.)

3.3.5 Association between SNP *PAI-1* SNP rs1799889 and PUD/UGIB

We tested the *PAI-1* SNP rs1799889 for association with case-control outcome status. This was done using logistic regression with covariates representing NSAID use (whether none;

non-aspirin NSAIDs; aspirin or both aspirin and non-aspirin NSAIDs), PPI use and gender. An analysis including only patients who used NSAIDs and developed ulcer as cases (n=259), and those that had an endoscopic diagnosis of PUD without NSAIDs use as control (n=255) was done; association was tested by logistic regression using likelihood ratio test (LRT) to compare models with and without covariate representing *PAI-1*. The test of association was not statistically significant for both models with covariates and without covariates (p=0.516, odds ratio 0.871; 95% CI 0.575, 1.321; without covariates and p=0.124 with covariates; odds ratio 1.371; 95% CI 0.917, 2.00). The Nagelkerke R^2 for model without covariates was 12.0%. These are summarised in table 3.4.

Furthermore, assuming an additive model, a repeat analysis in which the cases (n=514) were expanded to include ulcer patients with and without NSAID use, reported no association between *PAI-1* SNP and gastrointestinal complications of NSAID use (p=0.742, odds ratio 0.925; 95% CI 0.582 to 1.47). The Nagelkerke R^2 for model including NSAIDs use, *H. pylori* status, PPI use, gender, and *PAI-1* was 26%. A sensitivity analysis was done, including only cases without any evidence of *H. pylori* as cases (n=398); there was no association between SNP and binary ulcer status (p=0.124). However, an analysis including only *H. pylori* positive ulcer patients as cases (n=121), revealed a significant association between SNP and binary ulcer status (p=0.0001, OR 1.69; 95% CI 0.11, 0.247).

Further tests of association were undertaken for *PAI-1* SNP and upper GI bleeding status with patients who have upper GI bleeding as cases (n=94) and no upper GI bleeding as controls (n=662). Applying the genetic model described above, there was no association between *PAI-1* and upper GI bleeding status (p=0.198). Still assuming an additive mode of inheritance and applying the genetic model described above, association of SNP and ulcer

and bleeding status was tested by logistic regression with patients who had ulcer and GI bleed as cases (n=94) and ulcer with no bleed as control (n=420); the test of association did not show statistical significance (p=0.644). Table 3.4 presents the logistic regression of the non-genetic covariates while table 3.5 presents a summary of covariates tested against *PAI-1* and the association/outcome measures.

Table 3.4: Logistic regression for non-genetic covariates

Non-genetic covariates	n			
	Cases	Control	p	Odds ratio
No NSAID exposure	255	172	0.267	0.06; 95% CI 0.001-9.0
NSAID exposure	259	70	0.171	3.63; 95% CI 0.573-23.0
Aspirin exposure	179	59	0.158	1.57; 95% CI 0.84-2.94
Non-aspirin NSAID	80	11	0.346	2.27; 95% CI 0.41-12
PPI exposure	311	355	0.001	5.08 95% CI 2.73-9.47
Gender	514	242	0.001	1.76 95% CI 1.27-2.44
H. pylori	121	41	0.0001	1.69 95% CI 0.11, 0.25

Table 3.5: Association of NSAID and ulceration status with *PAI-1* SNP

Covariates tested against *PAI-1* SNP rs1799889		n			
		Cases	Control	*p*	OR
[$]Binary ulcer status		259	255	0.516	0.871; 95% CI 0.56, 1.32
[$]Upper GI bleed status		94	662	0.198	0.631; 95% CI 0.39, 1.13
[$]Ulcer and GI bleed status*		94	420	0.644	0.831; 95% CI 0.42, 1.85
Ulcer status, expanded cohort		514	242	0.742	0.930; 95% CI 0.58, 1.47
[$]Ulcer location:					
	GU	237	242	0.144	0.980; 95% CI 0.48, 1.60
	DU	228	242	0.170	0.857; 95% CI 0.54, 1.58
Gender		514	242	0.0001	1.790, 95% CI 1.30, 2.45
PPI use		311	355	0.001	3.560; 95% CI 1.95, 6.53

*Ulcer and bleed=case, ulcer no bleed=control; GU=gastric ulcer; DU=duodenal ulcer. [$]covariates include NSAIDs use, PPI use and gender.

3.4 Discussion

This work has examined the association of the *PAI-1* 4G/5G polymorphism (rs1799889) with the risk of NSAID-related gastrointestinal complications. Our study did not find any association between the *PAI-1* polymorphism and binary ulcer status (p=0.516).

The genotype frequencies of the 4G/5G polymorphism in our study population were 0.22 for 5G/5G; 0.52 for 4G/5G and 0.26 for the 4G/4G SNP. Also, the allelic frequencies of this SNP showed a statistically significantly (p=0.025) higher frequency of the 4G allele than the 5G allele; 0.54 and 0.46 respectively. Furthermore, there was no statistically significant (p>0.05) difference in the genotype and allele frequencies of the 4G/5G polymorphism between the case and control cohorts.

These results do not differ from previous data on the genotype and allele frequencies of this SNP in Caucasians (Dawson et al., 1991; Emonts et al., 2007). Similarly, among Asian populations, the genotype frequencies of the 4G/4G is also higher than that of the 5G/5G which has been reported to be 0.32 and 0.14 respectively (Kim et al., 2014). By contrast, in Black Africans, significantly higher genotype frequencies of the wild type allele (5G/5G=0.73) compared to the 4G/4G (0.0261) allele has been demonstrated (De Lange et al., 2013). This suggests a difference between our data and previous studies in other ethnicities on allele frequencies of *PAI-1* genotype.

The genetic variation at the *PAI-1* gene polymorphic locus is associated with differences in the levels of PAI-1 in the plasma of study subjects, with a single guanosine insertion deletion (4G/5G) polymorphism in the *PAI-1* promoter region playing an important role in the regulation of *PAI-1* gene expression (Dawson et al., 1991). The variant allele which occurred

at a higher frequency in our study population is associated with higher plasma PAI-1 levels, but various other factors such as hormones, growth factors, endotoxins, cytokines, and phorbol esters are known to modulate PAI-1 synthesis and secretion (Festa et al., 2003; Lijnen & Collen, 1995a).

This study did not show any association between *PAI-1* 5G/4G polymorphism and NSAID-related GI complication. Theoretically, an inactive PAI-1 protein would lead to failure in mucosal protection by PAI-1, and high PAI-1 level resulting from 5G/4G polymorphism would cause enhanced inhibition of tPA and uPA, which would consequently lead to the development of multiple thrombus (Fernandes et al., 2015; Sun et al., 2010) in the gastric microvasculature resulting in ischemia and a predisposition to ulcers from gastric mucosal necrosis (Wodzinski et al., 1993).

However, several factors could explain our finding. Firstly, the variant form of *PAI-1* rs1799889 SNP, 4G/4G, is associated with higher PAI-1 plasma levels in carriers of this variant genotype, a phenomenon that could compensate for the defect in the actions of the 4G/4G PAI-1 protein if these exist. In a mouse model, P*AI-1* knockout mice were shown to develop increased haemorrhagic lesions in response to NSAIDs, absolute ethanol and 50% ethanol compared to control mice in which there was targeted overexpression of *PAI-1* to gastric parietal epithelial cells (Kenny et al., 2013b). This would imply that the 4G/4G polymorphic variant is not associated with a loss of function of the PAI-1 protein. Secondly, studies have shown that despite the increased plasma PAI-1 level in carriers of the 4G/4G genotypes compared to carriers of the 5G/5G and the 4G/5G genotypes, the polymorphic variation did not affect the activity of the PAI-1 (De Lange et al., 2013). Thirdly, medications such as NSAIDs and PPI increase PAI-1 expression in humans. Aspirin and NSAID use is

associated with a 2-5 fold increase in the gastric PAI-1 mRNA relative abundance in the gastric biopsy samples of patients receiving these medications (Kenny et al., 2013b). Similarly, PPI may stimulate PAI-1 expression via the gastrin pathway (Nørsett et al., 2011), and both of these medications were used by most of our patients in the case and control cohorts. It is likely that use of NSAIDs and PPI in our study cohorts may have confounding effects in the interaction between the *PAI-1* polymorphism and binary ulcer status. This is supported by the observation that introducing the non-use of NSAIDs as an interaction term in our analysis, showed some trend toward association between *PAI-1* polymorphism and ulcer status, although not statistically significant (p=0.099).

Available evidence suggests a possible interaction of *H. pylori* with PAI-1 activity, an interaction that may interfere with the role of PAI-1 in mucosal protection. There is increased expression of uPA, uPAR and PAI-1 in the human gastric corpus in the presence of *H. pylori* infection. Also, in *H. pylori* infection there was an increase in the activity of cell-bound and the soluble uPA, and this increase was enhanced by *PAI-1* knockdown. Moreover, in the presence of *H. pylori* infection, there was increased epithelial cell proliferation mediated by uPA and enhanced by *PAI-1* knockdown (Kenny et al., 2008).

From the foregoing, it is likely that *H. pylori* infection modulates the function of the uPA and the PAI-1 system to drive gastric cancer development; however, the exact role of this interaction in the pathogenesis of NSAID ulcers is yet to be determined functionally. However, taken in conjunction with our results, it seems safe to conclude that *H. pylori* infection which was detected in our study subjects (cases=121 and control=41), could have interacted with 4G/5G polymorphism in several of the mechanisms highlighted above to

attenuate any significant association we would have found between binary ulcer status and this SNP.

Our study was the first to evaluate the relationship between *PAI-1* polymorphism and NSAID-induced gastrointestinal complications. Several studies have been investigating the association in the *PAI-1* 4G/5G polymorphism and other disease conditions such as recurrent pregnancy loss, outcome of sepsis, risk of otitis media, cardiovascular disease risk, abnormality of the fibrinolytic system and prognosis of gastric cancer (Cho et al., 2004; De Lange et al., 2013; Emonts et al., 2007; Helseth et al., 2015; Juhan-Vague et al., 1991; Li et al., 2015; Saely et al., 2008). However, there are no data on *PAI-1* 4G/5G polymorphism and its role in NSAID-related GI complications. Our sample size was fairly large which confers on it a reasonable effect size that should detect any association between this SNP and NSAID ulcer status. However, we do not know the magnitude of effect. A limitation of our study is that other *PAI-1* SNPs in linkage disequilibrium with the 4G/5G PAI-1 polymorphism such as the c.428G>T and c.429G>A (De Lange et al., 2013) were not genotyped and also we lacked samples (n=441) to genotype the entire cohort.

In conclusion, we have examined the role of *PAI-1* 4G/5G polymorphism in the pathogenesis of NSAID-related GI complications and found that there was no association between this single guanosine insertion deletion (4G/5G) polymorphism and NSAID-related GI complications. This underscores the need for further evaluation of various other pathways that may be relevant in NSAID-related ulcers.

CHAPTER 4

The role of the genetic variation in the *EYA1* gene in non-steroidal anti-inflammatory drug-induced upper gastrointestinal injury

Chapter 4

Chapter 4

Figures

Tables

4.1 Introduction

4.1.1 Previous *EYA1* GWAS report

A GWAS study on NSAID-related GI complications was conducted from July 2005 to June 2011 to unravel a possible genetic basis for NSAID-related GI complications in a Caucasian population (Bourgeois, S et al, unpublished data 2016). The study cohorts were classified as either cases or control, and the phenotypic traits tested were age, gender, history of duodenal or gastric ulcer disease, use of PPI, *H. pylori* infection, concurrent drug use, and NSAID use.

The report of the GWAS indicated that the "top hit" gene was located on the long arm of chromosome 8. A significant association ($p=1.14\times10^{-6}$, OR 2.01; CI 1.52-2.66) was identified between the *EYA1* SNP rs12678747 and NSAID-related GI injuries (see figure 4.1).

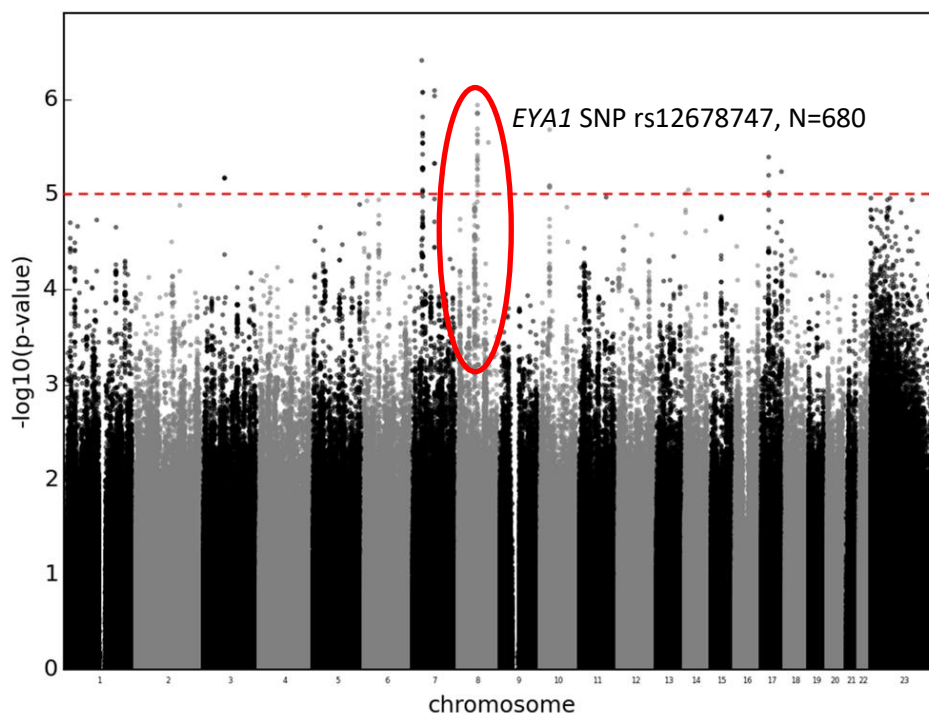Figure 4.1: Manhattan plot of GWAS study on NSAID-related GI complications (Bourgeois, S et al., unpublished data 2016). The plot shows that there are "top hit" genes on chromosome 7 and 8, 10 and 17 all having signals with $p<1\times10^{-5}$. The gene on chromosome 8 is the *EYA1* SNP rs12678747 with a p value of ($p=1.14\times10^{-6}$; OR 2.01, CI 1.52-2.66). The broken lines indicate the notional threshold for significance.

4.1.1.1 Aims and objectives

The aim of the study was to ascertain an association between the *EYA1* SNP rs12678747 and NSAID-related gastrointestinal complications and also to validate the report of the NSAID-related ulcer GWAS study. Furthermore, the aim was also to characterise the polymorphic variation in the expression of *EYA1* gene and protein expression in atypical gastrin secreting (AGS) cells and healthy human gastric epithelial cells.

To date, there has not been any study or a previous report of an association between *EYA1* gene and GI complications in NSAIDs users. Additionally, there is also no *EYA1* gene and protein expression data in gastric epithelial cells. The AGS cell is a validated *in vitro* model closely representing the human gastric mucosa in that it expresses both COX-1 and 2, with an increased expression of COX-2 upon stimulation (Hall et al., 2006). This was therefore used as the cell model of choice for further characterisation in terms of *EYA1* genotype, gene and protein expression.

4.1.2 Structure of EYA protein

As revealed by crystallographic work on pro-and eukaryotic haloacid dehydrogenase (HAD) phosphatases, all members of the HAD phosphatases superfamily share identical architecture of the active core, as shown in figure 4.2A. The modified Rossmann fold (figure 4.2A) contains the catalytic residues which consist of three α/β sandwiches, comprised of repeating α and β subunits (Burroughs et al., 2006).

A
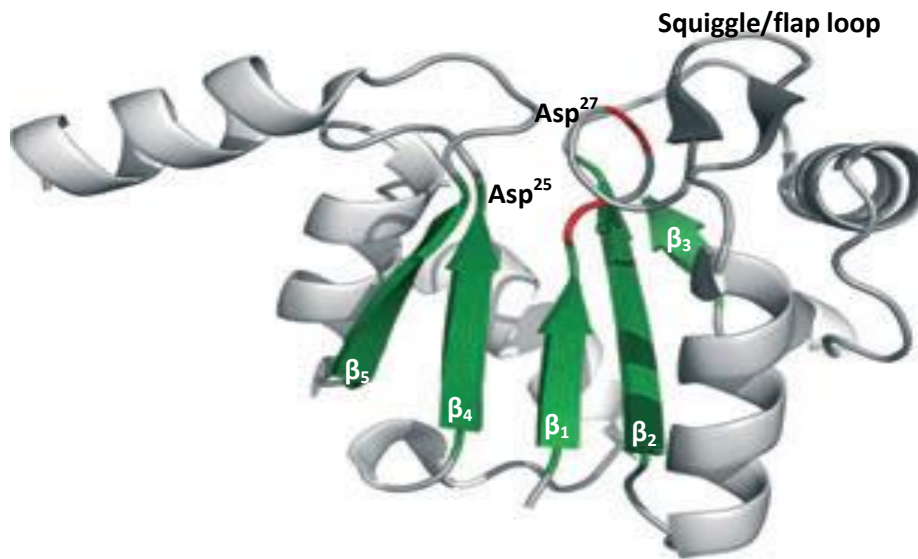
B

Figure 4.2: **A:** The Rossmann-like fold of HAD phosphatases formed by repeating β-α units (Seifried *et al*., 2012). β1-5: β subunits. **B**: A schematic view of the domain structure of Eya1-4. Domain structures of *Eya* and its RDGN partners. P/S/T=proline-serine-threonine rich domain; EYA D2= EYA domain 2.

As a member of the haloacid dehydrogenase (HAD) phosphatase superfamily, EYA proteins share a common active core with other HAD phosphatases, as revealed by crystallographic work on pro- and eukaryotic HAD phosphatases. However, unlike other protein tyrosine phosphatases (PTP), *EYA* does not utilise a cysteine residue in catalysis but rather, as a HAD phosphotransferase metalloenzyme, it employs an aspartate as a nucleophile and another conserved aspartate, two residues downstream as an acid catalyst (Rayapureddi et al., 2003). This confers some specificity to this class of proteins which could potentially be explored in the design of inhibitors.

The EYA proteins are composed of a highly conserved 271- amino acid C-terminal motif called the EYA domain (ED), which functions in EYA's interaction with other proteins (sine oculis in drosophila and six1 in vertebrates), and contains the phosphatase catalytic domain. By contrast, the N-terminal of EYA is highly divergent between EYA family members, and its proline-serine-threonine (PST) rich transactivation domain is indispensable for their function as coactivators of transcription. Embedded in the PST domain is a second moderately conserved domain called the EYA domain 2 (ED2) (Landgraf et al., 2010; Silver et al., 2003). These are schematically presented in figure 4.2B and the different protein isoforms of EYA1 are presented in table 4.1.

4.1.3 The Role of EYA in health and disease

EYA phosphatases play crucial role in a wide range of human diseases, from congenital birth defects, neurologic and cardiovascular disorders, to cancer (table 4.1). A role for *EYA1* in ulcerogenesis has not previously been described.

4.1.3.1 Putative role of EYA1 in NSAID-induced gastrointestinal complications

EYA1 dephosphorylates Tyrosine-142 on histone following DNA damage or breaks and this dephosphorylation is an important step required in the recruitment of the DNA repair complex MRN (figure 4.4). The MRN complex in humans are a group of complex proteins consisting of MRE11, RAD50 and NBS1, which bind to double strand DNA breaks (DSB) to initiate DNA repair process (Cook et al., 2009). The mechanism by which NSAID causes damage to gastric epithelial cells and the relationship to *EYA1* is unclear, but requires further investigation.

Table 4.1: Human *EYA* gene involvement in health and disease, modified from (Xu, 2013) and http://www.ncbi.nlm.nih.gov/gene/?term=eya2

Gene	Location	Target organs/tissues	Associated human disease	Cancers
EYA1	Chromosome 8	Ear, branchial arches, and craniofacial, sensory neurons, thymus, and parathyroid, kidney, ribs, skeleton, muscle	Branchial-oto-renal(BOR) syndrome, branchi-otic (BO) syndrome, cardiofacial syndrome (Seifried et al., 2013)	Acute leukemia Under expressed in gastric cancers (Nikpour et al., 2014)
EYA2	Chromosome 20	Muscle defects enhanced in EYA1/2 mutants	-	Ovarian cancer, dermoid tumors, colorectal cancer
EYA3	Chromosome 1	Decreased body size, respiratory, muscle and heart function	-	Breast cancer cell line
EYA4	Chromosome 6	Ear, Heart	Late-onset deafness (DFNA10), cardiomyopathy, holoprosencephaly (HPE) (Seifried et al., 2013)	Malignant peripheral nerve sheath tumours (Miller et al., 2010), sporadic and colitic neoplasia, Barrett's oesophagus and oesophageal adenocarcinoma

Unlike the *Drosophila melanogaster* which has one *EYA* gene, humans have four paralogs, designated as *EYA* 1-4. The name comes from their crucial function as components of a family of transcription factors called the 'retinal determination gene network' (RDGN). These genes play crucial roles in the development of various organs, are associated with some diseases and are also potential markers for various cancers.
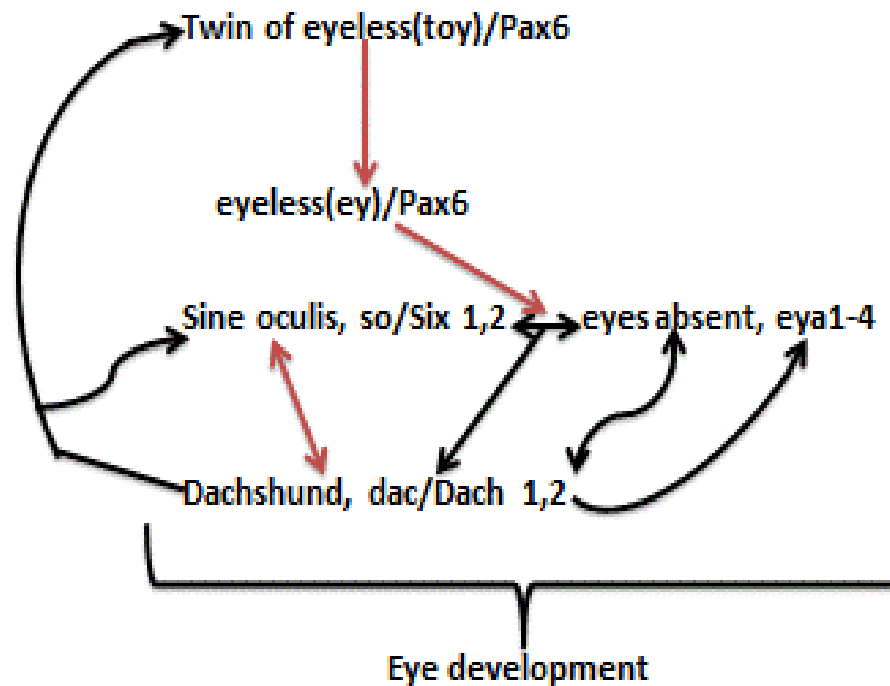
Figure 4.3: The retinal determination gene network (RDGN) regulatory network, modified from (Jemc & Rebay, 2007; Rebay et al., 2005). All members of the RDGN play crucial role in the *drosophilia* eye development, which is initiated by the "*toy*" gene leading to activation of the *SO/SIX/EYA* complex and the downstream effect of drosophila eye development. The red arrows indicate the initiation steps in the transcriptional hierarchy of the key regulators of the *drosophila* eye. The black and double arrows indicate feedback steps.

4.1.3.2 Role in cardiovascular disorder

*EYA*1 mutations have been reported in patients with cardio-facial syndrome- a unique association of asymmetric crying face with congenital cardiac defects (Shimasaki et al., 2004). Dilated cardiomyopathy as a result of a deletion mutation of *EYA*4 with associated heart failure and sensorineural loss has been reported. Biochemical analysis has demonstrated that following this deletion mutation, *EYA*4's role as a transcriptional co-activator may be impaired (Schönberger et al., 2005).

Deletion mutations of *EYA4* gene is linked to dilated cardiomyopathy type 1J with associated heart failure. This is thought to result from failure of the shortened arm of *EYA*4, produced by the deletion mutation, to bind *EYA4* and SIX proteins. In a study of the role of *EYA*4 in Zebra fish cardiac function, depleting *EYA4* in Zebra fish caused significant reduction in mean ventricular areas at end-systole and end-diastole, with contractile features suggestive of severely compromised ventricular function, associated regional wall motion abnormalities and attendant non-uniform ventricular contraction and retrograde blood flow (Schönberger et al., 2005).

4.1.3.3 Role in Cancers

There is increasing evidence of over expression of *EYAs* and their role in many adult cancers which positively correlates with increased tumour size and metastasis. *EYA2* has been implicated in breast and ovarian cancers while *EYA4* is involved in malignant peripheral nerve sheath tumours (Farabaugh et al., 2012; Miller et al., 2010; Pandey et al., 2010; Zhang et al., 2005).

More recently, *EYA1* has been reported to be under expressed in gastric carcinoma and this novel finding is being exploited for its use as a prognostic marker for this cancer (Nikpour et al., 2014). This finding sharply contrasts from reports of over expression of other *EYA* paralogs in various other cancers (Jemc & Rebay, 2007). The phosphatase activity of *EYA1* has been reported as an essential driving force for breast cancer proliferation via interaction with, and maintaining the levels of Cyclin D1 and also important in the induction of cell contact-independent growth of breast cancer cells (Wu et al., 2013). The underlying mechanism of *EYA's* role in cancer is that tyrosine phosphatase promotes cancer cell invasiveness and motility.

In ovarian and breast cancers, EYA2 is upregulated at the protein and RNA levels, partly as a result of genomic over amplification and this, in association with Six1 co-expression is a poor prognostic factor. EYA2 is a necessary cofactor for many of the metastasis promoting functions of Six1, and hence targeting Six1-Eya synergism may repress breast cancer progression (Farabaugh et al., 2012; Seifried et al., 2013). Expression of *EYA4* has been found to be approximately 100 fold higher in peripheral nerve sheath tumours than in normal nerve sheaths and inhibition of this expression using short hairpin RNA (shRNA) reduced cell adhesion and migration, and caused cell necrosis but had no effect on cell proliferation and apoptotic cell death (Miller et al., 2010).

4.1.3.4 Role in branchio-oto-renal syndrome

Mutations in the *EYA* gene cause a wide range of congenital disorders, due to their involvement in the formation of many organs and tissues (Seifried et al., 2013). Congenital anomalies which arise as a result of mutations in the *EYA1* gene, detectable in 40% of affected individuals, include Branchio-oto-renal syndrome (BOR1) and Branchio-otic syndrome (BOS1) (Seifried et al., 2013). The former is an autosomal dominant disorder characterized by hearing loss (in >90% of affected individuals), ear malformations, fistulae and cysts in the neck and renal abnormalities or total renal agenesis, while the later (BOS) has all features of BOR except renal involvement. Renal defects manifest in only about 6% of BOR patients as against the 93% that present with hearing loss.

Commonly occurring renal defects in BOR patients are malformation of the collecting system, polycystic, hypoplastic or complete renal agenesis (Chen et al., 1995; Heimler & Lieber, 1986) and these have also been observed in *EYA1* heterozygous animals (Xu et al., 1999).

The impairment of the function of the hair cells of the organ of Corti in the cochlea are known to generally cause sensorineural deafness, hence the phenotypic characteristics of individuals affected by dilated cardiomyopathy 1J (CMD1J) and deafness, autosomal dominant non-syndromic sensorineural deafness 10 (DFNA10) suggests that *EYA4* is crucial post developmentally for a normal functioning organ of Corti (Seifried et al., 2013).

4.1.4 The Biology of *EYA1* gene

EYA proteins belong to a novel family of proteins identified in many animals ranging from birds, frogs, and fish to higher vertebrates (Hoshiyama et al., 2007). Unlike *Drosophila melanogaster* which has only one *EYA* gene, humans have four paralogs, designated as *EYA* 1-4. The name comes from their crucial function as components of a family of transcription factors called the 'retinal determination gene network' (RDGN) (figure 4.3), derived from their role in *drosophila* eye development (Jemc & Rebay, 2007; Rebay et al., 2005). *EYA* has been reported to be present in some higher plants like rice and Arabidopsis (Takeda et al., 1999). In contrast to *SO/SIX* genes, *EYA* genes do not encode transcription factors but rather encodes proteins that interacts with DNA binding proteins such as So/six and other DNA-binding proteins (Xu, 2013).

4.1.4.1 EYA1 phosphorylase and transcription activity

EYA, as a member of a conserved network of transcription factors, named collectively as the 'retinal determination gene network' has been well characterized as transcriptional coactivators that function in consonance with So/Six proteins. This is crucial in many developmental contexts and also attributable to some disease, due in part to absent or

impaired *EYA* function (Jemc & Rebay, 2007). Analyses of the structure and function of both *EYA* and Gal4 DNA binding domain (Gal4DBD)-*EYA* fusion proteins has shown that the internal P/S/T rich region (figure 4.2B) is needed for transactivation. While ED2 is required for optimal activity *in vitro* and *in vivo*, it is not essential for transactivation. Six and Dach are known to be cofactors of *EYA* necessary for activation of transcription in cell based reporter gene assays and this is believed to explain the increase in ectopic eye formation when these cofactors are co-expressed with Eya in *Drosophila* (Silver et al., 2003)*.*

Alone, this family of proteins produces a low level of transcription, but transfection of *Six*/*So* and *EYA* produces tremendous activation of a number of reporter constructs. When Dach is added, an even greater transcriptional output is achieved (Ikeda et al., 2002; Silver et al., 2003). Following the identification of other binding partners for the Six family proteins, it is now thought that the Eya-Six-Dach complexes could have role as transcriptional activators and repressors. This opinion followed the finding that Six proteins act as transcriptional repressors on interaction with the Groucho (Gro) family corepressors (Silver et al., 2003).

EYA are also members of the phosphatase subgroup of the HAD superfamily, which constitutes about one-fifth of all human phosphatase catalytic subunits. HAD phosphatases differ from others in that while the tyrosine specific phosphatases use serine or cysteine nucleophiles for phosphoryl transfer, HAD phosphatases use aspartate residue in the active site for nucleophilic attack. Phosphatase activity is optimal at a pH of approximately 5.5 and $Mg^{2+,}$ $Ni^{2+,}$ $Co^{2+,}$ and Zn^{2+} are essential cofactors for optimal enzyme activity. The phosphatase activity of the HAD phosphatases is a two-step phosphoaspartyl transferase mechanism; the first being the attack of the aspartate nucleophile on the phosphoryl group

of the intermediate leading to the formation of a phosphoaspartyl enzyme intermediate and the displacement of the substrate leaving group. The second step is nucleophilic attack, by a water molecule, on the phosphoaspartyl intermediate and the release of free phosphate with regeneration of the catalytic aspartate (Seifried et al., 2013).

In vivo studies suggest that Eya phosphatase activity is important for the normal development of the eye in *Drosophila*. In BOR mutants, it's been observed that phosphatase activity is lost, and this strongly suggest that phosphatase activity is important for transcription factor activity of *EYA* and this may be contributory to the disease. Also in *Eya* HAD mutants, the ability to reverse Dach-Six-mediated repression is lost in *Eya* (Silver et al., 2003).

4.1.4.2 EYA phosphatase activity and histone dephosphorylation

Following genotoxic stress, *EYA1* and 3 can dephosphorylate Tyrosine-142 of histone (H2AX), and this is a decisive phosphorylation mark that discriminates between apoptotic or survival/DNA repair response of cells (figure 4.4). H2AX is an established substrate of *EYA* phosphatase, as wild type *EYA* has been observed to remove the phosphotyrosine mark of EYA, while the phosphatase-inactive mutant *EYA* has little or no effect (Cook et al., 2009).

The novel finding that EYA proteins are functional phosphatases, containing an embedded HAD phosphatase signature sequence was a major landmark in developmental biology because it indeed demonstrated for the first time, a transcription factor with an intrinsic phosphatase activity (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003).

Though EYA's phosphatase activity has earlier been noted to be very important in the transcriptional activation of some Six-dependent reporter genes, emerging evidence indicates that in Drosophila, down-regulating EYA phosphatase activity does not generally attenuate transcriptional output (Jemc & Rebay, 2007; Li et al., 2003). Despite these phosphatase activities of the EYAs, the establishment of a physiological target is yet to be fully elucidated (Seifried et al., 2013). From the fore going, bearing in mind the results of the GWAS, we propose biological plausibility for *EYA1* in NSAID-exposed mucosal epithelial cells that includes modulation of cell death/survival decisions which can lead to ulcerogenesis.
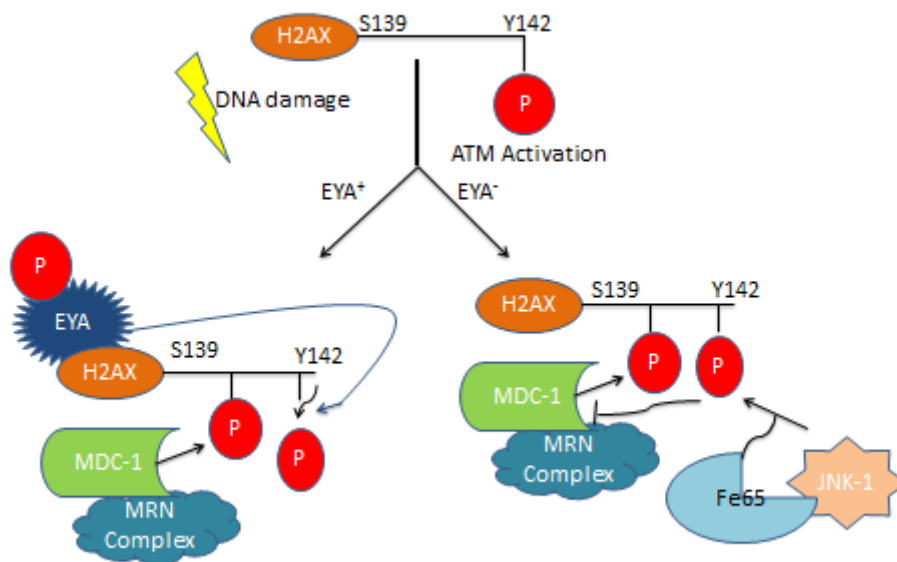

Figure 4.4: Proposed model for *EYA1* dephosphorylation of serine-139 phosphorylated histone following DNA damage adapted from Cook *et al.,* 2009. H2AX= histone; MRN complex=DNA ancillary repair factors.

4.2 Methods

4.2.1 Characterization of the association between *EYA1* SNP rs12678747 and GI complications in NSAID users

4.2.1.1 Patients recruitment and sample collection

Patient cohort, recruitment and sample collection is as described in sections 2.2.1 and 2.2.2.

4.2.1.2 Allelic discrimination for *EYA1* SNP rs12678747

Following genomic DNA extraction as described in section 2.2.4, genotyping of the *EYA1* SNP, rs12678747 was performed using TaqMan® SNP Genotyping assays according to manufacturer's instructions. Fluorescence and allelic discrimination were determined using the SDS software (version 2.2; Applied Biosystems, USA). The forward primer sequence of this assay is 5'CAGAGTATTAATAAGAACCACCTGGTCAA3', while the reverse primer sequence is 5'TGATGAGTTGTCTGTAATATACTACAGGAAGT3'. Details of the thermocycling conditions are as described in section 2.2.4.

4.2.1.3 Statistical analysis

Non-Caucasian patients and patients with missing genotype data were excluded from the analysis. Genotype frequencies were tested for Hardy-Weinberg equilibrium (HWE) in the control group only and in the sample as a whole, with a p value <0.01 assumed to indicate deviation from HWE.

In order to test for association between *EYA1* SNP rs12674747 and PUD (ulcer vs. no ulcer), two logistic regression models were fitted. The first (the 'baseline model') included covariates to represent aspirin use, proton pump inhibitors (PPI) use and gender. The second (the 'genetic model') was the same model, but with the addition of a covariate to represent *EYA1* SNP rs12674747. An additive mode of inheritance was assumed, with wild-type

homozygotes coded 0, variant heterozygotes coded 1 and variant homozygotes coded 2. The two models were compared using a likelihood ratio test to determine the association between rs12674747 and ulcer status. An odds ratio with 95% confidence intervals was computed for rs12674747. Further analyses were carried out using gastric ulcer and duodenal ulcer cases separately. The logistic regression analysis was repeated for the additional outcome measure of gastrointestinal bleeding. Tests were conducted to assess the association between rs12674747 and upper gastrointestinal bleeding (UGIB vs. no UGIB), and between rs12674747 and bleeding ulcers (bleeding ulcer vs. non-bleeding ulcer). Again, covariates were included to represent aspirin use, PPI use and gender. For all analyses, sensitivity analyses were conducted including only cases without any evidence of *H. pylori* as cases.

4.2.2 Characterization of *EYA1* gene expression in the peripheral blood mononuclear cells (PBMC) of healthy volunteers.

4.2.2.1 Recruitment and demographics of healthy volunteers

Following ethical approval, 30 healthy volunteers were recruited after informed consent was obtained from each participant. Inclusion criteria were absence of any medical condition and willingness to participate in the study. Approximately 15mls of whole blood was collected from each participant into 2 PAXgene tubes (Preanalytix QIAGEN, Germany) for RNA extraction and one EDTA tube for DNA analysis.

4.2.2.2 Blood RNA extraction from peripheral blood mononuclear cells (PBMCs)

Using PAXgene RNA extraction kit (Preanalytix QIAGEN, Germany), total RNA was extracted from PBMCs as described in the manufacturer's protocol. The RNA yield and quality was determined using NanoDrop spectrophotometer according to manufacturer's protocol. Samples with A260/280nM ratio higher than 1.8 were included in these experiments. These were stored at -80^{0}C for later use.

4.2.2.3 *EYA1* gene expression and allelic discrimination assay.

cDNA was synthesized from 100ng-500ng of RNA samples using high capacity reverse transcription kit as per manufacturer's instructions (Applied Biosystems, USA). RNA samples were thawed on ice and 20µl reaction containing 2µl of 10x RT buffer; 0.8 µl of 10x RT random primers; 2 µl of 25x (100nM) dNTP mix; 1 µl of Multiscribe Reverse Transcriptase; 1 µl of RNase inhibitor; 3.2 µl of nuclease-free water and 10 µl of RNA samples.

Reverse transcription was performed using the Veriti$^®$ Thermal cycler (Applied Biosystems, USA) programmed as follows: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes and then cooled rapidly to 4°C. cDNA was either stored at 4°C for short term use or at -20°C for later use.

The Taqman$^®$ gene expression assay (Applied Biosystems, USA) is a technology that utilizes oligonucleotide probes and primers that are sequence specific. To prepare a 20 µl reaction, the 20x Taqman gene expression assay for *EYA1* and beta actin genes were thawed on ice. The components of the reaction are 1 µl of 20x Taqman gene expression assay, 10 µl of gene expression master mix; 7 µl of RNase free water and 2 µl of cDNA, and these were added to

each well in a 398-well plates in triplicates and for quality controls, no template controls (NTC) were added. The endogenous control gene for this experiment is beta actin. The Applied Biosystems HT 7900 fast real-time PCR System (Applied Biosystems, USA) was used for the thermal cycling under the following conditions: 10 minutes at 95° C followed by 45 cycles of the denaturing step at 95°C for 15 seconds and an annealing step for 1 minute at 60°C. To determine the *EYA1* genotype of individual participants, genomic DNA was genotyped using the Taqman assay as described in section 4.2.1.2

4.2.3 Determination of *EYA1* gene expression in healthy gastric epithelial biopsy samples by qPCR

4.2.3.1 Patient recruitment and demographics

cDNA from corpus tissue biopsy and genomic DNA from100 sex and age-matched healthy control patients was kindly received from Professor Varro, University of Liverpool. Endoscopy and gastric epithelial biopsy sampling had previously excluded any pathology.

4.2.3.2 Real-time Polymerase chain reaction and allelic discrimination

The DNA samples were quantified using NanoDrop Spectrophotometry as per manufacturers' instruction and then normalized to a final concentration of 20ng/µl. The gene expression assay is as described in section 4.3.4.2. However, this experiment was performed in duplicate because the amount of sample was very limited. Allelic discrimination was then performed as described in section 4.2.4.

4.2.3.3 Gene Expression data analysis

Using the SDS software, version 2.2 (Applied Biosystems, USA), gene expression was quantified by the number of cycles at which the amplification curve crosses the threshold value; this is the point where the fluorescence signal surpasses the background signal. The comparative C_T method was used to determine the difference between the mean target C_T value for the *EYA1* gene and the mean control C_T value for beta actin in AGS cells and human embryonic kidney (HEK) cells.

Using the Livak and Schmittgen method, the level of expression of *EYA1* was determined (Livak & Schmittgen, 2001):

$$\Delta C_T = C_T \text{ (gene of interest)} - C_T \text{ (reference gene)}$$

The expression for each sample was then calculated using $2^{-\Delta C_T}$. The fold change was then determined using the formula: $2^{-\Delta C_T}$ (*EYA1* gene)/ $2^{-\Delta C_T}$ (Beta actin gene). One-way ANOVA was done to compare the mean relative expression of *EYA1* in different cells using Graphpad Prism, version 5.

4.2.4 Characterization of *EYA1* gene expression in AGS cells

4.2.4.1 AGS immortalized cell culture

Atypical gastrin secreting cells (AGS) were the generous gift of Prof Andrea Varro. AGS cells were seeded in Dulbecco's Modified Eagle's medium (DMEM) (supplied by Life Technologies) supplemented with 1% antibiotic/antimycotic, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) and cultured in a T75 flask in monolayer at 37° centigrade in a

humidified atmosphere of 95% O_2 and 5% CO_2. At 90% confluence, cells were passaged or cell pellets used in protein lysates. To determine the cell count, 10μl of AGS re-suspended in DMEM was placed in the counting chamber and cell viability determined by the Trypan blue exclusion test of cell viability using the Countess™ automated cell counter, following manufacturers' instruction (Invitrogen). Only cells that have viability ≥92% were passaged.

4.2.4.2 Extraction of total RNA from AGS cells

Total RNA was extracted from AGS cells when they achieved 100% confluence. Tryptinized AGS cells were re-suspended in PBS, transferred into a 2ml cryovial and 1.8mls of RNA stabilization solution, RNAlater®(Applied Biosystems) added to the cells and left in room temperature for 15 minutes, and then preserved at -80° C. RNA extraction from AGS cells suspended in RNAlater® was performed using the RNeasy® mini kit following the product's protocol (QIAGEN).

4.2.5   Characterization of EYA1 protein expression by Western blot

4.2.5.1 Preparation of protein lysates from AGS cells

RIPA buffer was supplemented with 1% protease inhibitor cocktail (both obtained from Sigma Aldrich, Poole, UK). Protein lysates of AGS cells were prepared by washing a 100% confluent flask of cultured cells twice with ice-cold PBS (Invitrogen, UK) and effluents were discarded. 1ml of PBS was then added to the flask and the monolayer of AGS cells were detached off the bottom of the flask as much as possible using a cell scraper. The PBS containing the detached cells was than pippeted into a 1.5ml eppendorf tube and spun for 2

minutes. The supernatant was discarded and 300μl of RIPA buffer was added to the pellet of cells and mixed and placed briefly in a Sonicator (for 10 secs) to break up the cells. The sonicated cells were spun at 13,000rpm for 20 minutes at 4° centigrade and the protein supernatant transferred into a new eppendorf tube and stored frozen at -80^oC.

4.2.5.2 Protein lysate quantification

AGS protein lysates were diluted with PBS 1:1, 1:10 and 1:100 and 10μl of sample was placed in each well in duplicates. The standard was prepared by dissolving 2mg of human serum albumin in 1ml of water and 10μl of standard concentrations of 0, 100, 200, 500, 1000, 1200, 1600 and 2000μg/ml was added in duplicate to a 96-well plate. Bradford reagent was diluted in water in a ratio of 1:5 and 250μl of the reagent was added to all wells containing samples and standard protein. The absorbance of the assay was determined in a plate reader at a wavelength of 595nM. The average absorbance was calculated and then a standard plot was used to determine sample concentration.

4.2.5.4 Western blot assay

The chemical and reagents required for this assay were: Running Buffer 1X (Invitrogen MOPS stock solution 20X); Transfer Buffer 1X (NuPAGE stock solution 20X); 10X Tris Buffered Saline (TBS) stock (80g NaCl, 2g KCl, and 30.3g Trizma Base, all dry reagents were obtained from Sigma Aldrich, dissolved in 800ml dH$_2$O, the pH adjusted to 7.4 with pure HCL and topped up to a final volume of 1L. 1X TBS/T was prepared by adding 100ml of 10X TBS + 1ml Tween 20 and topped up to 1L; and SDS stripping buffer (20ml 10% SDS, 12.5mL 1M Tris HCl pH 6.8,

700μl β-mercaptoethanol) added to 66.8ml of distilled water. The anti-EYA1 (product number ab85009) and anti-beta actin polyclonal primary antibodies (product number ab8227), and the goat anti-rabbit IgG horse raddish protein (secondary antibody, product numbers ab97051) were all obtained from abcam.

As per the Bradford Assay, 5, 10 and 20μg of AGS protein sample was added to 5μl of sample buffer (300μl of reducing agent and 700μl of sample buffer). This was heated to 85°C for 5 minutes and cooled rapidly on ice for 5-10 minutes and electrophoresed using precast gels (Invitrogen, UK) in x1 running buffer in the presence of antioxidant. Protein was then transferred from gel to nitrocellulose membrane (Invitrogen, UK) using the gel sandwich in x1 transfer buffer.

Non-specific binding was blocked overnight by adding 25mls of 5% non-fat milk powder (made by adding 5g of milk powder in 50ml of TBS/T) to each membrane in the cold room on a mixer. The membrane was incubated in the appropriate primary/secondary antibody for 1 hour at room temperature. The membrane was washed using TBS/T after incubation with the each antibody. 1ml of Chemiluminescence reagent was added to the membrane for 1 minute and exposed to Kodak film for 1 minute in a developer plate.

4.2.5.4 Protein densitometry data analysis

The films were scanned using the Quantity one 1-D analysis software (Biorad). The scanner was allowed to equilibrate for 5-10 minutes and following manufacturers' instruction, all films developed from the western blot experiment were scanned and the images saved. The density of the blot was determined using ImageJ software.

4.3 Results

4.3.1 Patients demographic data and data analysis

The demographic characteristics and patient distribution in the case and control cohorts is as presented in the flow diagram (figure 2.2) and table 2.2. About 1200 of the recruited 1300 patients were genotyped. The reasons for exclusion were due to withdrawal from the study for various reasons such as unknown NSAID status (n=25), non-white ethnicity (n=31), unknown ulcer status (n=6), and withdrawal (n=41). Of the 1197 that participated in this study, 66.8% had endoscopic confirmation of PUD while 33.2% had no ulcers on endoscopy. In the case cohort, UGIB was present in 23.0% and 14.4% of NSAID users and non-users respectively. A detailed clinical and socio-demographic characteristic of patients with and without ulcers is presented in figure 2.2 and table 2.2. A univariate analysis of non-genetic covariates was performed and the results are presented in table 2.3, chapter 2.

4.3.2. Association between *EYA1* polymorphism and GI ulceration

The *EYA1* SNP rs12678747 was tested for association with case-control outcome status. The association was tested using logistic regression with covariates representing NSAID use (whether none; non-aspirin NSAIDs; aspirin or both aspirin and non-aspirin NSAIDs), PPI use and gender. *EYA1* SNP, rs12678747 was in Hardy-Weinberg equilibrium ($p<0.05$). The allelic and genotype frequencies of *EYA1* SNP is as presented in Table 4.3; however, there are no available data on the genotype frequencies of this SNP in Caucasian population, making comparison impossible. The variant allele frequency from our data is 0.37, a value that is significantly different ($p<0.05$) than figures from databases which report the global minor allele frequency (GMAF) for this SNP as 0.4405 (NCBI). *EYA1* SNPs rs13260349 and

rs2380716 variant allele frequencies among Han Chinese non-syndromic orofacial clefts patients have been reported as 0.9 and 0.069 respectively (Zeng et al., 2013), which are also significantly (p<0.05) different from our data.

An analysis including only patients who used NSAIDs and developed ulcer as cases (n=466), and those with endoscopic diagnosis of PUD without NSAID use as control (N=334) was done; association was tested by way of logistic regression using likelihood ratio test (LRT) to compare models with and without covariate representing *EYA1*. The test of association was statistically significant for both models with covariate and without covariates (p=0.0007 without covariates and p=0.0003 with covariates; odds ratio 1.52, 95% CI; 1.21, 1.91). The pseudo R^2 value for the model including age, PPI use and gender was 16.9%. These are summarised in tables 4.2 and 4.4.

Assuming an additive model, a repeat analysis in which the cases (n=800) were expanded to include ulcer patients with and without NSAIDs use. There was no association between SNP and binary ulcer status (p=0.970, odds ratio 1.00; 95% confidence interval (CI) 0.82 to 1.23). The pseudo R^2 for model including NSAID use, *H. pylori* status, PPI use, gender and *EYA1* was 24.7%. A sensitivity analysis was done, including only cases without any evidence of *H. pylori* as cases (n=361); there was no association between SNP and binary ulcer status (p=0.961).

Further tests of association were undertaken for the *EYA1* SNP and upper GI bleeding status with patients who have upper GI bleeding as case (n=155) and no upper GI bleeding as control (n=1042). Applying the genetic model described above showed that there was no association between *EYA1* and upper GI bleeding status (p=0.514) while sensitivity analysis showed a p=0.9. Still, assuming an additive mode of inheritance and applying the genetic model described above, association of SNP and ulcer and bleeding status was tested by

logistic regression with patients who had ulcer and GI bleed as cases (n=155) and ulcer with no bleed as control (n=645). The test of association did not show statistical significant (p=0.297). Table 4.2 presents a summary of covariates tested against *EYA1* and the association/outcome measures.

Table 4.2: NSAID covariates tested against *EYA1* single nucleotide polymorphism

Covariates tested against *EYA1* SNP rs12678747		n		Test of association	
		Cases	Control	*p*	Odds ratio
NSAIDs ulcer vs No ulcer		466	334	0.0003	1.52; 95% CI 1.21, 1.91
H. pylori +NSAIDs ulcer		230	397	0.0004	1.63; 95% CI 1.24, 2.13
NSAIDs ulcer vs no ulcer in *H. pylori* positive patients^		230	56	0.0001	2.79; 95% CI 1.57, 4.95
$^{\$}$Ulcer location	GU	407	397	0.787	
	DU	313	397	0.192	
$^{\$}$Binary ulcer status		800	397	0.970	1.00; 95%CI 0.82, 1.23
$^{\$}$Upper GI bleed status		155	1042	0.514	
$^{\$}$Ulcer and GI bleed status*		155	645	0.297	

*Ulcer and bleed=case, ulcer no bleed=control; ^*H. pylori* positive patients only in both case and controls. GU=gastric ulcer; DU=duodenal ulcer. $^{\$}$covariates include NSAID use, PPI use and gender.

Table 4.3: Genotype and allelic frequencies for *EYA1* SNP rs12678747 for NSAID users and non-users

		Genotype frequency						Allele frequency			
Status	n	NSAIDs Users (n=588)			Non-users (n=609)			NSAIDs Users (n=588)		Non-users (n=609)	
		AA	AT	TT	AA	AT	TT	A	T	A	T
Ulcer	800	0.38	0.50	0.12	0.57	0.37	0.06	0.63	0.37	0.76	0.24
No ulcer	397	0.56	0.32	0.12	0.51	0.41	0.08	0.72	0.28	0.72	0.26
*UGIB	155	0.43	0.45	0.12	0.63	0.31	0.06	0.66	0.34	0.79	0.21
*No UGIB	1042	0.42	0.47	0.11	0.54	0.39	0.07	0.66	0.34	0.74	0.26
Ulcer with UGIB	155	0.43	0.45	0.12	0.63	0.31	0.08	0.66	0.34	0.79	0.21
Ulcer no UGIB	645	0.38	0.52	0.10	0.57	0.38	0.05	0.64	0.36	0.76	0.24
Status	n	Aspirin Users (n=426)			Non-aspirin users (n=771)			Aspirin-users (n=426)		Non users (n=771)	
		AA	AT	TT	AA	AT	TT	A	T	A	T
Ulcer	800	0.35	0.52	0.13	0.54	0.40	0.06	0.61	0.39	0.74	0.26
No ulcer	397	0.58	0.32	0.10	0.51	0.39	0.10	0.74	0.26	0.71	0.29
*UGI bleed	155	0.42	0.46	0.12	0.56	0.37	0.07	0.65	0.35	0.75	0.25
*No UGIB	1042	0.40	0.48	0.12	0.52	0.40	0.08	0.64	0.32	0.72	0.28
Ulcer with UGIB	155	0.42	0.45	0.13	0.55	0.37	0.08	0.64	0.36	0.74	0.26
Ulcer without UGIB	645	0.33	0.54	0.13	0.54	0.40	0.06	0.60	0.40	0.74	0.26

The genotype and allelic frequencies correlates with reported frequencies for the European population and there was no significant difference (p>0.05) between these frequencies in the case and control groups. UGIB= upper gastrointestinal bleeding; AA=homozygote wild type; AT= heterozygote variant; TT= homozygote variant; *the UGIB status of 2 patients was missing.

4.3.3 Association between *EYA1* polymorphism and location of ulcer

The site of the ulcer was distributed as follows in this cohort: gastric ulcer 407 (50.9%), duodenal ulcers 313 (39.1%), both gastric and duodenal ulcers (7.8%), and pyloric ulcers (2.0%). Test of association between SNP and site of ulcer was performed as described in section 4.3.2 but including only gastric ulcers as cases in the first analysis and then repeating the analysis with only duodenal ulcers as cases, and in both cases, no ulcers as control (n=397). Those with both gastric and duodenal ulcers were excluded from the analysis.

There was no association between *EYA1* SNP and gastric or duodenal ulcers with p=0.267 and p=0.157 respectively. Furthermore, gastric and duodenal ulcer sensitivity analysis which included only cases without any evidence of *H. pylori* infection as cases (n=211 for gastric ulcers and n=123 for duodenal ulcers) reported p>0.05 for both. These are presented in table 4.2.

4.3.4 Influence of *H. Pylori* infection

To explore the association between *EYA1* and ulcer status in the presence of *H. pylori* infection further, SNP was tested for association with case (NSAIDs ulcer and *H. pylori* infection, n=230) and controls (no ulcers, n=397) as described in section 4.3.2. Analysis repeated with and without covariates shows a significant association between *EYA1* and NSAIDs ulcer in the presence of H. pylori infection p=0.0007 and p=0.0004 respectively. The odds ratio for the second model (with covariates) was 1.63 (95% CI 1.24, 2.13). Including only *H. pylori* positive patients in both cases (n=230) and controls (n=56), using the same model resulted in p<0.0001 without covariates and p=0.0001 (odds ratio 2.79, 95% CI 1.57, 4.95) with covariates (table 4.2).

4.3.5 Association between *EYA1* polymorphism and GI complications in aspirin users

Analysis was done by excluding patients treated with non-aspirin NSAIDs, including only aspirin-treated patients. SNP was tested for association by logistic regression, with covariates to represent aspirin use, PPI use and gender as described in section 4.3.2. There was no significant association between *EYA1* SNP and binary ulcer status as shown in table 4.4 which presents all covariates tested and the outcome measures.

Table 4.4: Aspirin covariates tested against *EYA1* single nucleotide polymorphism

Covariates tested against *EYA1* SNP rs12678747	n		Test of association	
	Cases	**Control**	***p***	**Odds ratio**
$^$Binary ulcer status	800	397	0.568	1.06; 95% CI 0.87, 1.29
$^$Upper GI bleed status	155	1042	0.630	
$^$Ulcer and GI bleed status*	155	645	0.341	

*Ulcer and bleed=case, ulcer no bleed=control; ^*H. pylori* positive patients only in both case and controls. GU=gastric ulcer; DU=duodenal ulcer. $^$covariates include NSAID use, PPI use and gender.

4.3.6 Influence of concurrent use of steroids

The distribution of steroid users and non-users did not differ significantly between groups for the NSAIDs (p=0.44) and aspirin (p=0.1) cohorts. Hence, concurrent use of steroid was not a significant predictor of ulcer status when tested in the regression models. This data is presented in table 4.5 which also shows the percentage of steroid users in each subgroup.

Table 4.5: Concurrent use of steroids and NSAIDs according to ulcer status

Subgroups	Steroid users *n/N (%)
Ulcer and NSAID use	19/471 (4.0%)
Ulcer and no NSAID use	12/333 (3.6%)
No ulcer and NSAID use	7/123 (5.7%)
No ulcer and no NSAID use	7/279(2.5%)
Ulcer and aspirin use	8/329 (2.4%)
Ulcer and no aspirin use	23/475 (4.8%)
No ulcer and aspirin use	6/95 (6.3%)
No ulcer and no aspirin use	8/307 (2.6%)

*n= number of steroid users; N=number in subgroup

4.3.7 *EYA1* gene expression and correlation with genotype

4.3.7.1 Patient characteristics and demographics

100 cDNA and genomic DNA samples were provided by Professor Andrea Varro (University of Liverpool) for the determination of the role of *EYA1* polymorphism in its expression in the healthy gastric epithelium. 53.5% were males while 47% were females and the age range of participants was 19-88 years. The mean age for the males was 61.1±15.4 and 55.9±16.9 for the females. 34 samples were excluded as the gene expression data was incomplete due to insufficient sample. The allelic discrimination data matched with each patient's gene expression data are as presented in figure 4.5. The results show that the mean *EYA1* gene expression in the patients carrying the variant allele was not different from carriers of the wild type allele (p=0.3218). This data is in agreement with earlier work on *EYA1* (rs12678747) RNA sequencing (Carr et. al., -unpublished data) of 10 cases and 10 controls

(figure 4.6), which reported no statistically significant difference in the mean *EYA1* counts per million mapped areas between the wild and variant allele carriers in the control cohort (n=10); however, there was statistically significant difference (p=0.025) in the mean *EYA1* counts per million mapped areas in the ulcer case cohort between the wild-type (AA) and variant (AT/TT) *EYA1* rs12678747 alleles (Carr et. al; unpublished data).
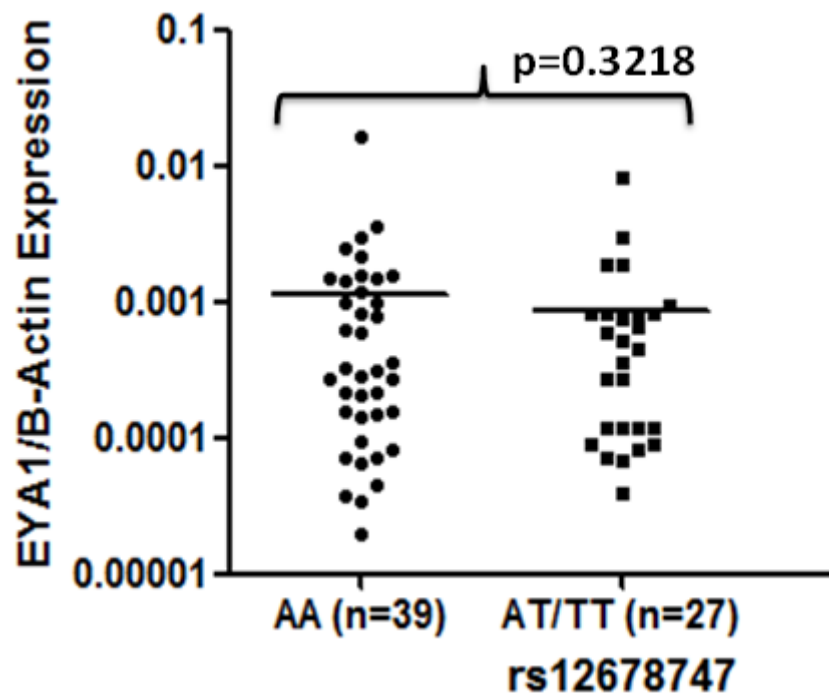

Figure 4.5: Mean relative gene expression of the wild type (AA) *EYA1* (rs12678747) and the variant (AT/TT) type. There was no statistically significant difference (p>0.05) between the mean relative gene expression in the wild type (AA) cohort and the variant type (AT/TT) cohort of patients with healthy gastric epithelial biopsy, this result agrees with the RNA sequencing data presented in figure 4.6.
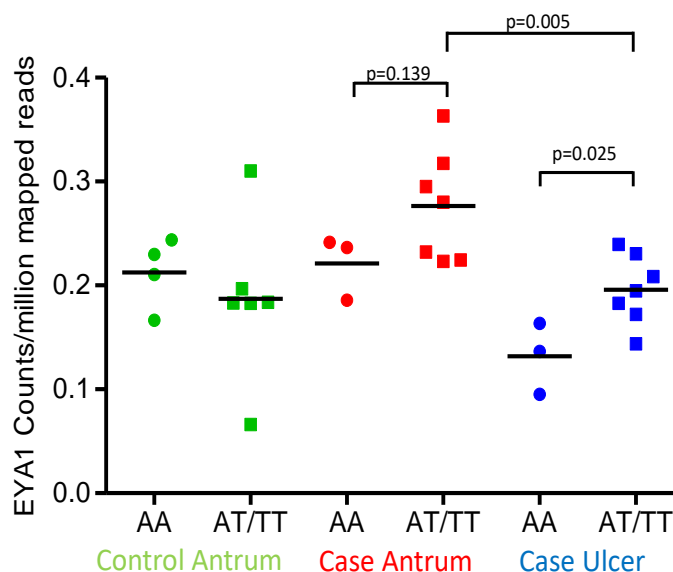
Figure 4.6: *EYA1* RNA sequencing data of gastric epithelial cells of 10 cases and controls grouped according to *EYA1* rs12678747 genotype. Based on *EYA1* genotype, there is a statistically significant difference in the *EYA1* counts per million mapped reads of the wild type genotype (AA) and the variant genotype (AT/TT) among the case cohort (n=10) but no statistically significant difference was observed in the controls (n=10).

4.3.7.2 Relative expression of *EYA1* in AGS cells

In search for a model to study NSAID-related gastric injury, we used the AGS cell line. AGS cells are known to behave similar to target cells and are therefore representative in terms of response to the anti-inflammatory and adverse effects of the NSAIDs (Hall et al., 2006). The abundance of endogenous basal expression of *EYA1* in AGS cells was determined as well as in HEK cells and healthy human gastric biopsy tissue. As shown in table 4.6, *EYA1* is expressed in AGS cells, HEK cells and healthy gastric biopsy samples with a relative expression of 4.86×10^{-6}, 6.7×10^{-4} and 1.0×10^{-3} respectively, relative to the standard/endogenous control, β-actin. Similar to the reports of lower *EYA1* gene expression in gastric cancer cells compared to the surrounding healthy epithelium (Nikpour et al.,

2014), we found a statistically significant (p<0.05) lower relative expression of *EYA1* in AGS cells compared to the gastric epithelium of healthy volunteers.

Table 4.6: Comparative analysis of *EYA1* relative gene expression for different cell types

	Mean CT				
Tissue type	EYA1	β-Actin	Average ΔC_T	Relative expression $(2^{-\Delta C_T})$	^P value
AGS	36.94	19.29	17.65	4.86×10^{-6}	
HEK	36.88	26.33	10.55	6.7×10^{-4}	*P<0.05
Healthy HGB	38.22	26.63	11.59	1.0×10^{-3}	*P<0.05

*AGS *EYA1* relative expression is significantly lower than the *EYA1* relative expression in human embryonic kidney cells (HEK) cells and healthy human gastric biopsy sample (HGB). This is in agreement with the reports of lower *EYA1* relative gene expression in human gastric cancer cells compared to adjacent normal epithelium (Nikpour et al., 2014). CT= cycle time.

4.3.8 Characterization of EYA1 protein expression in AGS cells by Western blot

The concentrations of the unknown samples were determined using the Bradford protein concentration assay, with standard concentrations of human serum albumin as standard. A curve of the concentration (y axis) and the optical densities (x axis) of the standard samples was constructed and the protein concentrations of the AGS cell lysates samples were calculated.

To further determine the level of expression of EYA1 protein in AGS cells, western blot was performed; this showed significant expression of the 65kDA EYA1 protein. There was significantly (p<0.05) lower relative expression of the *EYA1* protein (4.86×10^{-6}) compared to the endogenous β-actin (relative expression= 0.007289) (table 4.7 and figure 4.7).
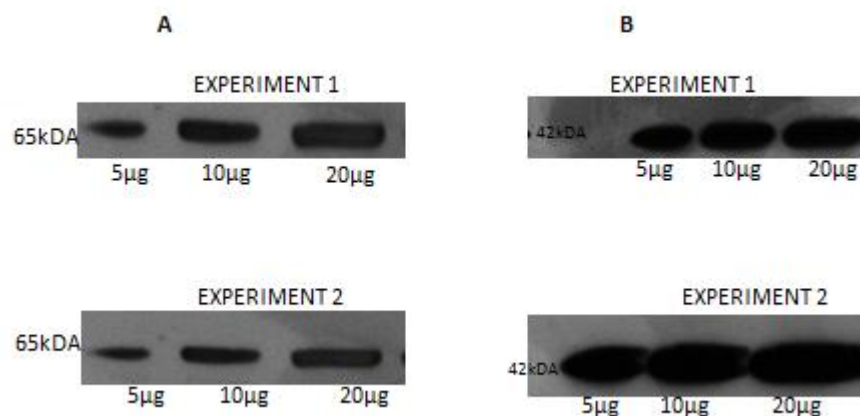
Figure 4.7: *EYA1* protein expression. Figure **A** is the blot of EYA1 protein from 5, 10 and 20µg of AGS cell lysates, while figure. **B** shows the blot for the endogenous β-actin protein expression in AGS cells.

Table 4.7: AGS EYA1 and β-actin protein densitometry data

	EYA1 protein density analysis			β-actin protein density analysis			
	Density(CNT/MM^2)			Density(CNT/MM^2)			Relative expression (EYA1/β-actin)
	Exp 1	Exp 2	Mean density±SE	Exp 1	Exp 2	Mean density±SE	
5µg sample	43851	36329	40091±5319	52096	56102	54099±2832	0.74
10µg sample	46316	39813	43065±4599	57239	57137	57188±72	0.75
20µg sample	53778	47879	50829±4171	68003	68201	68102±141	0.75

Relative EYA1 protein expression in AGS cells shows a relatively high expression of the protein despite a significantly low *EYA1* gene expression in AGS cells. EXP= experiment; SE= standard error.

4.4 Discussion

Genome-wide association studies which involve scanning of the whole genome and associating common genetic variants, SNPs and phenotypic characteristics/complex diseases of interest have now become feasible. A survey of the genome of a large cohort usually in a prospective study that provides detailed and meticulously collected information on the

phenotypes/clinical characteristics has the power to elucidate common genetic variants that are associated with complex diseases (Kottgen et al., 2008). This has been used to explore the genetic basis of various complex diseases like hypertension, diabetes, Crohn's and coeliac diseases as in the Wellcome Trust Case Control Consortium (2007). Despite some notable successes, linkage and candidate gene studies have often failed to deliver definitive results (2007). There has not been any previous GWAS on the toxicity of NSAID on the upper GI.

Our data shows that a case-control analysis including only patients with evidence of use of NSAIDs as cases (n=466), reveals a significant association between binary ulcer status and *EYA1* SNP ($p<0.005$, OR 1.52, 95% CI 1.21, 1.91). Bonferroni testing to correct for multiple testing did not change this association.

This is a very significant and novel finding because this association from our data validates the report of GWAS on NSAID-related GI complication in which *EYA1* is reported as the "top hit" gene in this study. Furthermore, expanding the case cohort (n=800) to include ulcer patients without NSAID use resulted in lack of association ($p=0.970$). This finding further implies that the *EYA1* SNP rs12678747 is associated with NSAID-related ulcer.

Whether this association is purely due to a treatment-gene interaction or it is in support of a plausible role of *EYA1* polymorphism in NSAID-related ulcers needs to be further elucidated. A statistically significant association ($p=0.0004$; odds ratio 1.63 95% CI 1.24, 2.13) was also noted when *H. pylori* positive patients with NSAID ulcers were tested as cases (n=230) against non-PUD patients as controls (n=397). The role of *H. pylori* infection in the pathogenesis of PUD has been well characterized. This association between *EYA1* SNP and this highly successful bacterial pathogen known to cause PUD on its own or in synergism

with NSAID use has not been previously reported. Moreover, this data may suggest a possible synergistic relationship between *EYA1* SNP and *H. pylori* in ulcerogenesis. *H. pylori* infection is associated with a state of chronic gastric inflammation leading to PUD and mucosal associated lymphoma or adenocarcinoma (Montecucco & Rappuoli, 2001; Peek Jr & Blaser, 2002).

Infection with wild type *H. pylori* has been reported to induce changes in the expression of a variety of host genes and proteins particularly proteins such as serine/threonine and tyrosine kinases (of which *EYA1* is a member), transcription factors, cell cycle-related factors, actin cytoskeletal signalling molecules and these are potential determinants of *H. pylori* virulence (Backert et al., 2005). Whether *H. pylori* infection of gastric epithelial cells interferes with the expression of *EYA1* in these cells needs to be explored further.

EYA1 belongs to the tyrosine kinases which as reported, are among the proteins whose expression in AGS cells are altered following infection with wild type *H. pylori*. If this alteration in protein expression leads to a down regulation of these kinases (Backert et al., 2005; Backert & Selbach, 2005), of which *EYA1* is a member, the resulting attenuation of *EYA1* activity could lead to a reduced ability of these epithelial cells to survive DNA damage, ultimately progressing to denudation of the mucosal surface and culminating in ulceration.

The above could explain the significant association we observed between *H. pylori* positive NSAID-induced ulcers and *EYA1*. The persistent association noticed when *H. pylori* positive patients as cases (n=230) were tested against *H. pylori* positive non PUD patients as control (n=56) may suggest that this interaction may be a true association of NSAIDs/*H. pylori* infection and *EYA1* SNP in the pathogenesis of ulcers and not merely a confounding variable.

There are, however, no previous reports on *EYA1* SNP and NSAID-related GI ulcer and bleeding. We are the first to report the MAF (0.36) of this SNP in a Caucasian population, and this is significantly ($p<0.05$) different from the global MAF reported as 0.44 (NCBI). Our data also shows that concurrent use of steroid is not a significant predictor of ulcer status.

Reports on the use of steroids as a risk factor in NSAID-related ulcers have been fraught with controversy (Valkhoff et al., 2012). While most studies implicate steroids as risk factors for NSAID-related ulcer, others report no association. Our data supports previous reports that there is no association between the use of steroid and NSAID-related ulcers (de Abajo & García Rodríguez, 2001). However our numbers are limited and since this was not our primary hypothesis, our findings should be treated with caution. Indeed, it has been reported that steroids in high doses produce gastric lesions in experimental animals and man via the inhibition of prostaglandin biosynthesis at the level of arachidonic acid elaboration from membrane phospholipids (Jama et al., 1975; Lancaster & Robert, 1978).

The major phenotypic consequence of loss of *EYA1* activity is accelerated apoptotic cell death in tissues and subsequent agenesis of target tissues (Xu et al., 1999). Whether this effect is seen in developed organs needs to be investigated. *EYA1* has been established as a protein tyrosine phosphatase, which acts to effect a damage-signal-dependent dephosphorylation of an histone (H2AX) carboxy-terminal tyrosine phosphate (Y142), promoting efficient DNA repair rather that apoptosis in response to genotoxic stress in mammalian cells (Cook et al., 2009).

Increased apoptotic response following evidence of increased serine 139 phosphorylation has been observed in the renal tubular cells of developing kidneys (Cook et al., 2009). Phosphorylation of H2AX by ATM/ATR phosphatidylinositol-3-OH kinase (PI (3) K-family

kinases at the nuclear level is now known to be an extraordinarily important component of apoptosis caused by a perturbation of the JNK/SAPK pathway (Lu et al., 2006). Furthermore, *EYA1* is recruited to H2AX foci that mark DNA double strand damage and while wild type *EYA1* efficiently removed this phosphotyrosine mark on H2AX, the phosphatase inactive mutant EYA1 proteins had little or no effect (Cook et al., 2009).

Similar to reports from other studies, we noted the higher prevalence of NSAID-related gastric ulcers (51.6%) compared to duodenal ulcers (38.5%) (Voutilainen et al., 2001). However, this contrasts from a study on the incidence of PUD in a Western population that reported a higher incidence of duodenal ulcers than gastric ulcers (Groenen et al., 2009). This may reflect the changing aetiologies in the pathogenesis of peptic ulceration (Musumba et al., 2013).

We have observed that despite a lower relative expression of *EYA1* gene in AGS and gastric epithelial biopsy cells, the protein expression was much higher, almost equivalent (0.75) to that of the endogenous control, β-actin. EYA1 proteins are transcriptional co-activators and this unexpected higher expression may suggest a role for this protein in the gastric epithelium.

Our study has several strengths; firstly, it is the first to report an association between the *EYA1* SNP rs12678747 and NSAID-related peptic ulceration. Hitherto, most of the data available on *EYA* genetic variation has focused on the brachio-oto-renal syndrome, non-syndromic orofacial cleft palate; congenital cardiomyopathy and potential roles in cancer pathogenesis. Our data has a large sample size (about 1200) improving the effect size greatly, hence enabling us to see the association reported here. The study design that involved use of endoscopy to diagnose PUD, UGIB, and ascertain presence or absence of *H.*

pylori increased the validity of our data. Our study cohort was exposed to a wide variety of NSAIDs including aspirin and this enabled us to examine any difference in the association between these NSAIDs and the *EYA1* SNP. We further expanded our control cohort to include two groups of patients - one with ulcer but no NSAID use and the second with no ulcer but use of NSAIDs confirmed. This approach would enable associations that are due to gene-by-treatment interaction rather than being a prognostic marker of NSAID-related ulcer to be unmasked (Musumba et al., 2013).

We are also the first to report the expression of EYA1 protein in the AGS and human gastric epithelial cells; prior to this, the only data available was a recent publication reporting a lower *EYA1* gene expression in gastric cancer cells compared to neighbouring epithelium (Nikpour et al., 2014). There has not been any previous work on the relative expression of *EYA1* gene based on the rs12678747 genotype.

The limitations of this study are that we evaluated only one SNP, rs12678747. Looking at other SNPs which are in close linkage disequilibrium with the *EYA1* SNP analysed in this study may provide further information. Secondly, we still do not know the exact mechanism by which *EYA1* predisposes to NSAID-related ulceration and this will need further study. Clearly, with our polymorphism which leads to milder effects on expression, we would not expect to see the congenital malformations that are observed in patients with severe loss of function mutations (Heimler & Lieber, 1986). Third, the non-significant difference in the mean gene expression between the wild type and the variant rs12678747 genotypes may be due to a low sample size; a repeat of this analysis with a higher sample size may be required to bring to a conclusion the exact relationship between *EYA1* gene expression and *EYA1*

genotype. However, it is also possible that the effect of the variant on expression may only

be manifest in the presence of disease rather than in normal tissue.

Chapter 5

A functional analysis of the role of *EYA1* in aspirin-induced gastric epithelial cell death

Chapter 5

5.1 Introduction

5.1.1 Aspirin-induced cell death in AGS- what is known?

Aspirin and other NSAIDs have been demonstrated to induce both the intrinsic and extrinsic pathways of apoptosis; mitochondrial release of cytochrome c and the activation of caspase-9 are observed with the intrinsic pathway, while the activation of caspase-8 induces the extrinsic pathway (Gu et al., 2005; Redlak et al., 2005; Zimmermann et al., 2000). Various modes/pathways of aspirin-related gastric epithelial cell death have been suggested. Table 5.1 presents a summary of available reports on aspirin-induced cell death in AGS cells.

5.1.2 Models for the study of cell death in gastric epithelium

Prior to the report by Hall and his colleagues, there existed no validated *in vitro* model for the assessment of the gastrointestinal toxicity of COX-2 inhibitors or NSAIDs, and hence models using human cell lines or human platelets and monocytes were the standard (Hall et al., 2006). Various systems that have been used to study the inhibitory activities of NSAIDs and natural compounds against COX-1 and COX-2 have been classified as follows: 1) systems that use animal enzymes, animal cells and cell lines; 2) models using human cells or human platelets and monocytes; and 3) newer and evolving models that behave similar to the target cells and are therefore representative of them in terms of response to the anti-inflammatory and adverse effects of the NSAIDs (Pairet & Van Ryn, 1998). For instance, while COX-2, mRNA and protein are both constitutively expressed and inducible at specific locations in both animals and man (Halter et al., 2001; O'Neill & Ford-Hutchinson, 1993),

COX-1 is constitutively expressed in most tissue and may be responsible for the synthesis of PG in normal gastric mucosa which is essential for the maintenance of mucosal homeostasis.

Therefore an appropriate *in vitro* model for assessing the gastrointestinal adverse effects of COX inhibitors should constitutively express both COX-1 and COX-2 in appropriately relative amounts and should express COX-2 in response to physiological stressors. AGS cells fulfil these criteria as they constitutively express both COX-1 and COX-2, with COX-1 expression approximately 4 times greater than COX-2 expression. Also hydrogen peroxide treatment of AGS resulted in a 27-fold increase in PG synthesis (Hall et al., 2006).

Using this model, it was feasible to rank compounds for potential for GI adverse effects as follows: rofecoxib <acetaminophen <nimesulide < celecoxib <salicylic acid < ibuprofen < aspirin < naproxen <indomethacin, from lowest to highest. This rank estimate of GI toxicity provided by using either A23187-mediated arachidonic acid release or addition of 100μM of arachidonic acid is associated with clinical rankings of NSAIDs gastropathy, with a lower therapeutic index observed in AGS models more accurately reflecting the clinical scenarios than older models utilizing enzymes or non-target cells (Hall et al., 2006).

Table 5.1: Studies on the mechanisms of aspirin-induced gastric epithelial cell death

Author	Title	Aspirin conc.	Treatment duration	High through-put screening method
(Leung et al., 2009)	Aspirin-induced mucosal cell death in Human gastric cells: Role of caspase-independent mechanism	40mM	2-4 hrs	Acridine orange-ethidium bromide(EtBr) assay
(Redlak et al., 2005)	Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells	40mM	1-4 hrs	Cell death ELISA kit
(Power et al., 2004)	Aspirin-induced mucosal cell death in Human Gastric cells: Evidence supporting an apoptotic mechanism	3-50mM	24 hrs	Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) assay
(Redlak et al., 2008)	Prevention of deoxycholate-induced gastric apoptosis by aspirin: Roles of NF-kB and PKC signalling	40mM	1 hr	Acridine orange-ethidium bromide (EtBr) assay
(Redlak et al., 2007)	Aspirin-Induced Apoptosis in Human Gastric Cancer Epithelial Cells: Relationship with Protein Kinase C Signaling	40mM	1-4hrs	Cell death ELISA Kit
(Gu et al., 2005)	Activation of the caspase-8/Bid and Bax pathways in aspirin-induced apoptosis in gastric cancer	1-4mM	1-3 days	MTT, acridine orange staining
(Zhou et al., 2001)	Non-steroidal anti-inflammatory drugs induce apoptosis in gastric cancer cells through up-regulation of bax and bak	1mM	0, 12, 24, 36, 48, 60	MTT, acridine orange staining
(Zhu et al., 1999)	Non-steroidal anti-inflammatory drug-induced apoptosis in gastric cancer cells is blocked by protein kinase C activation through inhibition of c-*myc*	0.1-10mM	1-3 days	Measurement of DNA content of cells by PI staining and FACS analysis to detect the $preG_1$ peak; 2) Agarose gel electrophoresis of genomic DNA to detect fragment; 3) AO staining to detect the typical morphological changes under fluorescence microscopy

5.1.3 Benzbromarone and benzarone as EYA inhibitors

Benzbromarone (BBR), a benzofuran derivative, is a potent uricosuric agent that increases the excretion of uric acid through the kidneys. It lowers serum urate and increases urate excretion in normal, hyperuricemic and gouty patients. In short and long term studies, benzbromarone reduces serum uric acid level by one-third to one-half, maintaining its efficacy for up to 8 years (Heel et al., 1977).

Benzbromarone and its metabolite benzarone are potent inhibitors of the EYA tyrosine phosphatase with selectivity for EYA over a representative of typical protein tyrosine phosphatase (PTP), PTP1B. Both are active in cellular assays where they have been observed to inhibit the EYA-promoted motility of mammary epithelial cells and endothelial cells. They are also known to attenuate angiogenesis observed in *in vitro*, *in vivo* and *ex vivo* assays (Tadjuidje et al., 2012). Benzbromarone also inhibited the EYA catalytic domain (ED) with similar potency. Other structurally related compounds were also tested for their potential EYA inhibitory action and it was found that those compounds which retain their basic scaffold of phenol and a benzofuran linked by carbonyl group had IC_{50} values similar to that of benzbromarone. The phenol moiety seems to be required for EYA binding and docking studies have revealed that the OH group forms H-bond with Tyr329 of EYA3. The 3, 5-dihalogenation of BBR gave rise to a compound with the highest binding affinity, while the product from the dehalogenation of benzarone had an affinity half of the parent compound, BBR towards EYA 3. Similar effects were observed with related compounds such as dimethylbenzarone a compound without halogen substituent but has 1-C alkyl side chain at position 2; and the mono-iodinated and di-iodinated derivatives of benzarone, respectively (Pandey et al., 2013; Tadjuidje et al., 2012). Also, removal of the two bromine atoms or

increasing the length of the aliphatic chain on the benzofuran did not significantly affect the inhibitory effects of these compounds. The compounds that lacked the benzofuran or the phenol group had the least inhibitory effect suggesting that both moieties are required for potent EYA inhibition (Tadjuidje et al., 2012). A synthetic hydroxylated BBR metabolite, 6-OHBBR had similar *in vitro* EYA3 inhibitory activity to that of benzarone and an IC_{50} more than twice that of BBR. On the contrary, the 5OH-BBR and the 1'OH-BBR were reported to be less effective in the inhibition of EYA3 (Pandey et al., 2013).

5.1.4 Plausible role of *EYA1* in apoptosis

DNA damage occurs in living cells as a result of interaction with different 'genotoxins', and following this, the decision to either commence DNA damage repair or apoptotic process is crucial. It has been reported that γ-H2AX is involved in the adjudication of the balance between these two outcomes, and phosphorylation of tyrosine 142 modulates the induction of functional apoptotic or repair complexes (Cook et al., 2009).

A vital function of H2AX S139 phosphorylation is to provide a docking site for DNA repair factors (such as mediator of DNA damage checkpoint protein 1, MDC1) near or at DNA double- strand breaks (Rogakou et al., 1999). A group has demonstrated that MDC1 binds directly to phosphorylated S139 of H2AX at the sites of double strand breaks; however, following Y142 phosphorylation, binding of repair factors to phosphorylated serine 139 mediated by MDC1 is inhibited while recruitment of pro-apoptotic factors especially JNK1 is activated (Stucki et al., 2005).

JNK1 is a stress-response kinase, which when activated by DNA damage, initiates a pro-apoptotic cascade of events: it translocates into the nucleus and phosphorylates substrates such as H2AX S139, an event believed to be critical for DNA degradation mediated by caspase activated DNA (CAD) in the apoptotic cells (Lu et al, 2006). In confirmation of the above, Cook and his colleagues demonstrated a robust interaction between transfected wild type H2AX and endogenous JNK1 in 293T cells in response to high dose radiation, an interaction noted to be markedly reduced in the case of the H2AX Y142F variant/mutant (Cook et al., 2009). Loss of EYA3 is reported to result in complete loss of MDC1-H2AX interaction. Thus following EYA3 knock down using siRNA, and testing for MDC1-H2AX interaction by co-immunoprecipitation, there was complete loss of this interaction (Cook et al, 2009).

There is evidence to suggest that in response to DNA damage, EYA is recruited to H2AX foci that mark double-strand breaks (figure 4.4), as chromatin immunoprecipitation analysis after 4-hydroxytamoxifen (4-OHT) induction of I-Ppol in 293T cells revealed that γH2AX and EYA3 were present, suggesting a direct role for EYA in the cellular response to genotoxic stress.

Three groups have independently identified EYA as a potential substrate for the DNA damage response kinases ATM/ATR, as inhibition of ATM/ATR function blocked the interaction between EYA1 or EYA3 and H2AX in response to ionizing radiation, noting that H2AX is an EYA tyrosine phosphatase substrate. This is supported by the fact that *EYA* has the ability to directly dephosphorylate H2AX phosphorylated on Y142 (Lavin & Kozlov, 2007; Matsuoka et al., 2007; Stokes et al., 2007).

Because *EYA1* is involved in the cell's decision to either proceed to apoptosis or DNA repair, loss of function involving this gene may modulate this decision-making process. The aim of this chapter was to investigate the mechanisms at play in aspirin-related gastric cell death and validate the possible role of the EYA1 protein in aspirin-induced apoptosis in gastric epithelial cells (AGS) by functionally ascertaining the effects of EYA chemical inhibition in this process.

5.2 Methods

5.2.1 Experimental techniques

5.2.1.1 Cell seeding and dosing

AGS cell line was cultured as described in section 4.2.4.1. At 90% confluence, cells were tryptinized and cell count done as described in the same section. 5,000; 7,500; 10,000; 15,000; 20,000 and 30,000 cells in 50μl of complete media per each well in a 96-well plate were seeded in triplicate, and incubated for 6 hours to recover from handling. These were then grown for 12 hours and MTT assay performed to determine optimal cell density (15,000cells/well) for all future experiments.

For all aspirin treatments, unless otherwise stated, 0, 0.2, 1, 2, 10, 20, 50, and 100mM of aspirin was dissolved in 0.7% DMSO and cells were treated for 30 minutes, 3 hours and 12 hours duration. Likewise, benzbromarone doses of 0, 0.4, 1, 4, 10, 40, 100, 200, 300, 350, and 400μM suspended in 0.1% DMSO were used in the treatment of AGS cells for similar durations as the aspirin treatment.

5.2.1.2 MTT assay

The compound (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), is readily taken up and reduced, in part by the action of dehydrogenase enzymes in metabolically active cells, to generate reducing equivalents such as NADH and NAPDH. The resulting intracellular precipitate, formazan salts can be solubilized and quantified by spectrophotometry (Berridge et al., 2005).

To determine the viability of AGS cells at each cell density, or following drug dosing, the MTT assay was performed. The reagents required in this experiment were 5M tetrazolyl blue tetrazolinium (MTT) solution, HBSS, 50% v/v dimethylformamide and 20% w/v sodium dodecyl sulphate. 5 M MTT solution was prepared by dissolving 5mg of tetrazolyl blue tetrazolinium in 1ml of HBSS; all chemicals and reagents were obtained from Sigma Aldrich.

Lysis buffer was prepared by adding 30mls of distilled water to 50mls of 50% v/v Dimethylformamide. 20g of 20% w/v sodium dodecyl sulphate (SDS) was added to the above solution and the bottle was given a gentle shake to dissolve. 20mls of distilled water was then added to the solution and to ensure that the SDS is completely dissolved, the solution was warmed in a water bath at 37^{0}C for 1 hour.

The media was removed carefully by aspiration, and 20µl of freshly prepared MTT solution added to each well containing AGS cells, adding also to empty wells, as negative controls and incubated for 3 hours, before 100 µl of lysis buffer was added to each well and the plate covered and incubated overnight. The plate was then shaken for 15 minutes and the absorbance read on a Beckman Coulter DTS 880 plate reader at 595nm using Apex software, version 1.0.0.14.

5.2.1.3 Caspase Glo assay

Caspase 3 and 7 are members of the cysteine aspartic acid-specific protease (caspase) family of effector proteins that play key roles in apoptosis in mammalian cells. The activity of these proteases can be measured by a homogenous luminescent assay, the caspase-Glo® 3/7 assay. This assay, optimized for caspase activity, luciferase activity and cell lysis contains the tetrapeptide sequence DEVD that provides the luminogenic caspase 3/7 substrate. Cleavage of the substrate produces a "glow type" luminescent signal, following the consumption of aminoluciferin by luciferase, which is proportional to the amount of caspase activity detected on a plate reader (Promega Corporation). To perform this assay, cells were treated as described in section 5.2.1.1. At the end of the treatment period, equal volume of caspase glo 3/7 assay was added to each well and the luminescence determined using Beckman Coulter DTS 880 plate reader after 30 minutes.

For all caspase glo 3/7 experiments, 2 sets of the experiments were set up, with one set treated with 20μM of Zvad and the other set not treated with Zvad. Three independent experiments were performed.

5.2.2   Characterization of the viability of AGS cells in different acid pH range

In a bid to mimic the internal milieu of the stomach (pH 1.5-3.5), we tried to ascertain the effect of growing the AGS cells under low pH as is found in the stomach. The pH of complete media was measured using a pH meter under sterile conditions and this was determined to be 8.3. The pH of the culture media was then reduced to 3.0, 4, 4.5, 5, 5.5, 6, 6.5 and 7 by titrating different volumes of concentrated hydrochloric acid in 10ml of complete media. The pH was then confirmed again using the pH meter.

15,000 cells in 50µl of media at different pH range were seeded per well in triplicate and cultured for 3 and 12 hours. At the end of the treatment time, MTT assay was performed as in section 5.2.1.2, and the absorbance determined in the plate reader. This experiment was performed in three replicates.

5.2.3   Characterisation of aspirin-induced AGS cell death mechanisms

To determine the dose of aspirin required for this experiment, we calculated the concentration of aspirin in the gastric juice for a typical low dose aspirin user (75-300mg). The volume of gastric juice in the stomach at each point in time is between 20-100mls (Steingoetter et al., 2015). Hence we used this range as the volume of gastric juice in order to determine the concentration of aspirin. The calculations are as follows:

Volume of gastric juice in the stomach = 20-100mls

Concentration=$\frac{no.of\ moles\ of\ solute (n)}{volume\ of\ solution\ in\ Litres}$ $=\frac{n}{v}$

Molecular weight of Aspirin = 180 g/mol ($C_9H_8O_4$)

75mg of Aspirin =0.075 g

No. of moles in 0.075g of aspirin = $\frac{weight}{Mol\ weight}$ = $\frac{0.075}{180}$ = 4.166 $x10^{-4}$ M = 0.416 mM

For 20mls gastric juice, C = $\frac{0.416}{0.02l}$ = 21mM/L

For 100mls gastric juice, C = $\frac{0.416}{0.1}$ = 4.2 mM/L

For 300mg of aspirin:

Number of moles= $\frac{weight}{Mol\ weight}$ = $\frac{0.3}{180}$ =1.66 x 10^{-3} = 1.66mM

Hence, for 20mls (0.02l) of gastric juice, aspirin concentration = $\frac{1.66}{0.02}$ = 83mM/L

Assuming gastric juice volume of 100mls (0.1L), the concentration of aspirin= $\frac{1.6}{0.1}$ = 16.7mM/L

All serial dilutions of drugs were confirmed using Molarity graph pad. This informed the choice of aspirin concentration between 0.2mM-100mM in testing its effects on cell viability. Prior to the viability assay, we determined the pH of complete media-containing aspirin at each aspirin concentration used in the experiments (see table 5.2).

Table 5.2: Aspirin concentrations used in the viability assays and their respective pH

Aspirin concentrations (mM) in media	pH of media at each aspirin concentration
0	8.30
0.2	8.20
1	8.20
2	8.08
10	7.79
20	7.37
50	6.40
100	3.98

Cell seeding and dosing are as described in section 5.2.1.1. A positive control (1µM Staurosporin) and 2 negative controls using 0.7% DMSO and cells in media (no drug) respectively, were also added to each experiment. MTT assay was then done as described in section 5.2.1.2.

To further elucidate the exact mechanism of cell death at play in this assay, caspase glo 3/7 assay was setup as described in section 5.2.1.3; however, another experiment was setup in which prior to treatment with aspirin, the AGS cells were pre-treated with 20µM of the caspase inhibitor, Zvad for 1 hour. Aspirin concentrations and duration of treatment are as described in section 5.2.1.1.

5.2.4   Characterisation of benzbromarone toxicity in AGS cells

Cell seeding and dosing are as described in section 5.2.1.1. Staurosporine (1µM) was used as a positive control. These drugs were dissolved in DMSO and added to complete media at a final DMSO concentration of 0.1%. Two negative controls that include cells in complete media only and cells in 0.1% DMSO without benzbromarone were also added. These were left to incubate for 30 minutes, 3 hours and 12 hours.

At the end of the treatment period, MTT assay was performed as described in section 5.2.1.1 to determine viability of the AGS at the these concentrations and treatment duration. Furthermore, to perform caspase glo 3/7 assay, benzbromarone was serially diluted to make x2 concentrations of the concentrations stated in section 5.2.1.2 and 50µl of the benzbromarone-containing media was added to AGS cells in 50 µl of media to give a final volume of 100 µl and a final concentration of 1, 4, 10, 40, 100, 200, 300, 350, and 400µM benzbromarone. Controls were added as above.

5.2.5 Assessment of benzbromarone modulation of aspirin-induced cell death.

To determine the effect of pre-treatment of AGS cells with benzbromarone prior to aspirin exposure, AGS cells were treated with 1, 2.5 and 5μM respectively of benzbromarone for 3 hours. Aspirin concentrations, treatment duration and controls in section 5.2.1.1 were used for this experiment and 3 independent experiments were performed. MTT assay was then performed at the end of each treatment period as described in section 5.2.1.2.

For the caspase glo 3/7 assay, aspirin concentrations, treatment duration and controls are as described in section 5.2.1.1. A second control was setup on the same plate but here, prior to treatment with aspirin, AGS was then exposed to Zvad for 1 hour after treatment with benzbromarone. Caspase Glo 3/7 assay was then performed as described in section 5.2.4 and luminescence taken on a plate reader.

5.2.6 Validating the aspirin-related cell death with PARP-cleavage using Western Blot

100,000 cells were seeded into each well in a 6-well plate in 1.5mls of complete media and left to grow. At 70% confluence, the cells were then treated with 10, 20 and 50mM concentrations of aspirin, adding 1μM staurosporine as positive control, in fresh media, for 12 hours. The negative control, 0.7% DMSO without aspirin was added. Cells that did not detach were scraped and centrifuged to form pellets. The pellets were then frozen at -80° C if to be used at a later date and protein lysates prepared from the pellets as described in section 4.2.5.1. Bradford and Western blot assays were then performed as described in section 4.2.5.2 and 4.2.5.3 respectively.

The primary and secondary antibodies were cleaved PARP (Asp214) rabbit mouse antibody (product number 5625) and anti-rabbit IgG, HRP-linked Antibody (product number 7074), respectively, while GAPDH was used as loading control and all were obtained from Cell Signalling Technology (CST), USA. The primary antibody detects endogenous levels of the large fragment (89kDa) of human PARP-1 protein produced by caspase cleavage, and does not recognise full length PARP-1 or other PARP isoforms (Cell Signalling Technology).

5.2.7 Statistical analysis

The MTT absorbance data was presented as a percentage of the negative control (0mM) and the mean ± SE for 3 replicate experiments was calculated. A one-way analysis of variance (1-way ANOVA) or the 2-way ANOVA (applying the Bonferroni post-tests analysis to compare means where applicable) was computed using the Prism Graphpad Software. Further test of significance of means was carried out using the Tukey's multiple comparison tests.

The data on the caspase activity were presented as the luminescence (RLU), and as above, the mean ± SE of 3 replicate experiments was calculated. Also the Tukey's multiple comparison tests was applied to determine if there are any difference in the observed means of the control and among the treatment group. All data were rounded off to one decimal place.

Results

5.3.1 Optimal cell density

We performed a pilot assay to determine the optimal density of AGS cells required for the viability experiments by seeding different numbers of AGS cells in triplicate in 96-well plates and incubated them for 12 hours. The mean absorbance for each cell density was determined and the cell density plotted against the absorbance.

The cell density with the highest absorbance (15,000cells/well) was taken to be the optimum cell density for further experiments. Cell viability declined at higher cell densities (figure 5.1).
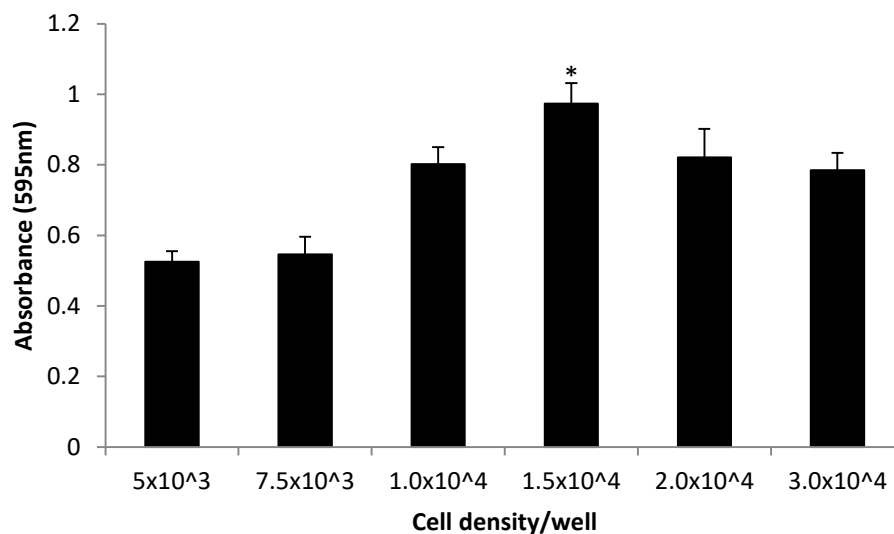
Figure 5.1: Optimal cell density for 96-well plate via MTT assay. There is steady increase in absorbance until 15,000cells/well after which the absorbance begins to drop at 20,000 cells/well (*Optimum cell density).

5.3.2 Effect of pH on the viability of AGS cells cultured in different acid pH ranges

The physiologic pH of the stomach has been determined to be in the range of 1.5-3.5 (Ivey, 1986). To mimic the pH of the stomach in the media in which the AGS cells were cultured, we determined the volume of concentrated/sterile hydrochloric acid required to bring the pH of complete media to lower pH values. The MTT assay was used to assess viability following a-12 hour incubation at different pH values as shown in figure 5.2.

We observed a significant ($p < 0.05$) decrease in viability of AGS cells from pH 6.5 and lower, with about 50% reduction in viability at pH 3.0. As a result of the finding of significant cell death with lower pH values, and bearing in mind that the aspirin solution is acidic (table 5.1), we decided to perform the viability assays at the pH (8.3) of complete media.
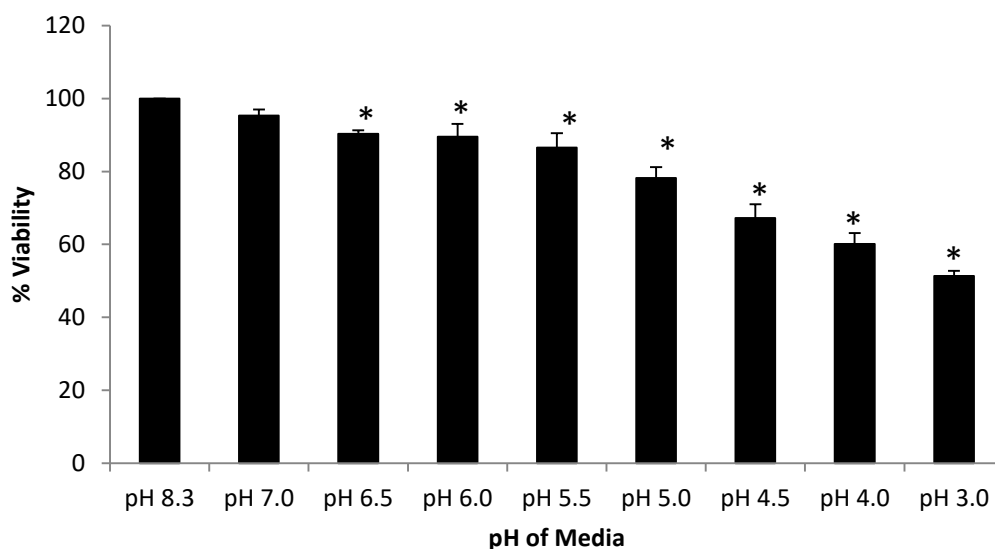
Figure 5.2: Decline in viability of AGS with decrease in pH of media. There was significant ($p < 0.05$, * indicates significant difference) decline in AGS cells viability as pH of the media falls, with a hallmark decline in viability of 50% at pH 3.0. We observed no significant ($p > 0.05$) difference in viability at pH 7.0 and 8.3.

5.3.3 Aspirin-induced AGS cell death

Following 30 minutes of incubation of AGS cell with aspirin, we noted a dose-dependent decline in the viability of AGS cells as determined by the MTT assay. The decrease in the cell viability was statistically significant ($p < 0.05$) at aspirin concentrations above 2mM; at 100mM, the viability of AGS exposed to aspirin dropped to about 6%. We also observed that for the 30 minute incubation, 1μM of the standard apoptosis inducer, staurosporine, caused a reduction in the viability of AGS cell comparable to the effect of 10mM and 20mM aspirin concentration ($p > 0.05$), while observing a more marked effect at 50 and 100mM aspirin concentration.

Table 5.3 presents the cell viability data and the statistical analysis, while figure 5.3 is a graphical representation of the aspirin-related dose dependent decrease in AGS cell viability.

Contrary to the observed significant decline in cell viability starting at 2mM for the 30 minute incubation period, following the treatment of AGS cells with aspirin for 3 hours, we noted a significant ($p < 0.05$) reduction in cell viability from 1mM aspirin concentration in a dose dependent manner. We also observed a time-dependent decline in the viability of AGS cells treated with 1μM staurosporine, when comparing the 30 minutes or 3 hours' time points with the 12 hours' time point.

However, aspirin-treated AGS cells did not show significant ($p > 0.05$) time dependent decline in cell viability, but rather a concentration-dependent effect (table 5.3). The finding of dose dependent decline in AGS cell viability supports previous reports of an increased percentage

of apoptotic cells when AGS cells were treated with increasing concentration of aspirin up to

50mM for 24 hours (Power et al., 2004).

Table 5.3: Comparing the effect of duration of aspirin exposure (time) on viability of AGS cells

Aspirin treatment for 30 minutes, 3 and 12 hours			
Concentration	Viability (%) of treated cells		
	3O min ± SE	3 Hrs ± SE	12Hrs ± SE
0mM	100±0	100.0±0.0	100.0±0.0
0.2mM	94.1±4.5	95.2±0.8	96.3±2.6
1mM	92.1±3.0	90.7±3.6	91.2±5.9
2mM	86.7±5.5	86.3±4.2	88.9±3.6
10mM	74.9±4.3	71.6±1.5	68.6±0.7
20mM	72.3±3.1	58.8±4.4	60.2±1.7
50mM	54.4±2.7	35.4±3.1	24.8±4.0
100mM	6.0±.4	5.1±1.3	5.6±0.2
Staurosporine (1µM)	79.3±3.2***	60.5±0.7	29.8±2.5***

There was no significant difference in the time-dependent effect of aspirin on the viability of AGS cells treated with aspirin. Tukey's comparison test was done, comparing 30 minutes and 3 hours; 30 minutes and 12 hours and 3 hours vs 12 hours. There was no significant difference between all aspirin doses and time duration while for staurosporine, there was significant difference ($p < 0.05$) between (shown as *** in the table) 30 minutes and 12 hours incubation period, respectively. SE= standard error; Hrs=hours; min=minutes.
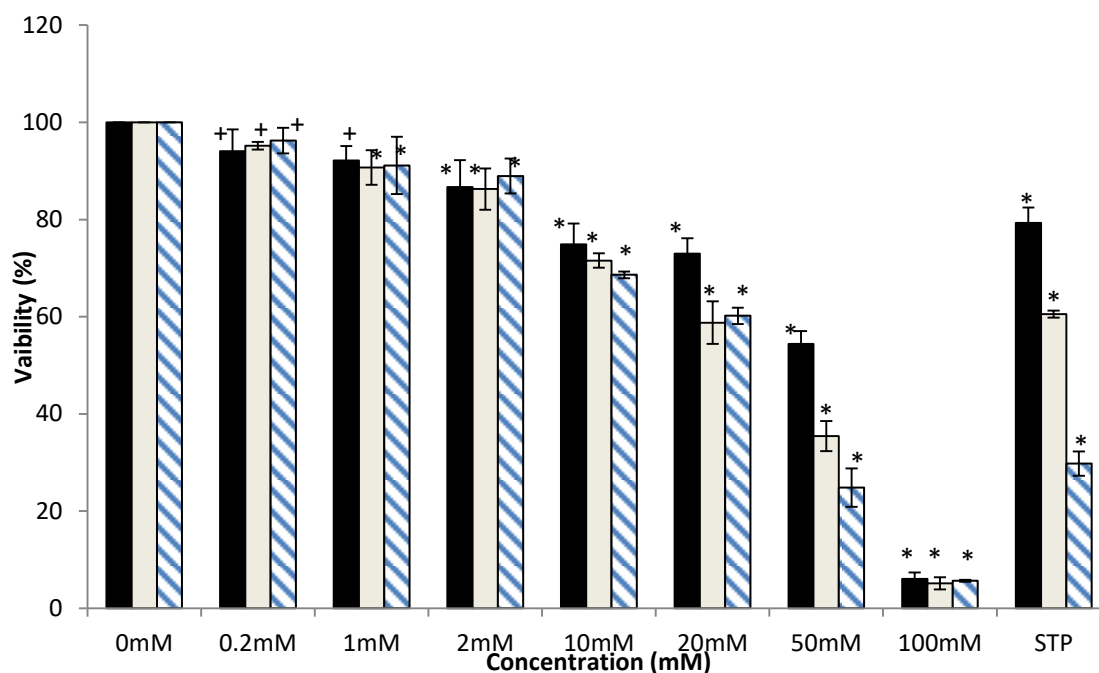
Figure 5.3: Shows the bar chart representing the dose-dependent decrease in the viability of cells treated with aspirin for 12 hours. STP = staurosporine (1µM), used as positive control and 0µM concentration is the negative control. There is no significant effect of longer duration of treatment except for the staurosporine treated-cells that shows a significant (p<0.05) difference between the 30 minutes and the 12 hour time points. The effect of aspirin on the viability of AGS cells is concentration dependent but not time dependent, even at the maximum concentration of 100mM. *indicates statistically significant reduction in cell viability (while +indicates no significant difference) at these doses compared to the negative control (0µM). Black solid= 30mintes; grey solid bar= 3 hours; oblique bar= 12 hours.

To further characterize the cell death pathway for aspirin-induced cell death, we determined caspase 3 and 7 activities of aspirin-treated cells after 30 minutes, 3 hours and 12 hours. For all treatment durations, we observed a dose-dependent decrease in the caspase activity of AGS cells. For the 30 minute incubation, the highest caspase activity for aspirin treated cells was observed at 0.2mM aspirin concentration.

There was also a significant (p <0.05) decrease in the caspase activity of cells that were pre-treated with ZVAD prior to aspirin treatment compared to cells that were not pre-treated

with aspirin. At the 12 hour time point, the positive controls (staurosporine-treated cells) had a remarkable increase in caspase activity, in excess of 40000 RLU. Similar to the earlier observed time-dependent decrease in the viability of staurosporine treated cells, we observed a time-dependent increase in the activities of caspase 3 and 7 confirming that staurosporine-induced cell death is time-dependent (Boulares et al., 1999). Additionally, aspirin-related cell death is not wholly caspase dependent. These data are presented in figure 5.4.
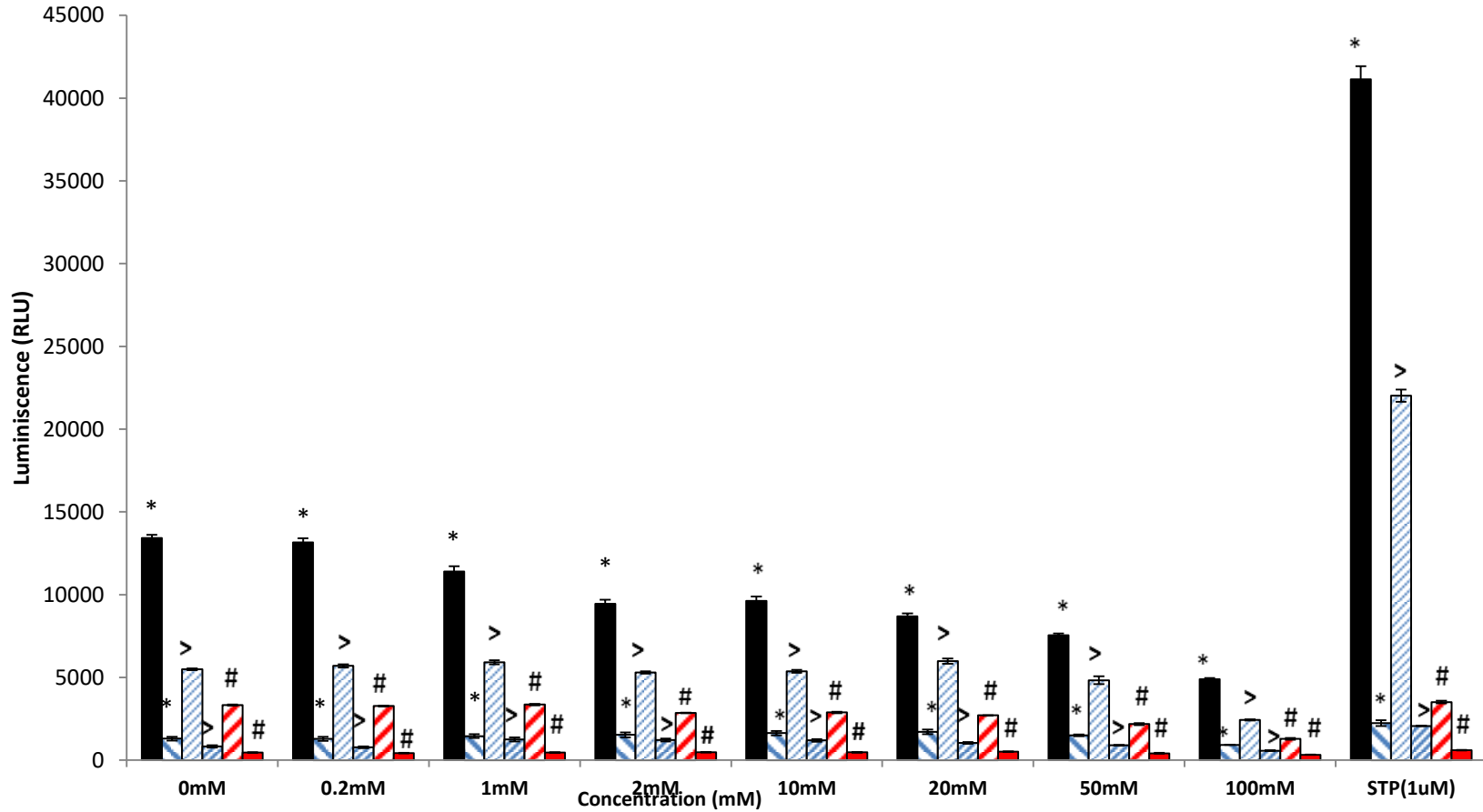
Figure 5.4: Summary data of caspase activity for all incubation periods. The longer the duration of aspirin treatment, the higher the caspase 3 and 7 activities in the groups that was not pre-treated with the caspase inhibitor, Zvad, while there was significant decrease (p<0.05) in the caspase activity of the controls pre-treated with Zvad compared to non-Zvad-treated cells. STP= Staurosporine, as positive control. Bars from left to right are aspirin only for 12 hours (*), aspirin and Zvad for 12 hours (*), aspirin only 3 hours (>), aspirin with Zvad for 3 hours (>), aspirin only 30 minutes (#) and aspirin with Zvad 30 minutes(#) assays.

5.3.4 Effect of caspase inhibition on the viability of AGS cells treated with aspirin

To further explore mechanisms underlying the aspirin-related cell death in AGS cells, we pre-treated AGS cells with the universal caspase inhibitor, ZVAD and compared the cell viability with ZVAD-untreated cells after 12 hours aspirin incubation.

Contrary to the expected prevention of apoptosis following caspase inhibition, the results did not show any significant ($p > 0.05$) difference in caspase activity between both groups (figure 5.5). This suggests that caspase inhibition was unable to "rescue" the aspirin treated cells from apoptosis. This finding is corroborated by the report that inhibition of caspase was unable to inhibit cell death in AGS cells treated with 40mM of aspirin for 4 hours (Leung et al., 2009).
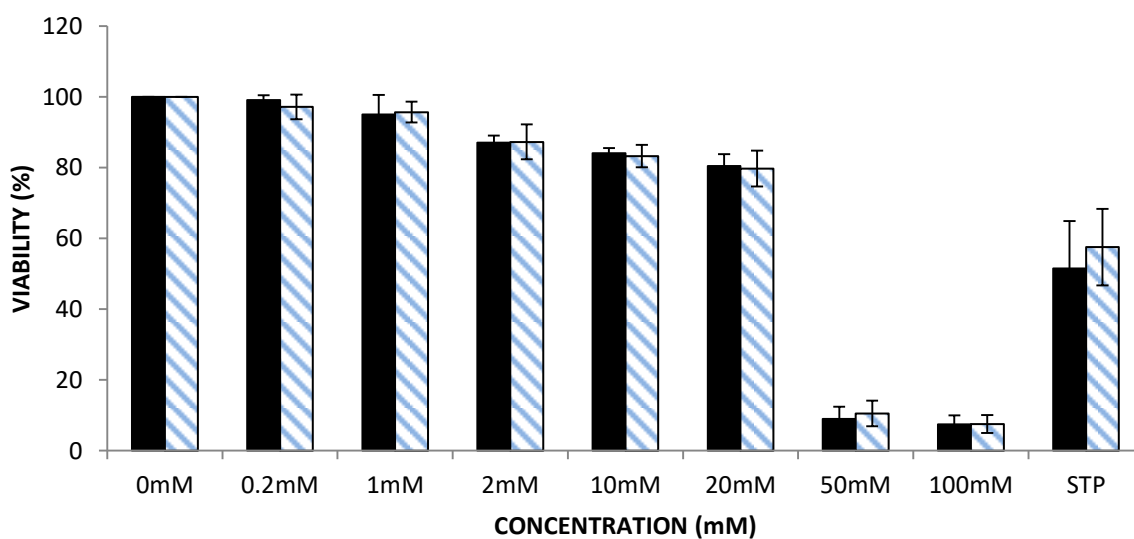
Figure 5.5: There was no significant ($p > 0.05$) difference between the mean viability of AGS cells pre-treated with ZVAD compared to those that were not pre-treated with ZVAD prior to treatment with aspirin. Solid bar= aspirin only treatment for 12 hours; dotted bar= cells pre-treated with Zvad before aspirin exposure.

5.3.5 Characterisation of benzbromarone cell toxicity in AGS cells

To determine the dose range of benzbromarone that would be appropriate for the pre-treatment of AGS cells before treatment with aspirin without causing significant toxicity to the cells, we performed a MTT viability assay by incubating the cells with different concentrations of benzbromarone for 30 minutes, 3 and 12 hours.

The results show a dose-dependent decrease in the viability of AGS cells from 0.4µM of benzbromarone; cell death became statistically significant ($p < 0.05$; 95% CI 1.9-29.0) at higher concentrations (>4uM) (figure 5.6). Also the reduction in viability caused by 1 µM of staurosporine, a standard inducer of apoptosis (Boulares et al., 1999), was comparable ($p > 0.05$; 95% CI -8.1-19.0) to the effect of 10 µM benzbromarone in the 30 minute incubation period.

The viability data are presented in table 5.4 and graphically presented in figure 5.6. The 3 and 12 hour incubation time experiments also showed similar trends in concentration-dependent decrease in the viability of the AGS cells. Moreover, there was a statistically significant ($p < 0.05$) time-dependent decrease in the viability of the cells from 200µM-400µM when comparing 30minutes and 3 hours with the 12 hours' time point (table 5.4 and figure 5.6).

We further observed an increased ability of staurosporine to cause significant ($p < 0.05$) decrease in cell viability at 12 hours compared to 30 minutes and 3 hours. This is suggested by the significant decrease ($p < 0.05$) in cell viability associated with exposure to staurosporine (a reduction in cell viability from 71.4% and 66.6%) at 30 minutes and 3 hours respectively to 25.5% at the 12hour time point.

Table 5.4: Effect of the duration of benzbromarone exposure on viability of AGS cells

Benzbromarone treatment for all time duration			
Viability(%) of treated cells			
Concentration	30mins ± SE	3Hrs ± SE	12Hrs ± SE
0µM	100.0±0.0	100.0±0.0	100.0±0
0.4µM	89.4±6.6	99.4±0.5	96.9±0.5
1µM	90.4±7.0	98.9±3.0	97.1±1.1
4µM	84.5±4.6	75.4±1.9	74.9±2.3
10µM	76.8±10.2	70.7±6.2	68.9±2.7
40µM	56.0±1.9	62.7±6.2	57.2±10.5
100µM	57.1±1.9	57.8±2.7	51.2±5.0
200µM	54.3±2.8	50.4±2.4	26.8±6.5**
300µM	53.9±1.7	49.7±0.5	24.2±4.4**
350µM	56.8±3.5	46.2±4.7	22.7±2.8**
400µM	55.1±2.0	44.2±5.3	22.4±3.3**
STP (1µM)	71.4±1.6	66.6±3.1	25.5±3.5**

Decrease in the viability of cells with increasing benzbromarone concentration. From 200µM concentrations and higher, there was significant reduction ($p<0.05$) in viability at 12 hours treatment (**) compared to 30 minutes and 3 hours. Also, at 12hours, there was a significant decrease ($p<0.05$) in viability of AGS cell treated with 1µM of the positive control staurosporine. STP = staurosporine, used as positive control and 0µM concentration is the negative control which contains 0.1% of DMSO.
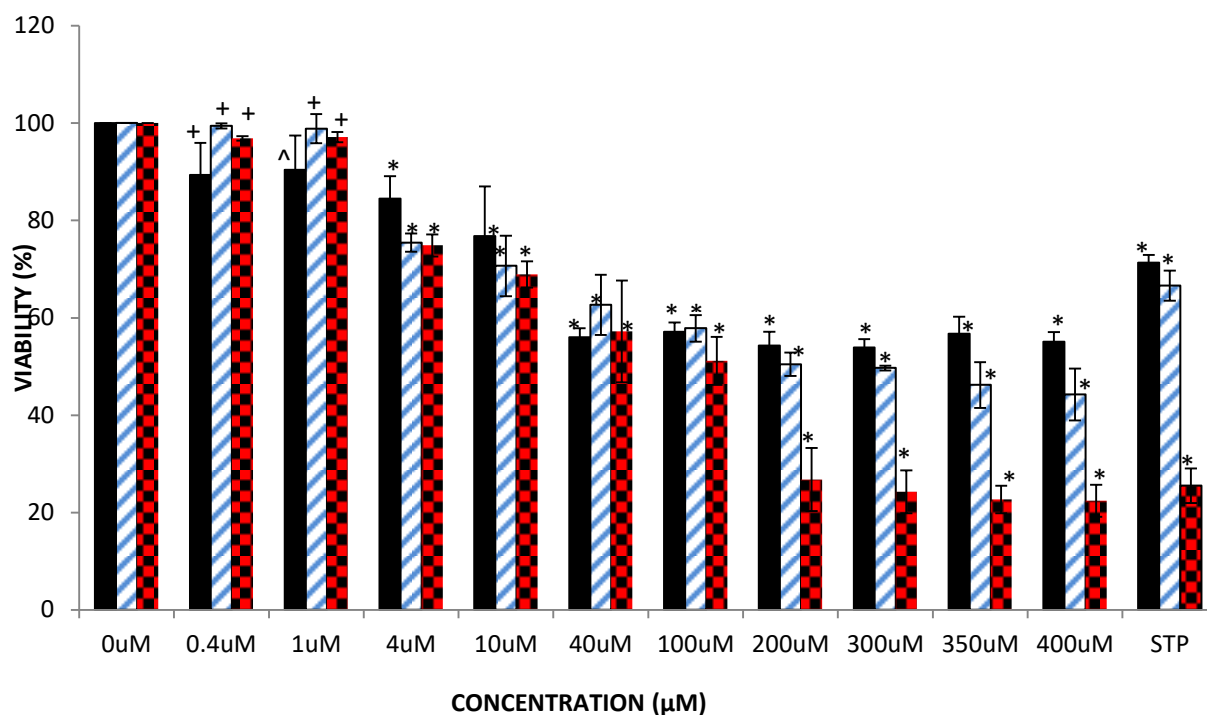
Figure 5.6: Time-dependent decrease in the viability of cells treated with benzbromarone for all treatment durations. STP = staurosporine (1µM), used as positive control and 0µM concentration is the negative control which contains 0.1% of DMSO. From 200µM concentrations and higher, there was a significant reduction (p<0.05) in viability at 12 hours compared to 30 minutes and 3 hours. Also, at 12hours there was a significant decrease (p<0.05) in viability of AGS cell treated with 1µM of the positive control staurosporine. *indicates statistically significant reduction in cell viability (while +indicates no significant difference) at these doses compared to the negative control (0µM); solid bar= benzbromarone for 30 minutes, oblique line bar= benzbromarone 3 hours, dotted bar= benzbromarone 12 hours.

The results we observed in the caspase glo 3/7 assay when AGS cells were treated with concentrations of benzbromarone similar to those used for MTT assay, showed a dose-dependent increase in caspase activity. Also there was a high caspase activity in the staurosporine-treated cells confirming that the finding of a dose-dependent increase in caspase 3/7 activity of AGS treated with benzbromarone was not incidental. For each

concentration of benzbromarone, there was a statistically significant difference (p<0.05) in the caspase 3/7 activities of the cells pre-treated with ZVAD compared with those not pre-treated. These data are presented in figure 5.7.
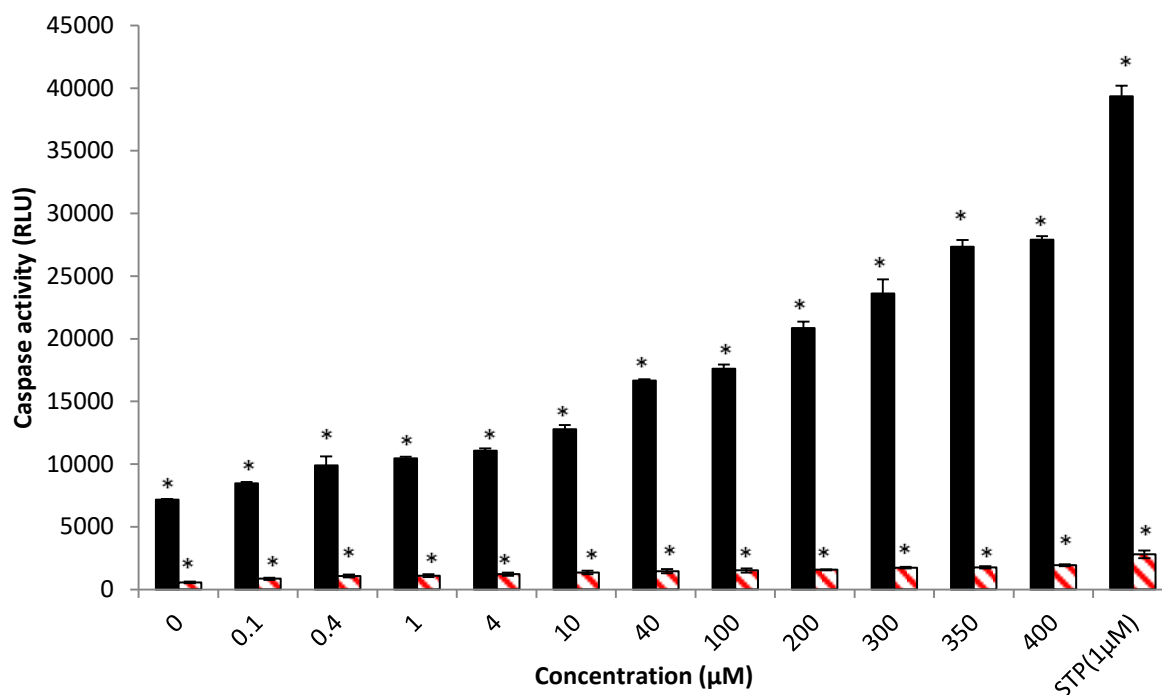
Figure 5.7: Graphical presentation of caspase activity in AGS cells treated with Benzbromarone with/without pre-treatment with ZVAD. There was a dose-dependent increase in caspase activity in the cells that were treated with benzbromarone, while there was significant difference (p<0.05) in the caspase activity of cells pre-treated with ZVAD and those not pre-treated. STP= Staurosporine as negative control; solid bar=benzbromarone without Zvad and oblique bar= benzbromarone with Zvad were all 12 hours assays.

5.3.6 Modulating effect of EYA1 inhibitor, benzbromarone on aspirin-related AGS death.

To determine the role of EYA1 inhibition on AGS cell death related to aspirin exposure, we pre-treated AGS cells with 1, 2.5 and 5 µM of benzbromarone for 3 hours prior to dosing the cells with aspirin for 12 hours. At all test doses, there was no significant (p>0.05) difference

in the viability of cells pre-treated with benzbromarone and the control. These are

presented in in figures 5.8-5.10.
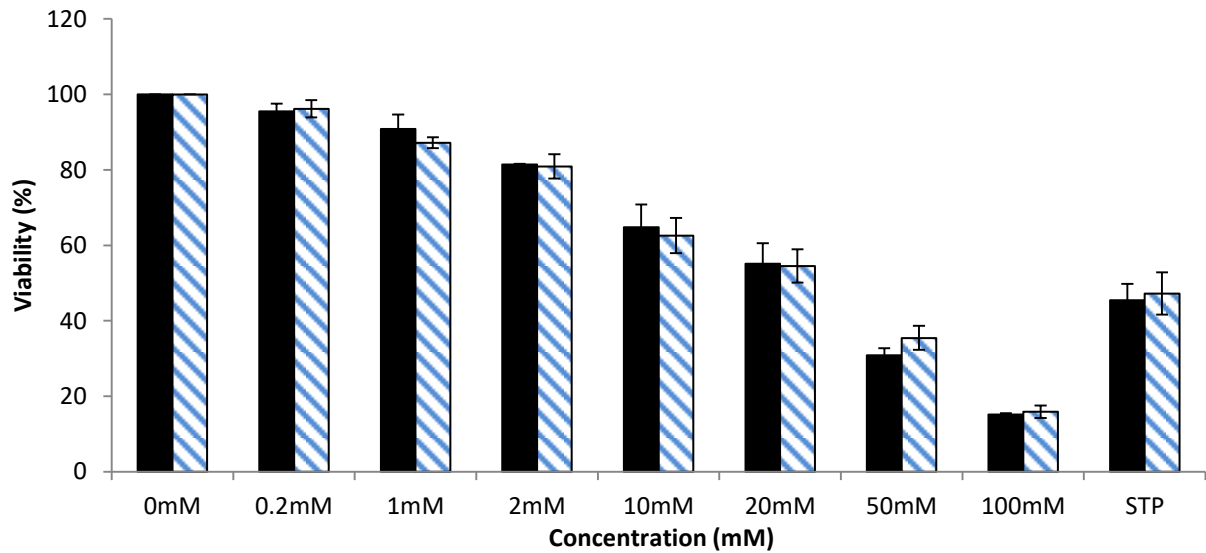
Figure 5.8: Pre-treating the cells with 1µM benzbromarone for 3 hours prior to aspirin exposure for 12 hours did not cause a significant decrease in the viability of aspirin-treated AGS cells. Solid bar= aspirin only 12 hours assay; dotted bar= benzbromarone only 12 hours assay; STP= staurosporine as negative control.
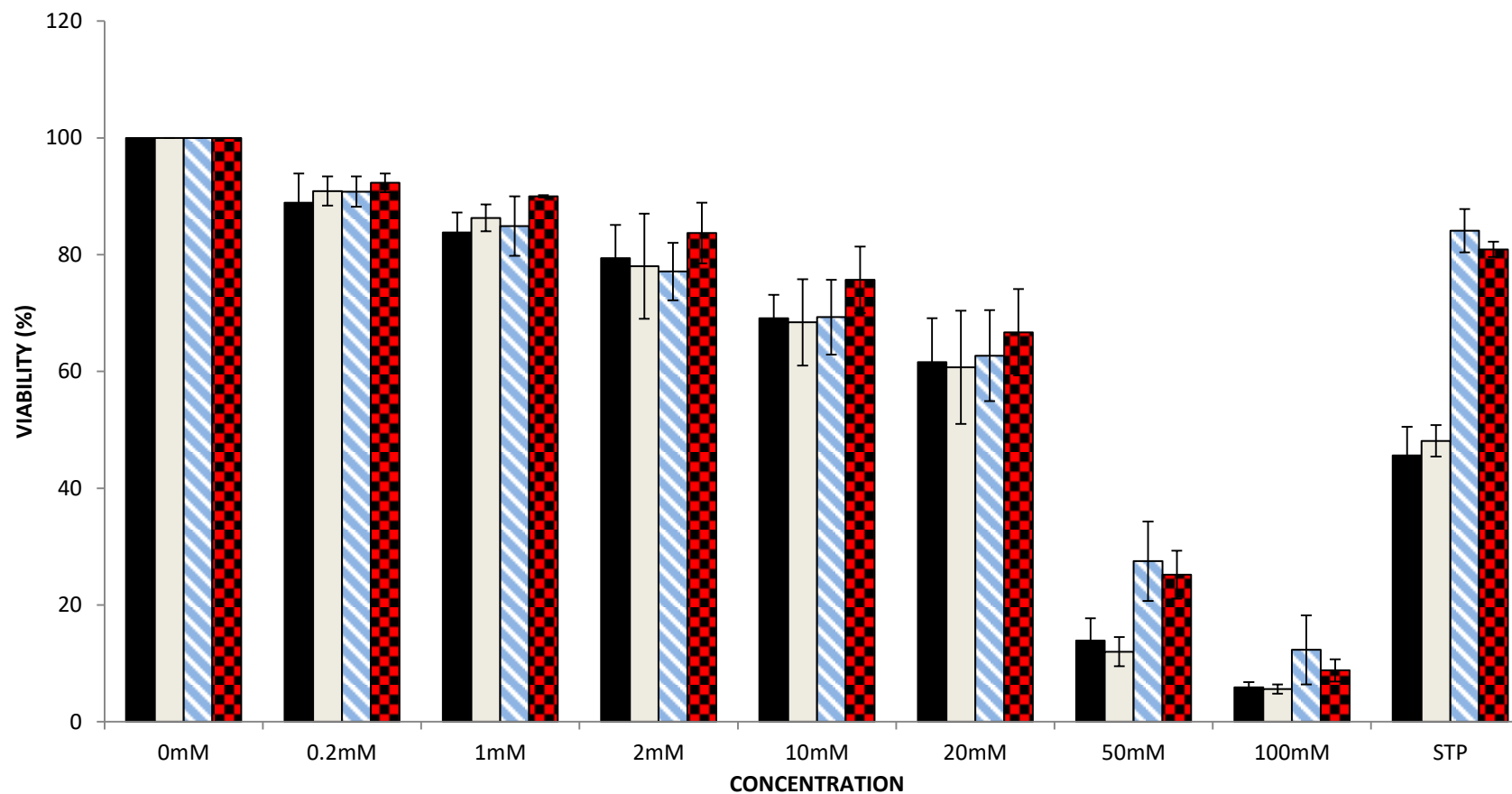
Figure 5.9: Pre-treating the cells with 2.5µM benzbromarone for 3 hours prior to aspirin exposure for 3 and 12 hours respectively, did not reveal any significant (p>0.05) effect in the ability of benzbromarone to modulate aspirin-related toxicity in AGS cells. Bars from left to right are aspirin only for 12 hours, aspirin and 2.5µM benzbromarone for 12 hours, aspirin only 3 hours, aspirin with 2.5µM benzbromarone for 3 hours.
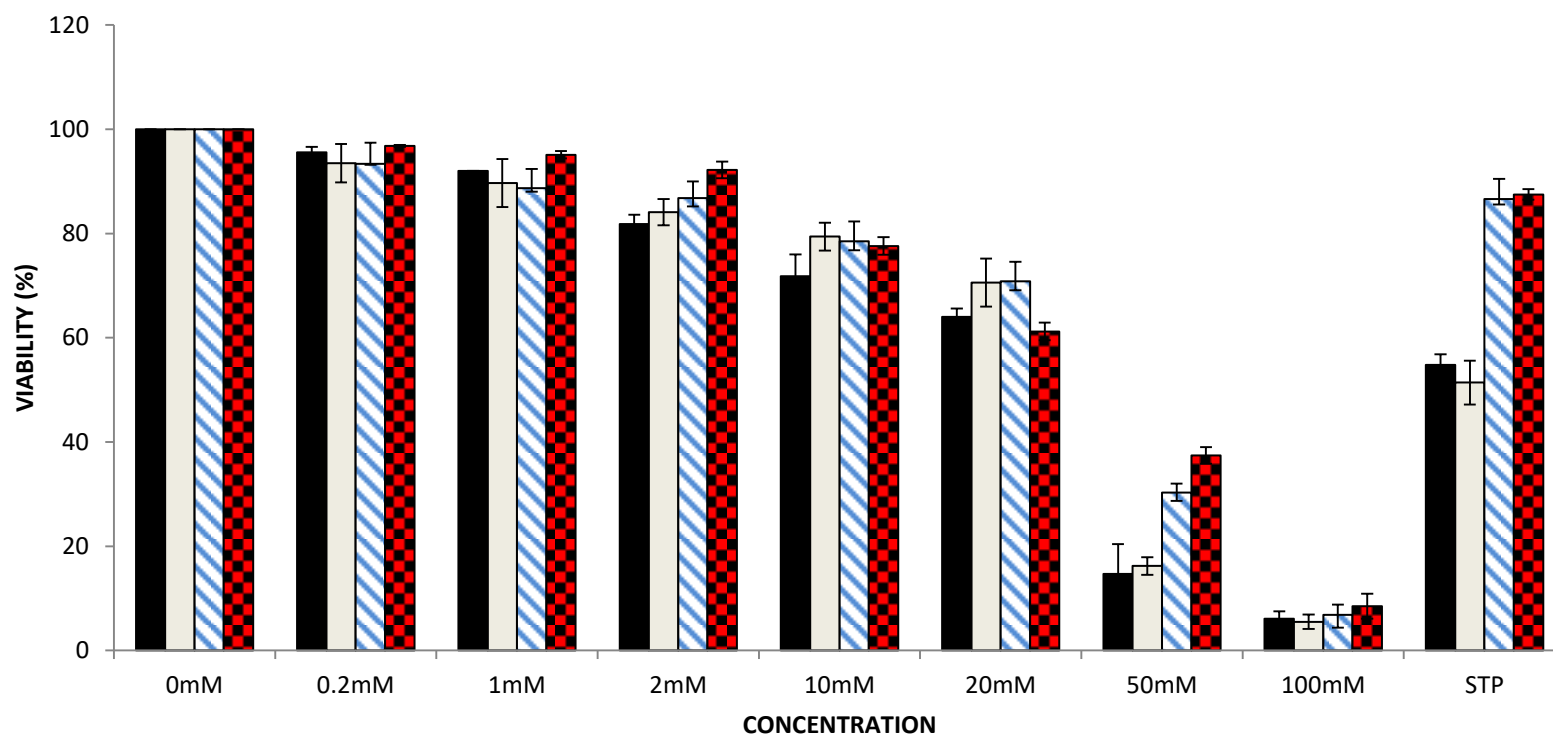
Figure 5.10: Pre-treating the cells with 5µM benzbromarone for 3 hours prior to aspirin exposure for 3 and 12 hours did not cause a significant decrease in the viability of aspirin-treated AGS cells, STP= staurosporine as positive control; bars from left to right are aspirin only for 12 hours, aspirin and 5µM benzbromarone for 12 hours, aspirin only 3 hours, aspirin with 5µM benzbromarone for 3 hours.

We then further explored the effect of EYA1 inhibition by benzbromarone on the caspase activity of AGS cells pre-treated with benzbromarone for 3 hours prior to aspirin exposure for 12 hours. The results of the experiments showed a decrease in caspase activity with increasing doses of aspirin but pre-treatment with benzbromarone did not alter this trend. However, this difference in caspase activity between ZVAD-treated cells and the untreated cells was lost at 50 and 100mM during the 12 hours treatment (Figure 5.11).
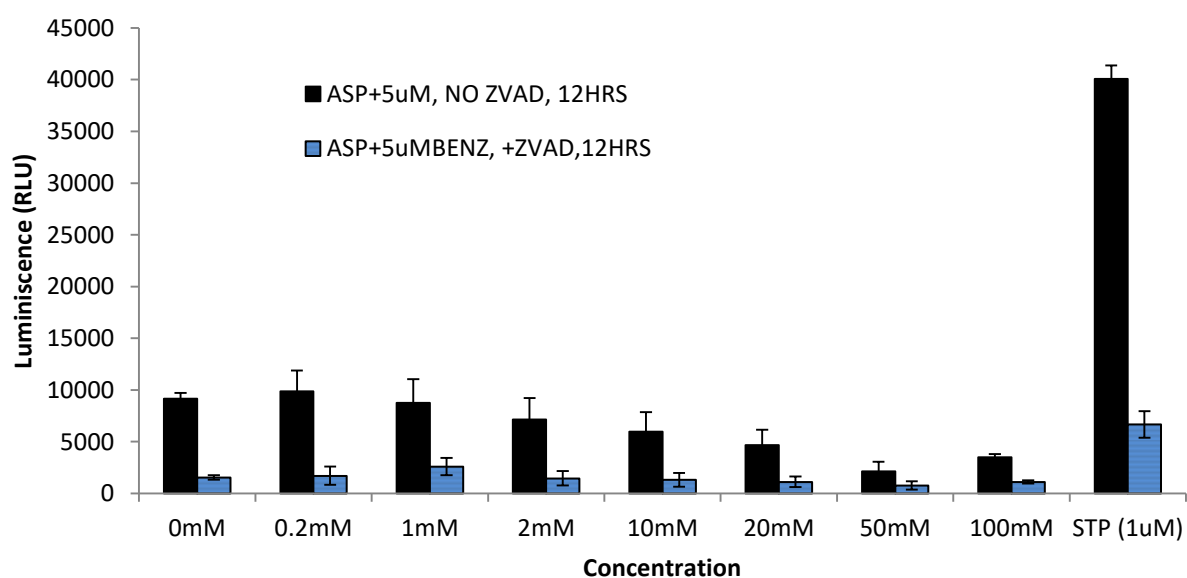
Figure 5.11: Dose dependent-decrease in caspase activity with increasing dose of aspirin in AGS cells pre-treated with 5µM of Benzbromarone, to inhibit EYA1. Staurosporine treatment for 12 hours is associated with significant increase in caspase activity compared with the cells that were pre-treated with caspase inhibitor, ZVAD. The significant difference in the caspase activity between ZVAD-treated cells and the untreated cells is lost at 50 and 100mM during the 12 hours treatment. Solid bar= aspirin+5µM benzbromarone, no Zvad, 12 hours assay; narrow horizontal bars= aspirin+5µM benzbromarone with Zvad 12 hours assay.

5.3.7 Validating the cell death pathway of aspirin-related cell death in AGS by PARP-cleavage using western blotting and cell morphology.

The finding of reduced viability of AGS cells and the aspirin-related reduction in caspase activity suggests that the cell death may not be caspase dependent. Hence, to investigate the cell death pathways involved, we decided to probe for proteins which are markers of apoptosis. One such protein is poly (ADP) ribosyl polymerase (PARP), an 89kDa protein cleaved from the intact protein between ASP214 and GLY215 residue by caspase 3 following activation of apoptosis. This revealed that PARP cleavage in aspirin-induced apoptosis is dose-dependent: we did not detect cleaved PARP at 0mM and 50mM, but this was detected at 10mM and 20mM, the positive control, and the 1µM staurosporine (figure 5.12).

This supports and also explains our earlier finding of higher caspase activity at lower doses of aspirin, than at 50mM and 100mM aspirin concentrations, suggesting that there is a role for caspase enzymes in aspirin-related cell death at lower doses but at higher doses, other modes of cell death that are non-caspase dependent come into play.

Cell morphology is shown in figure 5.13 A-E: this shows features of necrosis at 50mM (figure 5.13 D) and 100mM (figure 5.13 E) aspirin concentrations compared to lower aspirin concentration and the negative control (0mM). This is consistent with reports of various cell death mechanisms being involved in aspirin related-GI injury (Dikshit et al., 2006; Leung et al., 2009; Power et al., 2004).
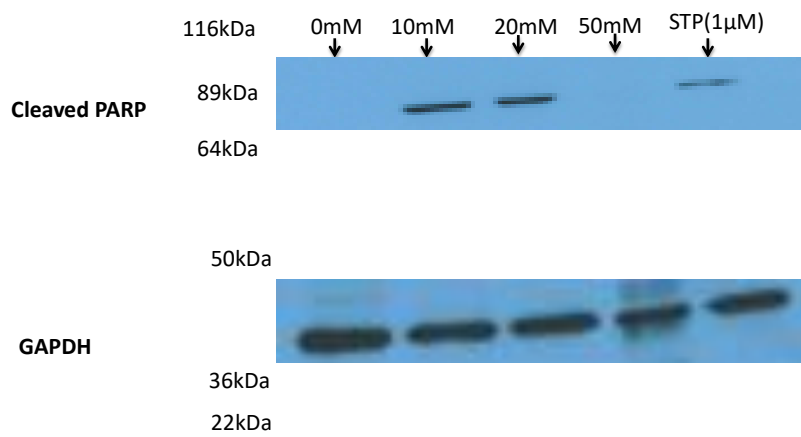
Figure 5.12: Western blots showing the 89kDa cleaved PARP in the AGS cells treated with staurosporine and aspirin at 10 and 20 mM but not at 0 and 50mM. The intact PARP (116kDa) was not detected. The lower blot shows the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detected for all concentrations of aspirin.
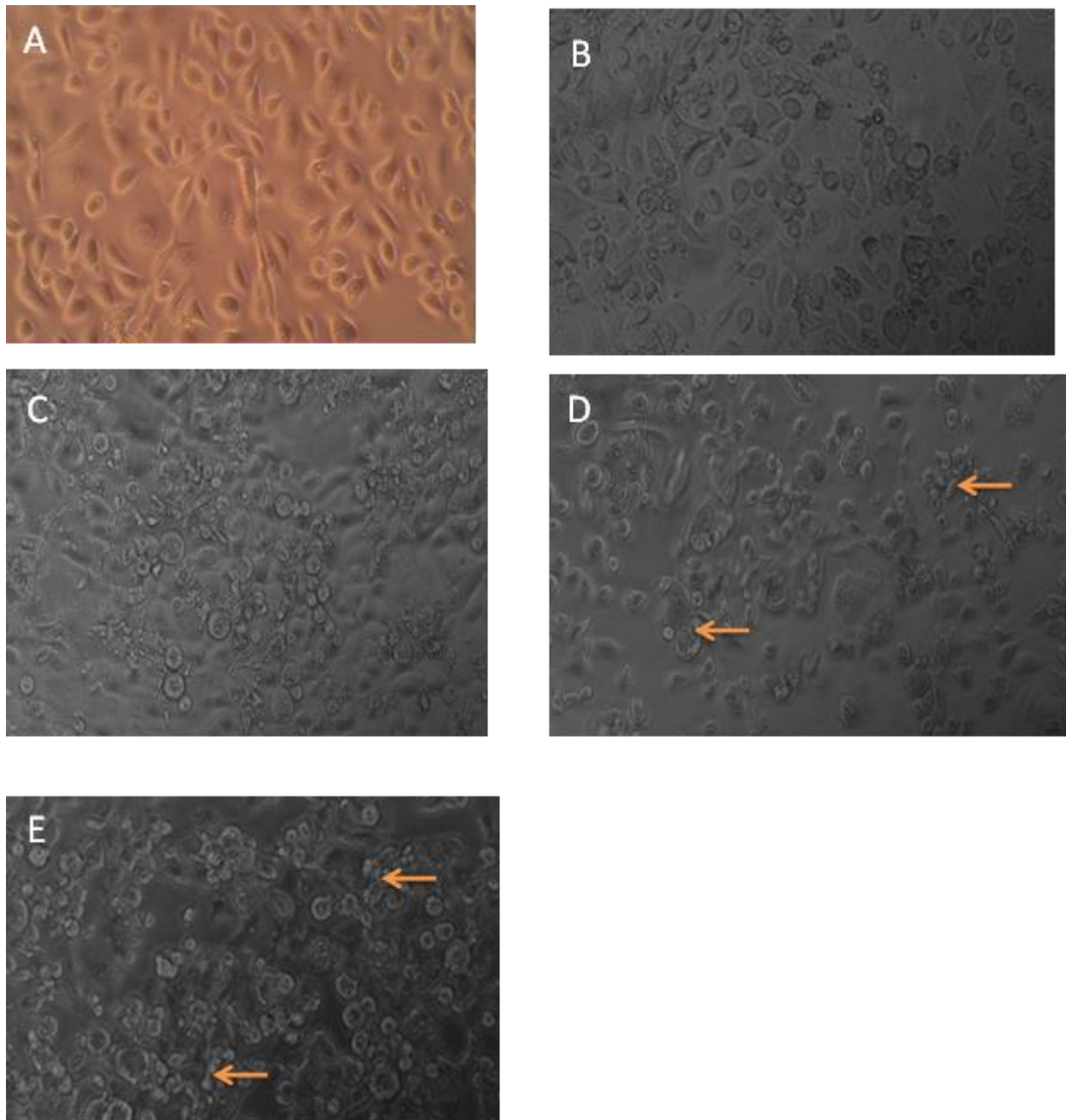
Figure 5.13: Micrographs of AGS cells (x20) exposed to (a) 0mM; (b) 10mM; (b) 20mM; (d) 50mM; and (e) 100mM aspirin. a) Viable cells with an intact plasma membrane and normal morphologic features; b and c) there are cells showing apoptotic morphology, with intact cell membrane and few necrotic cells (arrows); d and e) increasing presence of cells with evidence of cell swelling, loss of plasma membrane integrity and cellular disintegration, markers of necrosis.

5.4 Discussion

We have examined the mechanism of cell death of AGS cells treated with aspirin and observed that the viability of AGS cells reduces in a dose-dependent manner irrespective of the duration of treatment with aspirin. The likely explanation for this is that passage of aspirin into the cells in an acidic pH is enhanced; therefore, aspirin achieves a high intracellular concentration rapidly, enough to cause increased membrane permeability and damage by luminal hydrogen ions (Schoen & Vender, 1989) even within 30 minutes of exposure.

Similar to earlier reports that 2 tablets of aspirin dissolved in 100mls of water has a pH as low as 2.5 (Ivey, 1986), we note that 100mM of aspirin in complete culture media has a pH of 3.98, which brings the pH of the culture media to a level similar to the physiologic pH in the stomach, i.e. 2.5 (Schoen & Vender, 1989). Aspirin , as a weak acid has a pKa of 3.5 and exists in the non-ionized state in the acid environment of the stomach, but readily diffuses into the mucosal cells and in the intracellular pH of 7, is able to dissociate rapidly, leading to 'ion trapping' in the relatively more water soluble ionized form (Schoen & Vender, 1989).

The lack of the adaptive cytoprotection (Boku et al., 2001) as occurs in the *in vitro* cell model makes the cells more prone to direct injury from exposure to aspirin compared to the intact gastric epithelium. The roles of adaptive cytoprotection in preventing gastric epithelial damage have been discussed in chapter 1.

Various modes of aspirin-related apoptotic cell death have been reported (Gu et al., 2005; Power et al., 2004; Redlak et al., 2005) with some studies suggesting a non-caspase dependent mechanism (Leung et al., 2009). Similar to the report of a non-caspase

dependent mechanism underlying aspirin-related cell death; we have observed a reduction in caspase 3 and 7 activities in the AGS cells treated with aspirin in a dose-dependent manner, in a fashion that correlates with the increasing proportion of morphologically necrotic cells with increasing aspirin concentration. We found higher caspase activity in the negative control as well as at a lower aspirin concentration than in the highest aspirin concentration (100mM) used in our experiments suggesting that there are other mechanisms of cell death at play.

A possible explanation is that aspirin has been observed to cause inhibition of caspase activation; pre-treatment of AGS cells with aspirin completely prevented caspase 3, 6 and 9 activation following treatment with deoxycholate, an inducer of apoptosis in gastric epithelium (Redlak et al., 2008). Further to this, benzbromarone caused a dose dependent increase in caspase activity, which was lost when benzbromarone was co-incubated with aspirin.

The significant difference in caspase activity of the ZVAD treated cells and the non ZVAD pre-treated cells that were exposed to aspirin suggests that there is still some level of caspase 3 and 7 activity, and this caspase inhibition by aspirin cannot be said to be complete in our model. This finding is in agreement with reports that caspases are activated in virtually all forms of programmed cell death, and the process of caspase activation is not the sole determinant of life and death decisions in programmed cell death (Belmokhtar et al., 2001).

Furthermore, inhibition of caspase activity with zVAD did not show any difference in the viability of AGS cells treated with aspirin for 12 hours in both the zVAD pre-treated cells and the non-pre-treated cells. This implies that caspase inhibition may not always 'rescue' AGS

cells exposed to DNA double strand breaking stimuli, such as aspirin toxicity, from cell death. This observation corroborates reports that caspase inhibition does not necessarily prevent cell death, neither does its activation necessarily cause cell death (Perfettini & Kroemer, 2003); rather , caspase inhibition only shifts cells to a non-caspase dependent self-destructive mode of cell death via the mitochondrial pathway (Leung et al., 2009).

Various postulates behind the inability of caspase inhibition to confer complete protection against cellular injury include an unidentified inhibitor, such as zVAD, though able to block most caspases, may not be able to effectively neutralize all caspases (Lavrik et al., 2005). Secondly, there may be an inherent difficulty in obtaining high enough fractional inhibition to confer complete protection (Leung et al., 2009). The above findings suggest that besides caspase activation, there could be other biological processes involved that would explain aspirin-related gastric cell death that is wholly or partly caspase independent and is unable to be prevented by caspase inhibition (Budihardjo et al., 1999; Leung et al., 2009).

Moreover, mitochondrial toxicity following ion trapping leading to increased mitochondrial membrane permeability, which cannot be prevented by caspase inhibition, leads to release of mitochondrial proteins which are drivers of cell death pathways (Leung et al., 2009). Caspases are known to initiate the intracellular events that lead to apoptosis and one such event is the demolition of nuclear DNA via histone associated fragmentation (Nuñez et al., 1998; Oberhammer et al., 1993).

The apoptosis inducer staurosporine was relatively ineffective in causing reduction in cell viability at 30mins and 3 hours compared to 12 hours. At 30 minutes, 10µM of benzbromarone was equivalent to 1 µM of staurosporine in terms of its propensity to cause reduction in cell viability. To further buttress the above findings, the caspase 3 and 7

activities at 30 minutes and 3 hours were significantly ($p<0.05$) lower than at 12 hours. This corroborates the finding of a significant reduction in cell viability associated with treatment with staurosporine at longer time intervals than at shorter time intervals (Boulares et al., 1999). Hence unlike the aspirin-induced decline in AGS cell viability which is concentration-dependent, we note a significant time-dependent effect of staurosporine. Staurosporine toxicity has been reported to be time-dependent based on the type of cell line tested. It was observed to show a delay of up to 12 hours in causing cell death in one subline of mouse lymphocytic cell line, whereas significant cell death was noted after 3 hours in another mouse lymphocytic subline, and this has been attributed to the intactness of the caspase activation pathway (Belmokhtar et al., 2001).

Benzbromarone has been reported to cause inhibition of EYA in various experimental models. While exploring the plausible role of *EYA1* in apoptosis induced by the treatment of AGS cells with aspirin, we pre-treated AGS cells with the *EYA* chemical inhibitor benzbromarone, with the expectation that the inhibition of EYA1 would cause an increased cell death. Though we observed some decline in the cell viability of AGS cells pre-treated with 1 µM of benzbromarone at aspirin concentrations of 1-20mM during a 30 minutes treatment, this reduction in cell viability was not statistically significant ($p>0.05$). As we did not confirm inhibition of EYA by benzbromarone (Tadjuidje et al., 2012) *in vitro,* it becomes hard to tell whether this finding is truly the effect of EYA inhibition or due to the toxicity of the inhibitor. Of course, we also do not know whether benzbromarone has off-target effects.

To further explore the exact cell death mechanism involved in the observed aspirin-induced cell death, we probed the cleavage of PARP. AGS cells were treated with 10, 20 and 50mM

of aspirin, and 1µM staurosporine, including a negative control for 12 hours. The cell lysate was probed for PARP cleavage. Cleaved PARP on western blot was noted for 1µM staurosporine, 10 and 20mM aspirin but there was no PARP cleavage at 0mM and 50mM aspirin.

PARP, in the presence of NAD, catalyses the poly(ADP-ribosyl)ation of a number of nuclear proteins and its activation depends on binding to the ends of DNA strand breaks, with a possible depletion of cellular NAD and ATP (Berger, 1985; Berger & Petzold, 1985). Typically, in response to cell death, PARP is cleaved primarily by caspase 3 into two components; the 85kDa and the 24kDa subunits that contains the active site and the DNA binding domain of the enzyme, respectively, during drug-induced apoptosis in a variety of cells (Kaufmann et al., 1993; Nicholson et al., 1995; Tewari et al., 1995). This cleavage occurring between Asp214 and Gly215, is thought to prevent the depletion of intracellular ATP and NAD, thereby rescuing the cell from cell death (Berger, 1985). This caspase 3 cleavage of PARP is very well conserved in the PARP protein even in very distant species, underpinning the essential role of PARP cleavage in apoptosis (Rhun et al., 1998; Tewari et al., 1995).

The inability to demonstrate PARP cleavage, the very low caspase activity at 50mM aspirin concentration, the mixed morphotypes at 20mM (Northington et al., 2007) and the morphologic changes similar to that occurring during necrosis (figure 5.15, c and d) suggests that necrosis, rather than apoptosis is the cell death mechanism at play at higher aspirin concentrations. This is rather a local toxic effect associated with increased intracellular aspirin concentration and ion trapping leading to cell necrosis (Scheiman, 1996).

Our study has demonstrated that physiologic concentrations of aspirin cause apoptosis in a dose-dependent manner and at the high end dose similar to that used in the management

of mild musculoskeletal disorders; necrosis rather than apoptosis comes into play. Moreover, there are various other processes involved in the aspirin related cell death with PARP activation being one such mechanism.

The strengths of our study are that we have utilised validated screening methods in identifying the effect of aspirin on AGS cells and then went further to examine the exact cell death mechanism involved using specific cell death pathway assays. Secondly, we have used a validated *in vitro* gastric cell model in performing our experiments, as this will make translation of data from this study to clinical setting more acceptable (Hall et al., 2006).

The limitation of the MTT assay is that similar to ATP based assays, a number of other factors can inhibit mitochondrial reductases, and hence the conversion of tetrazolium salts is not a conclusive evidence of cell proliferation. It is also very susceptible to metabolic interference and may lead to false positive results; hence, *bona fide* markers of cell death must be used. This was however improved on by the use of further cell death validation assays to confirm our findings. The second limitation of our study, as earlier noted, was that we did not confirm benzbromarone inhibition of EYA (Tadjuidje et al., 2012). Thirdly, an *EYA* knockout experiment *was* not performed; this would have produced stronger evidence of loss of EYA function than chemical inhibition alone.

In conclusion, we have been able to demonstrate that there are multiple mechanisms at play in the cell death pathway associated with use of aspirin. One such mechanism is PARP cleavage, but there is still a role for caspase-dependent apoptosis depending on the concentration of aspirin used. Moreover, at very high concentrations there is a switch from an apoptotic cell death to necrosis.

Chapter 6

Final Discussion

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly used medications with an annual prescription of over 30 million for aspirin alone in England (National Health Service information centre, 2007). Also availability of these medications over the counter makes them more easily accessible than most other medications. NSAIDs are commonly used in the elderly in whom concomitant use of other drugs could increase the gastrointestinal toxicity of NSAIDs.

Gastrointestinal complications, related to NSAID use have been estimated to cause 3500 hospital admissions and 400 deaths per annum in the UK in those aged over 60 years, while over 60% of deaths from ADRs involving 1225 patients were attributable to NSAIDs (Langman, 2001; Pirmohamed et al., 2004). As revealed in our analysis, most of the patients with NSAID-related ulcers are in the older age group (>65years) who also have other co-morbidities and take concomitant medications that could significantly increase their risk of NSAID-related gastrointestinal complications. We know that age, co-morbidity and concomitant medications increase the risk of NSAID-related GI complication (Deighton et al., 2009; Valkhoff et al., 2012).

We did not show an association between the *UGT1A6* SNP rs1105879 and development of NSAID-related ulceration (p=0.052, odds ratio 1.21, 95% CI 1.00, 1.47), although this just missed statistical significance. This observation of a trend toward an association could be the result of the confounding effect of *H. pylori* infection, because following sensitivity analysis excluding cases with evidence of *H. pylori* infection negated any signal of association. Furthermore, excluding non-aspirin NSAID users from the analysis did not change the finding of a lack of association between *UGT1A6* SNP rs1105879 and NSAID-induced GI complications. Our finding is contrary to our expectation because a loss-of-

function polymorphism in UGT1A6, an important enzyme in the phase II metabolism of aspirin would be expected to lead to high plasma concentration of salicylic acid and ultimately to increased aspirin-related GI toxicity (Carlini et al., 2005; García-Martín, 2008). Previous studies aimed at establishing an association between *UGT1A6* SNP and NSAID-induced GI complications have reported no association (Shiotani et al., 2008; Shiotani et al., 2009; Van Oijen et al., 2005a; Van Oijen et al., 2009b).

Because of the trend toward association we observed in our study, and the small sample sizes of the 2 other previous studies in this area, we conducted a meta-analysis including our data and the 2 previous studies that passed our inclusion criteria; again there was no association (P=0.31, OR= 1.11, 0.91, 0.36) between this loss-of-function polymorphism and aspirin/NSAID-induced gastrointestinal complications. It appears safe to conclude that in terms of polymorphisms of aspirin/NSAIDs metabolizing enzymes, polymorphisms of *UGT1A6* are either not relevant genetic risk factors for the development of aspirin/NSAID-related GI complications or the effect size is very small which would require much larger sample sizes to uncover.

By contrast, polymorphisms in CYP2C, a cytochrome P450 enzyme involved in the metabolism of the acetylsalicylic acid and other NSAIDs led to a statistically significant association between this polymorphism and NSAID-related GI complications (Carbonell et al., 2010; Figueiras et al., 2016). Polymorphic expression of *UGT1A6* gene in the human gastric epithelium has been reported (Strassburg et al., 1998) and this could explain the isolated finding of an association between this SNP and duodenal ulcers (p=0.002) and a lack of association between gastric ulcers (p=0.302) and *UGT1A6* SNP. However, a sensitivity analysis excluding *H.pylori* infected cases resulted in the loss of this association between

duodenal ulcers and *UGT1A6* rs1105879 SNP. A limitation of this work is the small sample size. Also, because of a dearth of publications on this subject, we added studies conducted in non-Caucasian populations in our meta-analysis and this could introduce some variability in our study cohorts.

We have examined the role of a functional promoter polymorphism (rs1799889) of Plasminogen activator inhibitor gene (*PAI-1*) in NSAIDs-related gastrointestinal toxicity. There are reports of increased fibrinolytic activity in patients with bleeding peptic ulcers, which is partially decreased by increased plasma PAI-1 levels and by acid suppression (Vreeburg et al., 2001).

PAI-1 is believed to play a role in the protection of the gastric mucosa; there is increased expression of the plasminogen activator and increased fibrinolytic activity of tPA from biopsy specimens of peptic ulcer lesions with associated significantly elevated PAI-1 levels in the plasma of gastric ulcer patients with active ulcers compared to patients with inactive ulcers (Kurose et al., 1990). Also, it has been reported that in the presence of irritants of the gastric mucosa such as aspirin/NSAIDs and *H. pylori* infection, there is increased expression of PAI-1 mRNA, PAI-1, uPA and uPAR with associated inhibition of gastric epithelial proliferation (Kenny et al., 2008; Kenny et al., 2013a).

Therefore, one would speculate that polymorphisms of *PAI-1* rs1799889 SNP could be associated with an increased risk of NSAID-induced GI complications. Contrary to this, we could not find any association between *PAI-1 5G/4G* polymorphism and NSAID-related GI complications, despite a significantly higher frequency of the variant 4G allele, the occurrence of which did not differ between the case and control groups. Previous reports have associated the variant 4G/4G allele with higher plasma PAI-1 levels (Festa et al., 2003;

Lijnen & Collen, 1995a), and this higher plasma PAI-1 levels we speculated could compensate for the reduced activity of PAI-1, a phenomenon that could explain the lack of association between this SNP and NSAID-induced GI complications. NSAIDs and PPI cause increased PAI-1 expression in humans (Kenny et al., 2013a; Nørsett et al., 2011); as these medications were used by our cohorts it could have confounding effect on the interaction between this SNP and the ulcer status and may likely explain the observed lack of association.

This speculation is validated by the observation that introducing the non-use of NSAIDs as an interaction term in our analysis showed some association (p=0.099), though not statistically significant. Furthermore, available evidence supports an interference of *H. pylori* with the activity of PAI-1 and this is thought to modulate the role of PAI-1 in gastric mucosal protection (Kenny et al., 2008). Finally, the entire cohort was not genotyped because of lack of DNA samples and this could also be a possible reason for the observed lack of association.

Following the identification of *EYA1* as the "top hit" in the NSAIDs GWAS study, we examined the role of a functional polymorphism of *EYA1* in the pathogenesis of NSAID-related GI complications. We reported a significant association between binary ulcer status and *EYA1* SNP in a case-control analysis of patients with evidence of NSAID use. This finding is novel and very significant in that it has validated the report of the GWAS on NSAID-related GI complications. However, when the case cohort was expanded to include patients without evidence of use of NSAIDs, the significant association we detected earlier was negated. We speculate that the loss of association was likely due to a confounding effect of the inclusion of patients without any evidence of NSAID use in the case.

To further explore the functional consequence of this SNP association, we utilised an *in vitro* model using an immortalised gastric adenocarcinoma cell line (AGS). Here, we initially sought to determine whether aspirin exposure was associated with increased AGS cell death, and found that exposure of AGS cells to aspirin was associated with significant cell death ($p<0.05$) at concentrations <2mM. Also to model a loss of function *EYA1* polymorphism, we pre-treated AGS cells with an *EYA1* chemical inhibitor prior to treatment with various concentrations of aspirin, this data was not statistically significant (see section 5.3.6).

Furthermore, we also examined the difference, if any, in *EYA1* gene expression based on genotype in healthy human gastric biopsy samples. The mean *EYA1* gene expression among carriers of the wild type allele (AA) was not different from those carrying the variant allele (AT/TT). This data contrasts with the findings of *EYA1* RNA sequencing data and the rs12678747 genotype in gut biopsy tissue from ulcer and antrum tissue in NSAIDs ulcer patients (Carr et. al., 2011-unpublished data). This may suggest that the SNP shows functional effects in relation to diseased tissues rather than normal tissue. We of course do not know whether the SNP is truly the causal variant, or whether it is in linkage disequilibrium with another functional variant.

We also noted a significant association between NSAIDs ulcers in the presence of *H. pylori* infection, and *EYA1* SNP with/without covariates. *H. pylori* is associated with changes in the expression of the serine/threonine tyrosine kinases and transcription factors (Backert et al., 2005; Backert & Selbach, 2005) and this could possibly explain the effects we have seen in the presence of *H. pylori* infection. It would be worthwhile to examine the exact modulating role of this infection on the expression of *EYA1* gene. This is important because 54.4% and

14.9% of the cases and controls respectively were positive for *H. pylori* infection. We have earlier highlighted the interaction of *H. pylori* with NSAID use, serine/threonine tyrosine kinases and transcription factors, including the modulating role of this infection on the effect of *PAI-1* on gastric epithelial protection and proliferation. These multi-level interactions of *H.pylori* with both genetic and non-genetic factors in our study would have significant confounding effect on our data.

Another intriguing finding from our data was that in the gene expression assay, we observed that the expression of the endogenous control gene, β-actin, was significantly higher ($p<0.05$) than the expression of the *EYA1* in AGS cells; however, at the protein level we noted that the expression of EYA1 and β-actin was almost equivalent (0.75). Thus, despite a low expression of the gene, the protein expression is significantly up-regulated but the mechanisms of this are unclear. Different mechanistic hypotheses of aspirin-related GI injury have been presented and current evidence suggest that the systemic effect of aspirin may be more dominant in the pathogenesis of NSAID-induced GI complications (Sostres et al., 2010); however we note that the topical/direct effect of aspirin may be more significant in the development of aspirin-related GI complications than is currently known.

Firstly, we reported that polymorphism of aspirin metabolizing enzyme, UGT1A6 is not relevant to the development of these complications. Secondly, from our data, 100mM of aspirin in complete media has a pH of 3.98, and AGS cells cultured in complete media (normal pH 8.3) in which the pH has been adjusted to 4 using HCL, had a reduction in the viability of the cells by over 40%, even without aspirin exposure. This finding supports our observation that 300mg of aspirin (commonly given for acute coronary syndrome), in the physiologic state, achieves an intra-gastric concentration of about 83mM (based on our

estimates), and If extrapolated from our cell models, this could cause about 30-40% reduction in the viability of the cells. Therefore, we speculate that the direct effect of aspirin on gastric epithelial cells viability may be more significant and more relevant than is currently thought.

Previous studies have reported various mechanisms of aspirin-induced cell death (Leung et al., 2009; Power et al., 2004; Redlak et al., 2005; Redlak et al., 2007; Redlak et al., 2008); this is consistent with our finding that there were multiple modes of aspirin-induced cell death ongoing concurrently in the cell model depending on the concentration of aspirin used in treating the cells. Specifically, contrary to previous observations of a caspase 9 dependent cell death mechanism (Power et al., 2004; Redlak et al., 2005), we found a significant dose dependent decrease in the caspase 3 and 7 activity of the cells despite a significant fall in the viability of the cells compared to the controls, suggesting that there is a role for non-caspase dependent mechanisms in the cell death. Furthermore, caspase inhibition with the universal caspase inhibitor ZVAD did not "rescue" the AGS cells from aspirin-related decrease in cell viability, supporting our speculation that aspirin-related cell death may not be wholly caspase dependent.

To further validate the cell-death mechanism involved in this assay, we probed for PARP, a marker for apoptosis and found that the 89kDa protein was cleaved in the aspirin-treated cells in a dose-dependent manner, with PARP cleavage occurring in the positive control (1µM staurosporine), 10 and 20mM aspirin but not at 0 and 50mM of aspirin. This suggests that at lower doses of aspirin, apoptotic cell death occurs while at high doses of aspirin, non-apoptotic cell death occurs. This is necrotic cell death as revealed by morphologic features in AGS cells treated with high doses of aspirin (see figure 5.13). These confirmed

our earlier speculation that there is multiple cell-death mechanisms involved in aspirin-related AGS cell death. Furthermore, our data also confirms that though apoptosis may be occurring, the exact apoptotic mechanism may be non-caspase 3/7 dependent.

We have looked at the study from clinical, molecular and functional points of view. Our study has reported several novel findings and validated some previous reports. For instance, we have been able to validate the NSAIDs GWAS report in which *EYA1* was the candidate gene implicated in the NSAID-related gastrointestinal toxicity. Our pharmacogenomic study was conducted using a larger sample size; sensitive study design and data analysis structure that should detect any association between these polymorphisms and NSAID-related GI complications. For instance our work on the role of polymorphism in *UGT1A6* and NSAID-related gastrointestinal complications used a sample size 4-7 folds larger than the sample size of similar studies published previously.

In conclusion, as is the case with most complex diseases, the pharmacogenetics of NSAID-related gastrointestinal complications is difficult to elucidate fully because not only are the phenotypes complex but there are multiple factors involved including environmental, life style, co-morbid diseases and other patient-specific factors. Further work is needed in this area so that we fully understand the mechanisms and develop predictive and preventive strategies to prevent NSAID-induced gastric ulceration. This is important as current preventative strategies, for example use of proton pump inhibitors is not fully successful, and these drugs are associated with serious adverse reactions. It is likely that aspirin and other NSAIDs will be much more widely used in the future because of the increasing age of our population, as well as the expanding indications of aspirin-NSAID, for example in cancer chemoprevention.

References

(2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447**:** 661-678.

Abbot FV, & Palmour RM (1988). Morphine-6-glucuronide. Analgesic effects and receptor binding profile in rats. Life Sci 43.

Agundez JAG, Martinez C, Dolores P-S, Carballo M, Torres MJ, & Garcia'-Martin E (2009). Pharmacogenomics in Aspirin Intolerance. Current Drug Metabolism 10**:** 998-1008.

Ajani UA, Ford ES, Greenland KJ, Giles WH, & Mokdad AH (2006). Aspirin use among U.S. adults: Behavioral risk factor surveillance system. American Journal of Preventive Medicine 30**:** 74-77.

Ajayi FO, Sun H, & Perry J (2000). Adverse drug reactions: A review of relevant factors. Journal of Clinical Pharmacology 40**:** 1093-1101.

Alhawassi TM, Krass1 I, Bajorek BV, & Pont5 LG (2014). A systematic review of the prevalence and risk factors for adverse drug reactions in the elderly in the acute care setting. Clinical Interventions in Aging 9**:** 2079-2086.

Anders MW (1980). Metabolism of drugs by the kidney. Kidney International 18**:** 636-647.

Anglin R, Yuan Y, Moayyedi P, Tse F, Armstrong D, & Leontiadis GI (2014). Risk of upper gastrointestinal bleeding with selective serotonin reuptake inhibitors with or without concurrent nonsteroidal anti-inflammatory use: A systematic review and meta-analysis. American Journal of Gastroenterology 109**:** 811-819.

Antman EM, DeMets D, & Loscalzo J (2005). Cyclooxygenase inhibition and cardiovascular risk. Circulation 112**:** 759-770.

Aoi M, Aihara E, Nakashima M, & Takeuchi K (2004). Participation of prostaglandin E receptor EP4 subtype in duodenal bicarbonate secretion in rats. American Journal of Physiology - Gastrointestinal and Liver Physiology 287**:** G96-G103.

Araki H, Ukawa H, Sugawa Y, Yagi K, Suzuki K, & Takeuchi K (2000). The roles of prostaglandin E receptor subtypes in the cytoprotective action of prostaglandin E2 in rat stomach. Alimentary Pharmacology and Therapeutics, Supplement 14**:** 116-124.

Arisawa T, Tahara T, Shibata T, Nagasaka M, Nakamura M, Kamiya Y, *et al.* (2007). Association between genetic polymorphisms in the cyclooxygenase-1 gene promoter and peptic ulcers in Japan. International Journal of Molecular Medicine 20**:** 373-378.

Arky R, Davidson CS, & Weld F (1998). Physician's Desk Reference, 52nd edMedical Economics Company: Montvale, NJ.

Aronson JK, & Ferner RE (2003). Joining the DoTS: New approach to classifying adverse drug reactions. British Medical Journal 327**:** 1222-1225.

Ayuso P, Blanca-López N, Doña I, Torres MJ, Guéant-Rodríguez RM, Canto G, *et al.* (2013). Advanced phenotyping in hypersensitivity drug reactions to NSAIDs. Clinical and Experimental Allergy 43**:** 1097-1109.

Backert S, Gressmann H, Kwok T, Zimny-Arndt U, König W, Jungblut PR, *et al.* (2005). Gene expression and protein profiling of AGS gastric epithelial cells upon infection with Helicobacterpylori. Proteomics 5**:** 3902-3918.

Backert S, & Selbach M (2005). Tyrosine-phosphorylated bacterial effector proteins: The enemies within. Trends in Microbiology 13**:** 476-484.

Baigent C, Blackwell L, Emberson J, Collins R, & Peto R (2009). Aspirin in the primary prevention of vascular disease - ATT secretariat's reply. The Lancet 374**:** 879.

Baron JA, & Sandler RS (2000). Nonsteroidal anti-inflammatory drugs and cancer prevention. In Annual Review of Medicine. pp 511-523.

Baudhuin LM, Miller WL, Train L, Bryant S, Hartman KA, Phelps M, *et al.* (2010). Relation of ADRB1, CYP2D6, and UGT1A1 polymorphisms with dose of, and response to, carvedilol or metoprolol therapy in patients with chronic heart failure. American Journal of Cardiology 106**:** 402-408.

Baumgartner HK, Starodub OT, Joehl JS, Tackett L, & Montrose MH (2004). Cyclooxygenase 1 is required for pH control at the mouse gastric surface. Gut 53**:** 1751-1757.

Bayramoglu A, Gunes HV, Metintas M, Degirmenci I, Guler HI, Ustuner C, *et al.* (2014). Plasminogen activator inhibitor-1 and susceptibility to lung cancer: a population genetics perspective. Genet Test Mol Biomarkers 18**:** 587-590.

Becker ML, Pearson ER, & Tkáč I (2013). Pharmacogenetics of oral antidiabetic drugs. International Journal of Endocrinology 2013.

Becker ML, Visser LE, Trienekens PH, Hofman A, Van Schaik RHN, & Stricker BHC (2008). Cytochrome P450 2C9 *2 and *3 polymorphisms and the dose and effect of sulfonylurea in type II diabetes mellitus. Clinical Pharmacology and Therapeutics 83: 288-292.

Belmokhtar CA, Híllion J, & Segal-Bendirdjian E (2001). Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms. Oncogene 20: 3354-3362.

Belsey J (2010). The cost impact of NSAID-induced gastrointestinal adverse events. Managing Pain in Practice 1: 3-11.

Berg A, Redéen S, Grenegård M, Ericson AC, & Sjöstrand SE (2005). Nitric oxide inhibits gastric acid secretion by increasing intraparietal cell levels of cGMP in isolated human gastric glands. American Journal of Physiology - Gastrointestinal and Liver Physiology 289: G1061-G1066.

Berger NA (1985). Poly(ADP-ribose) in the cellular response to DNA damage. Radiation Research 101: 4-15.

Berger NA, & Petzold SJ (1985). Identification of minimal size requirements of DNA for activation of poly(ADP-ribose) polymerase. Biochemistry 24: 4352-4355.

Berndt G, Grosser N, Hoogstraate J, & Schröder H (2005). AZD3582 increases heme oxygenase-1 expression and antioxidant activity in vascular endothelial and gastric mucosal cells. European Journal of Pharmaceutical Sciences 25: 229-235.

Berridge MV, Herst PM, & Tan AS (2005). Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. In Biotechnology Annual Review. pp 127-152.

Bessone F (2010). Non-steroidal anti-inflammatory drugs: What is the actual risk of liver damage? World Journal of Gastroenterology 16: 5651-5661.

Bigler J, Whitton J, Lampe JW, Fosdick L, Bostick RM, & Potter JD (2001). CYP2C9 and UGT1A6 Genotypes Modulate the Protective effect of Aspirin on Colon Adenoma Risk. *Cancer Research* 61: 3566-3569.

Reference section

Bjarnason I (2013). Gastrointestinal safety of NSAIDs and over-the-counter analgesics. International Journal of Clinical Practice 67**:** 37-42.

Bjarnason I, Scarpignato C, Takeuchi K, & Rainsford KD (2007). Determinants of the short-term gastric damage caused by NSAIDs in man. Alimentary Pharmacology and Therapeutics 26**:** 95-106.

Bloom BS (1988). Direct medical costs of disease and gastrointestinal side effects during treatment for arthritis. The American Journal of Medicine 84**:** 20-24.

Bock KW, & Kohle C (2005). UDP-Glucoronosyltransrease 1A6:Structural, Functional and Regulatory aspects. Methods in Enzymology 400**:** 57-75.

Bock KW, & Lilienblum W (1994). Roles of Uridine diphosphate glucoronosyltransferase in chemical carcinogenesis Handbook Exp Pharmacol 112**:** 391-428.

Bock KW, Wiltfang J, Blume R, Ullrich D, & Bircher J (1987). Paracetamol as a test drug to determine glucuronide formation in man. Effects of inducers and of smoking. European Journal of Clinical Pharmacology 31**:** 677-683.

Boku K, Ohno T, Saeki T, Hayashi H, Hayashi I, Katori M*, et al.* (2001). Adaptive cytoprotection mediated by prostaglandin I2 is attributable to sensitization of CGRP-containing sensory nerves. Gastroenterology 120**:** 134-143.

Bolli R, Shinmura K, Tang XL, Kodani E, Xuan YT, Guo Y*, et al.* (2002). Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. Cardiovascular Research 55**:** 506-519.

Bolten WW (2001). Number needed to kill individual drug risk with NSAIDs [1]. Zeitschrift fur Rheumatologie 60**:** 288.

Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S*, et al.* (1999). Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. Journal of Biological Chemistry 274**:** 22932-22940.

Budihardjo I, Oliver H, Lutter M, Luo X, & Wang X (1999). Biochemical pathways of caspase activation during apoptosis. In Annual Review of Cell and Developmental Biology. pp 269-290.

Burroughs AM, Allen KN, Dunaway-Mariano D, & Aravind L (2006). Evolutionary Genomics of the HAD Superfamily: Understanding the Structural Adaptations and Catalytic Diversity in a Superfamily of Phosphoesterases and Allied Enzymes. Journal of Molecular Biology 361**:** 1003-1034.

Cadieux RJ (1989). Drug interactions in the elderly. How multiple drug use increases risk exponentially. Postgraduate Medicine 86**:** 179-186.

Cai X, Fang JM, Xue P, Song WF, Hu J, Gu HL*, et al.* (2014). The role of IVS14+1 G > A genotype detection in the dihydropyrimidine dehydrogenase gene and pharmacokinetic monitoring of 5-fluorouracil in the individualized adjustment of 5-fluorouracil for patients with local advanced and metastatic colorectal cancer: A preliminary report. European Review for Medical and Pharmacological Sciences 18**:** 1247-1258.

Calatayud S, Ramírez MC, Sanz MJ, Moreno L, Hernández C, Bosch J*, et al.* (2001). Gastric mucosal resistance to acute injury in experimental portal hypertension. British Journal of Pharmacology 132**:** 309-317.

Camin RMG, Cols M, Chevarria JL, Rosa García O, Carrerasc M, Lisbona JM*, et al.* (2015). Acute kidney failure secondary to a combination of renin-angiotensin system inhibitors, diuretics and NSAIDS. The "Triple Whammy". Nefrologia 35**:** 197-206.

Carbonell N, Verstuyft C, Massard J, Letierce A, Cellier C, Deforges L*, et al.* (2010). CYP2C9*3 loss-of-function allele is associated with acute upper gastrointestinal bleeding related to the use of NSAIDs other than aspirin. Clinical Pharmacology and Therapeutics 87**:** 693-698.

Carlini LE, Meropol NJ, Bever J, Andria ML, Hill T, Gold P*, et al.* (2005). UGT1A7 and UGT1A9 polymorphisms predict response and toxicity in colorectal cancer patients treated with capecitabine/irinotecan. Clinical Cancer Research 11**:** 1226-1236.

Caudle KE, Rettie AE, Whirl-Carrillo M, Smith LH, Mintzer S, Lee MT*, et al.* (2014). Clinical pharmacogenetics implementation consortium guidelines for CYP2C9 and HLA-B genotypes and phenytoin dosing. Clinical Pharmacology and Therapeutics 96**:** 542-548.

Cayla G, Macia JC, Roubille F, Rabesendratana H, Piot C, Schved JF*, et al.* (2008). Repeated high doses of clopidogrel in 2 cases of pharmacological resistance. Circulation Journal 72**:** 2098-2100.

Cayla G, Montalescot G, & Collet JP (2010). Ticagrelor to prevent restenosis: New drug for an old hypothesis? Arteriosclerosis, Thrombosis, and Vascular Biology 30**:** 2320-2322.

Chattopadhyay I, Bandyopadhyay U, Biswas K, Maity P, & Banerjee RK (2006). Indomethacin inactivates gastric peroxidase to induce reactive-oxygen-mediated gastric mucosal injury and curcumin protects it by preventing peroxidase inactivation and scavenging reactive oxygen. Free Radical Biology and Medicine 40: 1397-1408.

Chen A, Francis M, Ni L, Cremers CWRJ, Kimberling WJ, Sato Y, *et al.* (1995). Phenotypic manifestations of branchiootorenal syndrome. American Journal of Medical Genetics 58: 365-370.

Cho SH, Ryu CH, & Oh CK (2004). Plasminogen Activator Inhibitor-1 in the Pathogenesis of Asthma. Experimental Biology and Medicine 229: 138-146.

Ciotti M, Marrone A, Potter C, & Owens IS (1997). Genetic polymorphism in the human UGT1A6 (planar phenol) UDP-glucuronosyltransferase: Pharmacological implications. Pharmacogenetics 7: 485-495.

Collet JP, & Montalescot G (2004). Rebound coronary events after withdrawal of prophylactic anti-thrombotic therapy. The cardiologist's point of view. Sang Thrombose Vaisseaux 16: 403-411.

Collet JP, Montalescot G, Blanchet B, Tanguy ML, Golmard JL, Choussat R, *et al.* (2004). Impact of prior use or recent withdrawal of oral antiplatelet agents on acute coronary syndromes. Circulation 110: 2361-2367.

Cook PJ, Ju BG, Telese F, Wang X, Glass CK, & Rosenfeld MG (2009). Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. Nature 458: 591-596.

Cosan D, Kurt E, Kurt H, Degirmenci I, Kuçukarabaci B, Metintas M, *et al.* (2009). Plasminogen activator inhibitor type-1 gene 4G/5G polymorphism in Turkish adult patients with asthma. Genetic testing and molecular biomarkers 13: 543-546.

Court MH (2010). Interindividual variability in hepatic drug glucuronidation: Studies into the role of age, sex, enzyme inducers, and genetic polymorphism using the human liver bank as a model system. Drug Metabolism Reviews 42: 202-217.

Court MH, Duan SX, Von Moltke LL, Greenblatt DJ, Patten CJ, Miners JO, *et al.* (2001). Interindividual variability in acetaminophen glucuronidation by human liver microsomes: Identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms. Journal of Pharmacology and Experimental Therapeutics 299: 998-1006.

Crews KR, Gaedigk A, Dunnenberger HM, Klein TE, Shen DD, Callaghan JT*, et al.* (2012). Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines for codeine therapy in the context of cytochrome P450 2D6 (CYP2D6) genotype. Clin Pharmacol Ther 91**:** 321-326.

Cryer B (2005). NSAID-associated deaths: The rise and fall of NSAID-associated GI mortality. American Journal of Gastroenterology 100**:** 1694-1695.

Danan G, & Benichou C (1993). Causality assessment of adverse reactions to drugs-I. A novel method based on the conclusions of international consensus meetings: Application to drug-induced liver injuries. Journal of Clinical Epidemiology 46**:** 1323-1330.

Darling RL, Romero JJ, Dial EJ, Akunda JK, Langenbach R, & Lichtenberger LM (2004). The effects of aspirin on gastric mucosal integrity, surface hydrophobicity, and prostaglandin metabolism in cyclooxygenase knockout mice. Gastroenterology 127**:** 94-104.

Davies EC, Green CF, Taylor S, Williamson PR, Mottram DR, & Pirmohamed M (2009). Adverse drug reactions in hospital in-patients: A prospective analysis of 3695 patient-episodes. PLoS ONE 4.

Dawson S, Hamsten A, Wiman B, Henney A, & Humphries S (1991). Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. Arteriosclerosis, Thrombosis, and Vascular Biology 11**:** 183-190.

de Abajo FJ, & García Rodríguez LA (2001). Risk of upper gastrointestinal bleeding and perforation associated with low-dose aspirin as plain and enteric-coated formulations. BMC Clinical Pharmacology 1.

De Berardis G, Lucisano G, D'Ettorre A, Pellegrini F, Lepore V, Tognoni G*, et al.* (2012). Association of aspirin use with major bleeding in patients with and without diabetes. JAMA - Journal of the American Medical Association 307**:** 2286-2294.

De Lange Z, Rijken DC, Hoekstra T, Conradie KR, Jerling JC, & Pieters M (2013). In black south africans from rural and urban communities, the 4G/5G PAI-1 polymorphism influences PAI-1 activity, but not plasma clot lysis time. PLoS ONE 8.

Declerck PJ, De Mol M, Alessi MC, Baudner S, Paques EP, Preissner KT*, et al.* (1988). Purification and characterization of a plasminogen activator inhibitor 1 binding protein from

human plasma. Identification as a multimeric form of S protein (vitronectin). Journal of Biological Chemistry 263**:** 15454-15461.

Deighton C, O'Mahony R, Tosh J, Turner C, & Rudolf M (2009). Management of rheumatoid arthritis: summary of NICE guidance. BMJ (Clinical research ed) 338.

Dellas C, & Loskutoff DJ (2005). Historical analysis of PAI-I from its discovery to its potential role in cell motility and disease. Thrombosis and Haemostasis 93**:** 631-640.

Deng G, Royle G, Wang S, Crain K, & Loskutoff DJ (1996). Structural and functional analysis of the plasminogen activator inhibitor-1 binding motif in the somatomedin B domain of vitronectin. Journal of Biological Chemistry 271**:** 12716-12723.

Derry S, & Loke YK (2000). Risk of gastrointestinal haemorrhage with long term use of aspirin: Meta-analysis. British Medical Journal 321**:** 1183-1187.

Dey I, Lejeune M, & Chadee K (2006). Prostaglandin E 2 receptor distribution and function in the gastrointestinal tract. British Journal of Pharmacology 149**:** 611-623.

Dieval J, Nguyen G, Gross S, Delobel J, & Kruithof EKO (1991). A lifelong bleeding disorder associated with a deficiency of plasminogen activator inhibitor type 1. Blood 77**:** 528-532.

Dikman A, Sanyal S, Von Althann C, Whitson M, Desai J, Bodian C*, et al.* (2009). A randomized, placebo-controlled study of the effects of naproxen, aspirin, celecoxib or clopidogrel on gastroduodenal mucosal healing. Alimentary Pharmacology and Therapeutics 29**:** 781-791.

Dikshit P, Chatterjee M, Goswami A, Mishra A, & Jana NR (2006). Aspirin induces apoptosis through the inhibition of proteasome function. Journal of Biological Chemistry 281**:** 29228-29235.

Dömötör A, Kereskay L, Szekeres G, Hunyady B, Szolcsányi J, & Mózsik G (2007). Participation of capsaicin-sensitive afferent nerves in the gastric mucosa of patients with Helicobacter pylori-positive or-negative chronic gastritis. Digestive Diseases and Sciences 52**:** 411-417.

Doña I, Blanca-López N, Torres MJ, García-Campos J, García-Núñez I, Gómez F*, et al.* (2012). Drug hypersensitivity reactions: Response patterns, drug involved, and temporal variations in a large series of patients. Journal of Investigational Allergology and Clinical Immunology 22**:** 363-371.

Dorado P, Gallego A, Peñas-Lledó E, Terán E, & Llerena A (2014). Relationship between the CYP2C9 IVS8-109A>T polymorphism and high losartan hydroxylation in healthy Ecuadorian volunteers. Pharmacogenomics 15**:** 1417-1421.

Dupont DM, Madsen JB, Kristensen T, Bodker JS, Blouse GE, Wind T*, et al.* (2009). Biochemical properties of plasminogen activator inhibitor-1. Frontiers in Bioscience 14**:** 1337-1361.

Edwards IR, & Aronson JK (2000). Adverse drug reactions: Definitions, diagnosis, and management. Lancet 356**:** 1255-1259.

Egan KM, Wang M, Lucitt MB, Zukas AM, Puré E, Lawson JA*, et al.* (2005). Cyclooxygenases, thromboxane, and atherosclerosis: Plaque destabilization by cyclooxygenase-2 inhibition combined with thromboxane receptor antagonism. Circulation 111**:** 334-342.

Egeblad M, & Werb Z (2002). New functions for the matrix metalloproteinases in cancer progression. Nature Reviews Cancer 2**:** 161-174.

Emonts M, Wiertsema SP, Veenhoven RH, Houwing-Duistermaat JJ, Walraven V, De Groot R*, et al.* (2007). The 4G/4G plasminogen activator inhibitor-1 genotype is associated with frequent recurrence of acute otitis media. Pediatrics 120**:** e317-e323.

EU (2010). Directive 2010/84/EU of the European Parliamentand of the Council. In: UNION E.P.A.T.C.O.T.E. (ed). .

Falvella FS, Cheli S, Martinetti A, Mazzali C, Iacovelli R, Maggi C*, et al.* (2015). DPD and UGT1A1 deficiency in colorectal cancer patients receiving triplet chemotherapy with fluoropyrimidines, oxaliplatin and irinotecan. British Journal of Clinical Pharmacology 80**:** 581-588.

Farabaugh SM, Micalizzi DS, Jedlicka P, Zhao R, & Ford HL (2012). Eya2 is required to mediate the pro-metastatic functions of Six1 via the induction of TGF-B signaling, epithelial-mesenchymal transition, and cancer stem cell properties. Oncogene 31**:** 552-562.

Fay WP, Parker AC, Condrey LR, & Shapiro AD (1997). Human plasminogen activator inhibitor-1 (PAI-1) deficiency: Characterization of a large kindred with a null mutation in the PAI-1 gene. Blood 90**:** 204-208.

Fernandes AB, Lima LM, Sousa MO, Toledo VDPC, Kazmi RS, Lwaleed BA*, et al.* (2015). Impaired fibrinolysis in angiographically documented coronary artery disease. Advances in Hematology 2015.

Festa A, D'Agostino Jr R, Rich SS, Jenny NS, Tracy RP, & Haffner SM (2003). Promoter (4G/5G) plasminogen activator inhibitor-1 genotype and plasminogen activator inhibitor-1 levels in blacks, hispanics, and non-hispanic whites: The Insulin Resistance Atherosclerosis Study. Circulation 107**:** 2422-2427.

Figueiras A, Estany-Gestal A, Aguirre C, Ruiz B, Vidal X, Carvajal A*, et al.* (2016). CYP2C9 variants as a risk modifier of NSAID-related gastrointestinal bleeding: a case-control study. Pharmacogenet Genomics 26**:** 66-73.

Fiorucci S, & Santucci L (2011). Hydrogen sulfide-based therapies: focus on H2S releasing NSAIDs. Inflammation & allergy drug targets 10**:** 133-140.

Fiorucci S, Santucci L, & Distrutti E (2007). NSAIDs, coxibs, CINOD and H2S-releasing NSAIDs: What lies beyond the horizon. Digestive and Liver Disease 39**:** 1043-1051.

Forrest JA, Finlayson ND, & Shearman DJ (1974). Endoscopy in gastrointestinal bleeding. Lancet 2**:** 394 -397.

Fortun PJ, & Hawkey CJ (2005). Nonsteroidal antiinflammatory drugs and the small intestine. Current Opinion in Gastroenterology 21**:** 169-175.

Frank J (2005). Diagnosis and management of G6PD deficiency. Am Fam Physician 72**:** 1277-1282.

Friedman MA, Woodcock J, Lumpkin MM, Shuren JE, Hass AE, & Thompson LJ (1999). The safety of newly approved medicines: Do recent market removals mean there is a problem? Journal of the American Medical Association 281**:** 1728-1734.

Furuta T, Ohashi K, Kamata T, Takashima M, Kosuge K, Kawasaki T*, et al.* (1998). Effect of Genetic Differences in Omeprazole Metabolism on Cure Rates for Helicobacter pylori Infection and Peptic Ulcer. Annals of Internal Medicine 129**:** 1027-1030.

GarcÍA-CortÉS M, Lucena MI, Pachkoria K, Borraz Y, Hidalgo R, Andrade RJ*, et al.* (2008). Evaluation of Naranjo Adverse Drug Reactions Probability Scale in causality assessment of drug-induced liver injury. Alimentary Pharmacology & Therapeutics 27**:** 780-789.

García-Martín E (2008). Interethnic and intraethnic variability of NAT2 single nucleotide polymorphisms. Current Drug Metabolism 9**:** 487-497.

García Rodríguez LA, Hernández-Díaz S, & de Abajo FJ (2001). Association between aspirin and upper gastrointestinal complications: systematic review of epidemiologic studies. British Journal of Clinical Pharmacology 52**:** 563-571.

Gheissari A, Rajyaguru V, Kumashiro R, & Matsumoto T (1990). Gastrointestinal hemorrhage in end stage renal disease patients. International Surgery 75**:** 93-95.

Ghemtio L, Soikkeli A, Yliperttula M, Hirvonen J, Finel M, & Xhaard H (2014). SVM classification and CoMSIA modeling of UGT1A6 interacting molecules. Journal of Chemical Information and Modeling 54**:** 1011-1026.

Gils A, & Declerck PJ (2004a). Plasminogen activator inhibitor-1. Current Medicinal Chemistry 11**:** 2323-2334.

Gils A, & Declerck PJ (2004b). The structural basis for the pathophysiological relevance of PAI-I in cardiovascular diseases and the development of potential PAI-I inhibitors. Thrombosis and Haemostasis 92**:** 22-34.

Goldkind L, & Laine L (2006). A systematic review of NSAIDs withdrawn from the market due to hepatotoxicity: Lessons learned from the bromfenac experience. Pharmacoepidemiology and Drug Safety 15**:** 213-220.

Goldkind L, & Simon LS (2006). Patients, their doctors, nonsteroidal anti-inflammatory drugs and the perception of risk. Arthritis Research and Therapy 8.

Gomes E, Cardoso MF, Praça F, Gomes L, Mariño E, & Demoly P (2004). Self-reported drug allergy in a general adult Portuguese population. Clinical and Experimental Allergy 34**:** 1597-1601.

Gomes ER, & Demoly P (2005). Epidemiology of hypersensitivity drug reactions. Current Opinion in Allergy and Clinical Immunology 5**:** 309-316.

Gretzer B, Maricic N, Respondek M, Schuligoi R, & Peskar BM (2001). Effects of specific inhibition of cyclo-oxygenase-1 and cyclo-oxygenase-2 in the rat stomach with normal mucosa and after acid challenge. British Journal of Pharmacology 132**:** 1565-1573.

Groenen MJM, Kuipers EJ, Hansen BE, & Ouwendijk RJT (2009). Incidence of duodenal ulcers and gastric ulcers in a Western population: Back to where it started. Canadian Journal of Gastroenterology 23**:** 604-608.

Gu Q, De Wang J, Xia HHX, Lin MCM, He H, Zou B*, et al.* (2005). Activation of the caspase-8/Bid and Bax pathways in aspirin-induced apoptosis in gastric cancer. Carcinogenesis 26**:** 541-546.

Gulløv AL, Koefoed BG, & Petersen P (1999). Bleeding during warfarin and aspirin therapy in patients with atrial fibrillation: The AFASAK 2 Study. Archives of Internal Medicine 159**:** 1322-1328.

Hall AJ, Tripp M, Howell T, Darland G, Bland JS, & Babish JG (2006). Gastric mucosal cell model for estimating relative gastrointestinal toxicity of non-steroidal anti-inflammatory drugs. Prostaglandins Leukotrienes and Essential Fatty Acids 75**:** 9-17.

Halter F, Tarnawski AS, Schmassmann A, & Peskar BM (2001). Cyclooxygenase 2 - Implications on maintenance of gastric mucosal integrity and ulcer healing: Controversial issues and perspectives. Gut 49**:** 443-453.

Halushka MK, Walker LP, & Halushka PV (2003). Genetic variation in cyclooxygenase 1: Effects on response to aspirin. Clinical Pharmacology and Therapeutics 73**:** 122-130.

Hanioka N, Takeda Y, Jinno H, Tanaka-Kagawa T, Naito S, Koeda A*, et al.* (2006). Functional characterization of human and cynomolgus monkey UDP-glucuronosyltransferase 1A6 enzymes. Chemico-Biological Interactions 164**:** 136-145.

Hartwig SC, Siegel J, & Schneider PJ (1992). Preventability and severity assessment in reporting adverse drug reactions. American Journal of Hospital Pharmacy 49**:** 2229-2232.

Hatazawa R, Tanaka A, Tanigami M, Amagase K, Kato S, Ashida Y*, et al.* (2007). Cyclooxygenase-2/prostaglandin E 2 accelerates the healing of gastric ulcers via EP 4 receptors. American Journal of Physiology - Gastrointestinal and Liver Physiology 293**:** G788-G797.

Hayashi R, Tahara T, Shiroeda H, Matsue Y, Minato T, Nomura T*, et al.* (2012). Association of genetic polymorphisms in IL17A and IL17F with gastro-duodenal diseases. Journal of Gastrointestinal and Liver Diseases 21**:** 243-249.

Hedman J, Kaprio J, Poussa T, & Nieminen MM (1999). Prevalence of asthma, aspirin intolerance, nasal polyposis and chronic obstructive pulmonary disease in a population-based study. International Journal of Epidemiology 28**:** 717-722.

Heel RC, Brogden RN, Speight TM, & Avery GS (1977). Benzbromarone. A review of its pharmacological properties and therapeutic use in gout and hyperuricaemia. Drugs 14**:** 349-366.

Heimler A, & Lieber E (1986). Branchio-oto-renal syndrome: Reduced penetrance and variable expressivity in four generations of a large kindred. American Journal of Medical Genetics 25**:** 15-27.

Helseth R, Seljeflot I, Opstad T, Solheim S, Freynhofer M, Arnesen H*, et al.* (2015). Genes expressed in coronary thrombi are associated with ischemic time in patients with acute myocardial infarction. Thrombosis Research 135**:** 329-333.

Hendset M, Hermann M, Lunde H, Refsum H, & Molden E (2007). Impact of the CYP2D6 genotype on steady-state serum concentrations of aripiprazole and dehydroaripiprazole. European Journal of Clinical Pharmacology 63**:** 1147-1151.

Hicks JK, Swen JJ, Thorn CF, Sangkuhl K, Kharasch ED, Ellingrod VL*, et al.* (2013). Clinical Pharmacogenetics Implementation Consortium guideline for CYP2D6 and CYP2C19 genotypes and dosing of tricyclic antidepressants. Clin Pharmacol Ther 93**:** 402-408.

Hinz B, Kraus V, Pahl A, & Brune K (2000). Salicylate metabolites inhibit cyclooxygenase-2-dependent prostaglandin E2 synthesis in murine macrophages. Biochemical and Biophysical Research Communications 274**:** 197-202.

Hirschberg M (1902). Erfahrungen über die behandlung und heilung der Lepra im riga'schen Städtischen Leprosorium. Dermatology 9**:** 626-640.

Hoekstra T, Geleijnse JM, Schouten EG, & Kluft C (2004). Plaminogen activator inhibitor-type 1: Its plasma determinants and relation with cardiovascular risk. Thrombosis and Haemostasis 91**:** 861-872.

Hoffmann S, He S, Jin M, Ehren M, Wiedemann P, Ryan SJ*, et al.* (2005). A selective cyclic integrin antagonist blocks the integrin receptors αvβ3 and αvβ5 and inhibits retinal pigment epithelium cell attachment, migration and invasion. BMC Ophthalmology 5.

Reference section

Hoffmann S, Hofbauer LC, Scharrenbach V, Wunderlich A, Hassan I, Lingelbach S, *et al.* (2004). Thyrotropin (TSH)-induced production of vascular endothelial growth factor in thyroid cancer cells in vitro: Evaluation of TSH signal transduction and of angiogenesis-stimulating growth factors. Journal of Clinical Endocrinology and Metabolism 89**:** 6139-6145.

Holzer P (1998). Neural injury, repair, and adaptation in the GI tract II. The elusive action of capsaicin on the vagus nerve. American Journal of Physiology - Gastrointestinal and Liver Physiology 275**:** G8-G13.

Holzer P (2007). Role of visceral afferent neurons in mucosal inflammation and defense. Current Opinion in Pharmacology 7**:** 563-569.

Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, & Mizushima T (2003). Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. Journal of Biological Chemistry 278**:** 12752-12758.

Hoshiyama D, Iwabe N, & Miyata T (2007). Evolution of the gene families forming the Pax/Six regulatory network: Isolation of genes from primitive animals and molecular phylogenetic analyses. FEBS Letters 581**:** 1639-1643.

Hutt AJ, Caldwell J, & Smith RL (1986). The metabolism of aspirin in man: A population study. Xenobiotica 16**:** 239-249.

Ikeda K, Watanabe Y, Ohto H, & Kawakami K (2002). Molecular interaction and synergistic activation of a promoter by Six, Eya, and Dach proteins mediated through CREB binding protein. Molecular and Cellular Biology 22**:** 6759-6766.

Ishii Y, Mioshi A, Watanabe R, Tsuruda K, Tsuda M, Yamagushi-Nagamatsu Y, *et al.* (2001). Simultaneous expression of Guinea pig UDP-glucoronosyltransferase 2B21 and 2B22 in COS7 cells enhances   UDP-glucoronosyltransferase 2B21-catalysed morphine -6-glucoronide formation mol Pharm 60**:** 1040-1048.

Isik SR, Karakaya G, Celikel S, Demir AU, & Kalyoncu AF (2009). Association between asthma, rhinitis and NSAID hypersensitivity in chronic urticaria patients and prevalence rates. International Archives of Allergy and Immunology 150**:** 299-306.

Ivey KJ (1986). Gastrointestinal intolerance and bleeding with non-narcotic analgesics. Drugs 32**:** 71-89.

Jama RH, Perlman MH, & Matsumoto T (1975). Incidence of stress ulcer formation associated with steroid therapy in various shock states. The American Journal of Surgery 130**:** 328-331.

Jankovic SM, Aleksic J, Rakovic S, Aleksic A, Stevanovic I, Stefanovic-Stoimenov N*, et al.* (2009). Nonsteroidal antiinflammatory drugs and risk of gastrointestinal bleeding among patients on hemodialysis. Journal of Nephrology 22**:** 502-507.

Jemc J, & Rebay I (2007). The eyes absent family of phosphotyrosine phosphatases: Properties and roles in developmental regulation of transcription, pp 513-538.

Jenkins C, Costello J, & Hodge L (2004). Systematic review of prevalence of aspirin induced asthma and its implications for clinical practice. British Medical Journal 328**:** 434-437.

Juhan-Vague I, Alessi MC, & Vague P (1991). Increased plasma plasminogen activator inhibitor 1 levels. A possible link between insulin resistance and atherothrombosis. Diabetologia 34**:** 457-462.

Kamataki T, Yokoi T, Yamayoshi Y, Nakamura K, Kushida H, Fujit KI*, et al.* (1996). Strategic proposals to avoid toxic drug interactions during new drug development: Molecular toxicology proposals. Journal of Toxicological Sciences 21**:** 305-308.

Kasper L, Sladek K, Duplaga M, Bochenek G, Liebhart J, Gladysz U*, et al.* (2003). Prevalence of asthma with aspirin hypersensitivity in the adult population of Poland. Allergy: European Journal of Allergy and Clinical Immunology 58**:** 1064-1066.

Kato S, Aihara E, Yoshii K, & Takeuchi K (2005). Dual action of prostaglandin E2 on gastric acid secretion through different EP-receptor subtypes in the rat. American Journal of Physiology - Gastrointestinal and Liver Physiology 289**:** G64-G69.

Katori M, Nishiyama K, Ueno A, & Suzuki Y (1990). Possible role of endogenous prostaglandins against ethanol injury in rat stomach. Journal of Clinical Gastroenterology 12**:** S25-S31.

Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, & Poirier GG (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. Cancer Research 53**:** 3976-3985.

Kelly JP, Kaufman DW, Jurgelon JM, Sheehan J, Koff RS, & Shapiro S (1996). Risk of aspirin-associated major upper-gastrointestinal bleeding with enteric-coated or buffered product. Lancet 348: 1413-1416.

Kenny S, Duval C, Sammut SJ, Steele I, Pritchard DM, Atherton JC, *et al.* (2008). Increased expression of the urokinase plasminogen activator system by Helicobacter pylori in gastric epithelial cells. American Journal of Physiology - Gastrointestinal and Liver Physiology 295: G431-G441.

Kenny S, Steele I, Lyons S, Moore AR, Murugesan SV, Tiszlavicz L, *et al.* (2013a). The role of plasminogen activator inhibitor-1 in gastric mucosal protection. American Journal of Physiology - Gastrointestinal and Liver Physiology 304: G814-G822.

Kenny S, Steele I, Lyons S, Moore AR, Murugesan SV, Tiszlavicz L, *et al.* (2013b). The role of plasminogen activator inhibitor (PAI)-1 in gastric mucosal protection. American Journal of Physiology - Gastrointestinal and Liver Physiology.

Kim HK, Kim JI, Kim JK, Han JY, Park SH, Choi KY, *et al.* (2007). Preventive effects of rebamipide on NSAID-induced gastric mucosal injury and reduction of gastric mucosal blood flow in healthy volunteers. Digestive Diseases and Sciences 52: 1776-1782.

Kim JJ, Choi YM, Lee SK, Yang KM, Paik EC, Jeong HJ, *et al.* (2014). The PAI-1 4G/5G and ACE I/D Polymorphisms and Risk of Recurrent Pregnancy Loss: A Case-Control Study. American Journal of Reproductive Immunology 72: 571-576.

Koike T, Shimada T, Fujii Y, Chen G, Tabei K, Namatame T, *et al.* (2007). Up-regulation of TFF1 (pS2) expression by TNF-α in gastric epithelial cells. Journal of Gastroenterology and Hepatology (Australia) 22: 936-942.

Komoike Y, Nakashima M, Nakagiri A, & Takeuchi K (2003). Prostaglandin E receptor EP1 subtype but not prostacyclin IP receptor involved in mucosal blood flow response of mouse stomachs following barrier disruption. Digestion 67: 186-194.

Kosone T, Takagi H, Kakizaki S, Sohara N, Horiguchi N, Sato K, *et al.* (2006). Integrative roles of transforming growth factor-α in the cytoprotection mechanisms of gastric mucosal injury. BMC Gastroenterology 6.

Kotani T, Kobata A, Nakamura E, Amagase K, & Takeuchi K (2006). Roles of cyclooxygenase-2 and prostacyclin/IP receptors in mucosal defense against ischemia/reperfusion injury in mouse stomach. Journal of Pharmacology and Experimental Therapeutics 316: 547-555.

Kottgen A, Kao WHL, Hwang SJ, Boerwinkle E, Yang Q, Levy D*, et al.* (2008). Genome-wide association study for renal traits in the Framingham Heart and Atherosclerosis risk in Communities Studies. BMC Medical Genetics 9.

Kowalski ML, Asero R, Bavbek S, Blanca M, Blanca-Lopez N, Bochenek G*, et al.* (2013). Classification and practical approach to the diagnosis and management of hypersensitivity to nonsteroidal anti-inflammatory drugs. Allergy: European Journal of Allergy and Clinical Immunology 68**:** 1219-1232.

Kowalski ML, Makowska JS, Blanca M, Bavbek S, Bochenek G, Bousquet J*, et al.* (2011). Hypersensitivity to nonsteroidal anti-inflammatory drugs (NSAIDs) - Classification, diagnosis and management: Review of the EAACI/ENDA and GA2LEN/HANNA. Allergy: European Journal of Allergy and Clinical Immunology 66**:** 818-829.

Krishnaswamy S, Hao Q, Al-Rohaimi A, Hesse LM, von Moltke LL, & Greenblatt DJ (2005a). UDP glucuronosyltransferase (UGT) 1A6 pharmacogenetics: I. Identification of polymorphisms in the 5'-regulatory and exon 1 regions, and association with human liver UGT1A6 gene expression and glucuronidation. Journal of Pharmacology and Experimental Therapeutics 313**:** 1331.

Krishnaswamy S, Hao Q, Al-Rohaimi A, Hesse LM, von Moltke LL, & Greenblatt DJ (2005b). UDP glucuronosyltransferase (UGT) 1A6 pharmacogenetics: II. Functional impact of the three most common nonsynonymous UGT1A6 polymorphisms (S7A, T181A, and R184S). Journal of Pharmacology and Experimental Therapeutics 313**:** 1340-1346.

Krishnaswamy S, Hao Q, Al-Rohaimi A, Hesse LM, Von Moltke LL, Greenblatt DJ*, et al.* (2005c). UDP glucuronosyltransferase (UGT) 1A6 pharmacogenetics: I. Identification of polymorphisms in the 5'-regulatory and exon 1 regions, and association with human liver UGT1A6 gene expression and glucuronidation. Journal of Pharmacology and Experimental Therapeutics 313**:** 1331-1339.

Krishnaswamy S, Hao Q, Al-Rohaimi A, Hesse LM, Von Moltke LL, Greenblatt DJ*, et al.* (2005d). UDP glucuronosyltransferase (UGT) 1A6 pharmacogenetics: II. Functional impact of the three most common nonsynonymous UGT1A6 polymorphisms (S7A, T181A, and R184S). Journal of Pharmacology and Experimental Therapeutics 313**:** 1340-1346.

Kruithof EKO (1988a). Plasminogen activator inhibitor type 1: Biochemical, biological and clinical aspects. Fibrinolysis 2**:** 59-70.

Kruithof EKO (1988b). Plasminogen activator inhibitors - A review. Enzyme 40**:** 113-121.

Kuehl GE, Bigler J, Potter JD, & Lampe JW (2006). Glucuronidation of the aspirin metabolite salicylic acid by expressed UDP-glucuronosyltransferases and human liver microsomes. DRUG METABOLISM AND DISPOSITION 34**:** 199-202.

Kumar Singh B, Pathan RA, Pillai KK, Haque SE, & Dubey K (2010). Diclofenac sodium, a nonselective nonsteroidal anti-inflammatory drug aggravates doxorubicin-induced cardiomyopathy in rats. Journal of Cardiovascular Pharmacology 55**:** 139-144.

Kurose I, Maruyama K, Suzuki M, & et a (1988) *Oxyradicalmediated activation of t-PA in the rat acute gastric mucosal injury with special references to microvascular damage.* Nihon-Igakukan: Tokyo

Kurose I, Miura S, Suematsu M, Asako H, Serizawa H, Ogata H*, et al.* (1990). Possible involvement of tissue-type plasminogen activator in gastric ulcer formation. Biochemical evaluation using biopsy specimens from gastric mucosa. Digestive Endoscopy 2**:** 49-53.

Kvasz M, Allen IE, Gordon MJ, Ro EY, Estok R, Olkin I*, et al.* (2000). Adverse drug reactions in hospitalized patients: A critique of a meta-analysis. MedGenMed : Medscape general medicine 2.

Lampe JW, Bigler J, Horner NK, & Potter JD (1999). UDP-glucuronosyltransferase (UGT1A1* 28 and UGT1A6* 2) polymorphisms in Caucasians and Asians: relationships to serum bilirubin concentrations. Pharmacogenetics and Genomics 9: 341.

Lanas A, Bajador E, Serrano P, Fuentes J, Carreño S, Guardia J*, et al.* (2000). Nitrovasodilators, low-dose aspirin, other nonsteroidal antiinflammatory drugs, and the risk of upper gastrointestinal bleeding. New England Journal of Medicine 343**:** 834-839.

Lanas A, Fuentes J, Benito R, Serrano P, Bajador E, & Sáinz R (2002). Helicobacter pylori increases the risk of upper gastrointestinal bleeding in patients taking low-dose aspirin. Alimentary Pharmacology and Therapeutics 16**:** 779-786.

Lanas A, García-Rodríguez LA, Arroyo MT, Gomollón F, Feu F, González-Pérez A*, et al.* (2006). Risk of upper gastrointestinal ulcer bleeding associated with selective cyclo-oxygenase-2 inhibitors, traditional non-aspirin non-steroidal anti-inflammatory drugs, aspirin and combinations. Gut 55**:** 1731-1738.

Lanas A, Wu P, Medin J, & Mills EJ (2011). Low Doses of Acetylsalicylic Acid Increase Risk of Gastrointestinal Bleeding in a Meta-Analysis. Clinical Gastroenterology and Hepatology 9**:** 762-768.e766.

Lancaster C, & Robert A (1978). Intestinal lesions produced by prednisolone: prevention (cytoprotection) by 16,16-dimethyl prostaglandin E2. The American journal of physiology 235**:** E703-708.

Langman MJS (2001). Ulcer complications associated with anti-inflammatory drug use. What is the extent of the disease burden? Pharmacoepidemiology and Drug Safety 10**:** 13-19.

Lanza FL, Chan FKL, Quigley EMM, Inadomi J, Baroni D, Bernstein D*, et al.* (2009). Guidelines for prevention of NSAID-related ulcer complications. American Journal of Gastroenterology 104**:** 728-738.

Lares-Asseff I, Juárez-Olguín H, Flores-Pérez J, Guillé-Pérez A, & Vargas A (2004). Pharmacokinetics and metabolic rates of acetyl salicylic acid and its metabolites in an Otomi ethnic group of Mexico. Biological and Pharmaceutical Bulletin 27**:** 706-709.

Larsen R, Hansen MB, & Bindslev N (2005). Duodenal secretion in humans mediated by the EP4 receptor subtype. Acta Physiologica Scandinavica 185**:** 133-140.

Laurence D, & Carpenter J (eds) (1998). *A dictionary of pharmacology and allied topics*. Elsevier, : Amsterdam.

Lavin MF, & Kozlov S (2007). ATM activation and DNA damage response. Cell Cycle 6**:** 931-942.

Lavrik IN, Golks A, & Krammer PH (2005). Caspase: Pharmacological manipulation of cell death. Journal of Clinical Investigation 115**:** 2665-2672.

Lazarou J, Pomeranz BH, & Corey PN (1998). Incidence of adverse drug reactions in hospitalized patients: A meta- analysis of prospective studies. Journal of the American Medical Association 279**:** 1200-1205.

Leakey JEA, Hume R, & Burchell B (1987). Development of multiple activities of UDP-glucuronyltransferase in human liver. Biochemical Journal 243**:** 859-861.

Leung AM, Redlak MJ, & Miller TA (2009). Aspirin-Induced Mucosal Cell Death in Human Gastric Cells: Role of a Caspase-Independent Mechanism. Digestive Diseases and Sciences 54**:** 28-35.

Reference section

Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, & Moore PK (2007). Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. Free Radical Biology and Medicine 42**:** 706-719.

Li X, Liu Y, Zhang R, Tan J, Chen L, & Liu Y (2015). Meta-analysis of the association between plasminogen activator inhibitor-1 4G/5G polymorphism and recurrent pregnancy loss. Medical science monitor : international medical journal of experimental and clinical research 21**:** 1051-1056.

Li X, Oghi KA, Zhang J, Krones A, Bush KT, Glass CK*, et al.* (2003). Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. Nature 426**:** 247-254.

Lichtenberger LM, Zhou Y, Dial EJ, & Raphael RM (2006). NSAID injury to the gastrointestinal tract: Evidence that NSAIDs interact with phospholipids to weaken the hydrophobic surface barrier and induce the formation of unstable pores in membranes. Journal of Pharmacy and Pharmacology 58**:** 1421-1428.

Lijnen HR (2005). Pleiotropic functions of plasminogen activator inhibitor-1. Journal of Thrombosis and Haemostasis 3**:** 35-45.

Lijnen HR, & Collen D (1995a). 1 Mechanisms of physiological fibrinolysis. Bailliere's Clinical Haematology 8**:** 277-290.

Lijnen HR, & Collen D (1995b). Preface. Bailliere's Clinical Haematology 8**:** ix-x.

Livak KJ, & Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25**:** 402-408.

López-Rodríguez R, Novalbos J, Gallego-Sandín S, Román-Martínez M, Torrado J, Gisbert JP*, et al.* (2008). Influence of CYP2C8 and CYP2C9 polymorphisms on pharmacokinetic and pharmacodynamic parameters of racemic and enantiomeric forms of ibuprofen in healthy volunteers. Pharmacological Research 58**:** 77-84.

Lu C, Zhu F, Cho Y-Y, Tang F, Zykova T, Ma W-y*, et al.* (2006). Cell Apoptosis: Requirement of H2AX in DNA Ladder Formation, but Not for the Activation of Caspase-3. Molecular Cell 23**:** 121-132.

MacDonald CJ, Ciolino HP, & Yeh GC (2004). The Drug Salicylamide Is an Antagonist of the Aryl Hydrocarbon Receptor That Inhibits Signal Transduction Induced by 2,3,7,8-Tetrachlorodibenzo-p-dioxin. Cancer Research 64**:** 429-434.

Mackenzie PI, Gregory PA, Garner-stephen DA, Lewinsky RH, Jorgeson BR, Nishiyama T*, et al.* (2003). Regulation of UDP glucuronosyltransferase genes. *CurrDrug Metab* 4**:** 249-257.

Mackenzie PI, Mojarrabi B, Meech R, & Hansen A (1996). Steroid UDP glucuronosyltransferases: Characterization and regulation. Journal of Endocrinology 150**:** S79-S86.

Manganelli G, Fico A, Martini G, & Filosa S (2010). Discussion on pharmacogenetic interaction in g6pd deficiency and methods to identify potential hemolytic drugs. Cardiovascular and Hematological Disorders - Drug Targets 10**:** 143-150.

Martin GR, & Wallace JL (2006). Gastrointestinal inflammation: A central component of mucosal defense and repair. Experimental Biology and Medicine 231**:** 130-137.

Martin JH, Begg EJ, Kennedy MA, Roberts R, & Barclay ML (2001). Is cytochrome p450 2c9 genotype associated with NSAID gastric ulceration? British Journal of Clinical Pharmacology 51**:** 627-630.

Martínez C, Blanco G, Ladero JM, García-Martín E, Taxonera C, Gamito FG*, et al.* (2004). Genetic predisposition to acute gastrointestinal bleeding after NSAIDs use. British Journal of Pharmacology 141**:** 205-208.

Mashita Y, Taniguchi M, Yokota A, Tanaka A, & Takeuchi K (2006). Oral but not parenteral aspirin upregulates COX-2 expression in rat stomachs: A relationship between COX-2 expression and PG deficiency. Digestion 73**:** 124-132.

Matsuoka S, Ballif BA, Smogorzewska A, McDonald Iii ER, Hurov KE, Luo J*, et al.* (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316**:** 1160-1166.

McGreavey LE, Turner F, Smith G, Boylan K, Bishop DT, Forman D*, et al.* (2005). No evidence that polymorphisms in CYP2C8, CYP2C9, UGT1A6, PPAR delta and PPAR gamma act as modifiers of the protective effect of regular NSAID use on the risk of colorectal carcinoma. Pharmacogenetics and Genomics 15**:** 713-721.

Reference section

Meyer UA (1992). Interindividual differences in the reaction to drugs and toxic chemicals. Therapeutische Umschau 49**:** 97-101.

Meyer UA (2000). Pharmacogenetics and adverse drug reactions. Lancet 356**:** 1667-1671.

Miller SJ, Lan ZD, Hardiman A, Wu J, Kordich JJ, Patmore DM*, et al.* (2010). Inhibition of eyes absent homolog 4 expression induces malignant peripheral nerve sheath tumor necrosis. Oncogene 29**:** 368-379.

Mönnikes H, Van Der Voort IR, Wollenberg B, Heymann-Mönnikes I, Tebbe JJ, Alt W*, et al.* (2005). Gastric perception thresholds are low and sensory neuropeptide levels high in Helicobacter pylori-positive functional dyspepsia. Digestion 71**:** 111-123.

Montecucco C, & Rappuoli R (2001). Living dangerously: How helicobacter pylori survives in the human stomach. Nature Reviews Molecular Cell Biology 2**:** 457-466.

Moore N, Lecointre D, Noblet C, & Mabille M (1998). Frequency and cost of serious adverse drug reactions in a department of general medicine. British Journal of Clinical Pharmacology 45**:** 301-308.

Motawi TK, Abd Elgawad HM, & Shahin NN (2007). Modulation of indomethacin-induced gastric injury by spermine and taurine in rats. Journal of Biochemical and Molecular Toxicology 21**:** 280-288.

Mourad AA, & Bahna SL (2014). Hypersensitivities to non-steroidal anti-inflammatory drugs. Expert Review of Clinical Immunology 10**:** 1263-1268.

Munzel PA, Bruck M, & Bock KW (1994). Tissue-specificconstitutive and inducibleexpressionof rat phenol UDP-glucoronosyltransferase. *Biochem Pharmacol* 47**:** 1445-1448.

Musumba C, Pritchard DM, & Pirmohamed M (2009). Review article: Cellular and molecular mechanisms of NSAID-induced peptic ulcers. Alimentary Pharmacology and Therapeutics 30**:** 517-531.

Musumba CO, Jorgensen A, Sutton L, Van Eker D, Zhang E, O'Hara N*, et al.* (2013). CYP2C19*17 gain-of-function polymorphism is associated with peptic ulcer disease. Clinical Pharmacology and Therapeutics 93**:** 195-203.

Nagano Y, Matsui H, Muramatsu M, Shimokawa O, Shibahara T, Yanaka A, *et al.* (2005). Rebamipide significantly inhibits indomethacin-induced mitochondrial damage, lipid peroxidation, and apoptosis in gastric epithelial RGM-1 cells. Digestive Diseases and Sciences 50**:** S76-S83.

Nagar S, Zalatoris JJ, & Blanchard RL (2004). Human UGT1A6 pharmacogenetics: identification of a novel SNP, characterization of allele frequencies and functional analysis of recombinant allozymes in human liver tissue and in cultured cells. Pharmacogenetics and Genomics 14**:** 487.

Naranjo CA, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts EA*, et al.* (1981). A method for estimating the probability of adverse drug reactions. Clinical Pharmacology and Therapeutics 30**:** 239-245.

http://www.ncbi.nlm.nih.gov/snp/?term=rs12678747. [Online] [Accessed: 21/06/2015 2015].

Needs CJ, & Brooks PM (1985). Clinical pharmacokinetics of the salicylates. Clinical Pharmacokinetics 10**:** 164-177.

Negovan A, Voidăzan S, Pantea M, Moldovan V, Bataga S, Cozlea L*, et al.* (2015). AGT A-20C (rs5050) gene polymorphism and ulcer occurrence in patients treated with low-dose aspirin: A case-control study. Revista Romana de Medicina de Laborator 23**:** 179-188.

Netzer P, Halter F, Ma TY, Hoa N, Nguyen N, Nakamura T*, et al.* (2003). Interaction of hepatocyte growth factor and non-steroidal anti-inflammatory drugs during gastric epithelial wound healing. Digestion 67**:** 118-128.

Ng W, Wong WM, Chen WH, Tse HF, Lee PY, Lai KC*, et al.* (2006). Incidence and predictors of upper gastrointestinal bleeding in patients receiving low-dose aspirin for secondary prevention of cardiovascular events in patients with coronary artery disease. World Journal of Gastroenterology 12**:** 2923-2927.

Nguyen T, Chai J, Li A, Akahoshi T, Tanigawa T, & Tarnawski AS (2007). Novel Roles of Local Insulin-Like Growth Factor-1 Activation in Gastric Ulcer Healing: Promotes Actin Polymerization, Cell Proliferation, Re-Epithelialization, and Induces Cyclooxygenase-2 in a Phosphatidylinositol 3-Kinase-Dependent Manner. The American Journal of Pathology 170**:** 1219-1228.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, *et al.* (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376**:** 37-43.

Nikpour P, Emadi-Baygi M, Emadi-Andani E, & Rahmati S (2014). EYA1 expression in gastric carcinoma and its association with clinicopathological characteristics: a pilot study. Medical oncology (Northwood, London, England) 31**:** 955.

Nishio H, Terashima S, Nakashima M, Aihara E, & Takeuchi K (2007). Involvement of prostaglandin E receptor EP3 subtype and prostacyclin ip receptor in decreased acid response in damaged stomach. Journal of Physiology and Pharmacology 58**:** 407-421.

Nishiyama K, Katori M, Ueno A, Ohno T, & Saigenji K (1992). Suppression of myoelectrical activity of gastric smooth muscle by endogenous gastric prostaglandin E2. Digestive Diseases and Sciences 37**:** 1002-1008.

Nørsett KG, Steele I, Duval C, Sammut SJ, Murugesan SVM, Kenny S, *et al.* (2011). Gastrin stimulates expression of plasminogen activator inhibitor-1 in gastric epithelial cells. American Journal of Physiology - Gastrointestinal and Liver Physiology 301**:** G446-G453.

Northington FJ, Zelaya ME, O'Riordan DP, Blomgren K, Flock DL, Hagberg H, *et al.* (2007). Failure to complete apoptosis following neonatal hypoxia-ischemia manifests as "continuum" phenotype of cell death and occurs with multiple manifestations of mitochondrial dysfunction in rodent forebrain. Neuroscience 149**:** 822-833.

Nuñez G, Benedict MA, Hu Y, & Inohara N (1998). Caspases: The proteases of the apoptotic pathway. Oncogene 17**:** 3237-3245.

O'Neill GP, & Ford-Hutchinson AW (1993). Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. FEBS Letters 330**:** 157-160.

Oberhammer F, Wilson JW, Dive C, Morris LD, Hickman JA, Wakeling AE, *et al.* (1993). Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO Journal 12**:** 3679-3684.

Oh GS, Pae HO, Lee BS, Kim BN, Kim JM, Kim HR, *et al.* (2006). Hydrogen sulfide inhibits nitric oxide production and nuclear factor-κB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radical Biology and Medicine 41**:** 106-119.

Ohno T, Hattori Y, Komine R, Ae T, Mizuguchi S, Arai K, *et al.* (2008). Roles of Calcitonin Gene-Related Peptide in Maintenance of Gastric Mucosal Integrity and in Enhancement of Ulcer Healing and Angiogenesis. Gastroenterology 134**:** 215-225.

Oijen mGHV, Hulbers S, Wilbert HMP, Drenth JPH, Laheij RJF, Verheugt FWA, *et al.* (2005). Polymorphisms in genes encoding acetylsalicylic acid metabolizing enzymes are unrelated to upper gastrointestinal health in cardiovascular patients on acetylsalicylic acid. Br J Clin Pharmacol 60**:** 623-628.

Oitate M, Hirota T, Murai T, Miura SI, & Ikeda T (2007a). Covalent binding of rofecoxib, but not other cyclooxygenase-2 inhibitors, to allysine aldehyde in elastin of human aorta. Drug Metabolism and Disposition 35**:** 1846-1852.

Oitate M, Hirota T, Takahashi M, Murai T, Miura SI, Senoo A, *et al.* (2007b). Mechanism for covalent binding of rofecoxib to elastin of rat aorta. Journal of Pharmacology and Experimental Therapeutics 320**:** 1195-1203.

Okada K, Inamori M, Imajo K, Chiba H, Nonaka T, Shiba T, *et al.* (2009). Clinical study of upper gastrointestinal bleeding associated with low-dose aspirin in Japanese patients. Hepato-Gastroenterology 56**:** 1665-1669.

Okajima K, & Harada N (2006). Regulation of inflammatory responses by sensory neurons: Molecular mechanism(s) and possible therapeutic applications. Current Medicinal Chemistry 13**:** 2241-2251.

Oosterhuis M, Van De Kraats G, & Tenback D (2007). Safety of aripiprazole: High serum levels in a CYP2D6 mutated patient [10]. American Journal of Psychiatry 164**:** 175.

Pairet M, & Van Ryn J (1998). Experimental models used to investigate the differential inhibition of cyclooxygenase-1 and cyclooxygenase-2 by non-steroidal anti-inflammatory drugs. Inflammation Research 47**:** S93-S101.

Palmer RMJ, Rees DD, Ashton DS, & Moncada S (1988). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochemical and Biophysical Research Communications 153**:** 1251-1256.

Pan MR, & Hung WC (2002). Nonsteroidal anti-inflammatory drugs inhibit matrix metalloproteinase-2 via suppression of the ERK/Sp1-mediated transcription. Journal of Biological Chemistry 277**:** 32775-32780.

Pandey RN, Rani R, Yeo EJ, Spencer M, Hu S, Lang RA*, et al.* (2010). The Eyes Absent phosphatase-transactivator proteins promote proliferation, transformation, migration, and invasion of tumor cells. Oncogene 29**:** 3715-3722.

Pandey RN, Wang TS, Tadjuidje E, McDonald MG, Rettie AE, & Hegde RS (2013). Structure-activity relationships of benzbromarone metabolites and derivatives as EYA inhibitory anti-angiogenic agents. PLoS ONE 8.

Patel KJ, Kedia MS, Bajpai D, Mehta SS, Kshirsagar NA, & Gogtay NJ (2007). Evaluation of the prevalence and economic burden of adverse drug reactions presenting to the medical emergency department of a tertiary referral centre: A prospective study. BMC Clinical Pharmacology 7.

Patrono C, & Dunn MJ (1987). The clinical significance of inhibition of renal prostaglandin synthesis. Kidney International 32**:** 1-12.

Pazhayattil GS, & Shirali AC (2014). Drug-induced impairment of renal function. International Journal of Nephrology and Renovascular Disease 7**:** 457-468.

Peek Jr RM, & Blaser MJ (2002). Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nature Reviews Cancer 2**:** 28-37.

Perfettini JL, & Kroemer G (2003). Caspase activation is not death. Nature Immunology 4**:** 308-310.

Perini RF, Ma L, & Wallace JL (2003). Mucosal repair and COX-2 inhibition. Current Pharmaceutical Design 9**:** 2207-2211.

Perrone V, Conti V, Venegoni M, Scotto S, Esposti LD, Sangiorgi D*, et al.* (2014). Seriousness, preventability, and burden impact of reported adverse drug reactions in Lombardy emergency departments: A retrospective 2-year characterization. ClinicoEconomics and Outcomes Research 6**:** 505-514.

PharmGKB (2015). CPIC genes/DrugsPharmGKB https://www.pharmgkb.org/cpic/pairs

Piazuelo E, Fuentes J, Garcfa-González MA, Jiménez P, & Lanas A (2008). A case-control study of the association between polymorphisms of the endothelial nitric oxide synthase and glycoprotein IIIa genes and upper gastrointestinal bleeding in users of low-dose aspirin. Clinical Therapeutics 30**:** 121-130.

Reference section

Pilotto A, Seripa D, Franceschi M, Scarcelli C, Colaizzo D, Grandone E*, et al.* (2007). Genetic Susceptibility to Nonsteroidal Anti-Inflammatory Drug-Related Gastroduodenal Bleeding: Role of Cytochrome P450 2C9 Polymorphisms. Gastroenterology 133**:** 465-471.

Pirmohamed M (2010). Pharmacogenetics of idiosyncratic adverse drug reactions. In Handbook of Experimental Pharmacology. pp 477-491.

Pirmohamed M (2011). Pharmacogenetics: past, present and future Drug discovery today 16**:** 852-861.

Pirmohamed M, Breckenridge AM, Kitteringham NR, & Park BK (1998). Fortnightly review: Adverse drug reactions. British Medical Journal 316**:** 1295-1298.

Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ*, et al.* (2004). Adverse drug reactions as cause of admission to hospital: Prospective analysis of 18 820 patients. British Medical Journal 329**:** 15-19.

Pirmohamed M, & Park BK (2001). Genetic susceptibility to adverse drug reactions. Trends in Pharmacological Sciences 22**:** 298-305.

Plouin PF (2006). Prevalence and etiologies of endocrine hypertension. Best Practice and Research: Clinical Endocrinology and Metabolism 20**:** vii-viii.

Power JJ, Dennis MS, Redlak MJ, & Miller TA (2004). Aspirin-induced mucosal cell death in human gastric cells: Evidence supporting an apoptotic mechanism. Digestive Diseases and Sciences 49**:** 1518-1525.

Radominska-Pandya A, Czernik PJ, Little JM, Battaglia E, & Mackenzie PI (1999). Structural and functional studies of UDP-glucuronosyltransferases. Drug Metabolism Reviews 31**:** 817-899.

Rane A, Sjöqvist F, & Orrenius S (1973). Drugs and fetal metabolism. Clinical Pharmacology and Therapeutics 14**:** 666-672.

Rayapureddi JP, Kattamuri C, Steinmetz BD, Frankfort BJ, Ostrin EJ, Mardon G*, et al.* (2003). Eyes absent represents a class of protein tyrosine phosphatases. Nature 426**:** 295-298.

Rebay I, Silver SJ, & Tootle TL (2005). New vision from Eyes absent: Transcription factors as enzymes. Trends in Genetics 21**:** 163-171.

Redlak MJ, Power JJ, & Miller TA (2005). Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells. American Journal of Physiology - Gastrointestinal and Liver Physiology 289: G731-G738.

Redlak MJ, Power JJ, & Miller TA (2007). Aspirin-induced apoptosis in human gastric cancer epithelial cells: Relationship with protein kinase C signaling. Digestive diseases and sciences 52: 810-816.

Redlak MJ, Power JJ, & Miller TA (2008). Prevention of Deoxycholate-Induced Gastric Apoptosis by Aspirin: Roles of NF-κB and PKC Signaling. Journal of Surgical Research 145: 66-73.

Relling MV, Gardner EE, Sandborn WJ, Schmiegelow K, Pui CH, Yee SW, *et al.* (2011). Clinical pharmacogenetics implementation consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. Clinical Pharmacology and Therapeutics 89: 387-391.

Reuben A, Koch DG, & Lee WM (2010). Drug-induced acute liver failure: Results of a U.S. multicenter, prospective study. Hepatology 52: 2065-2076.

Rhun YL, Kirkland JB, & Shah GM (1998). Cellular responses to DNA damage in the absence of poly(ADP-ribose) polymerase. Biochemical and Biophysical Research Communications 245: 1-10.

Robert A, Nezamis JE, & Lancaster C (1983). Mild irritants prevent gastric necrosis through 'adaptive cytoprotection' mediated by prostaglandins. American Journal of Physiology - Gastrointestinal and Liver Physiology 8: G113-G121.

Rogakou EP, Boon C, Redon C, & Bonner WM (1999). Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol 146: 905-916.

Rostom A, Goldkind L, & Laine L (2005). Nonsteroidal anti-inflammatory drugs and hepatic toxicity: A systematic review of randomized controlled trials in arthritis patients. Clinical Gastroenterology and Hepatology 3: 489-498.

Routledge PA (2012). Safe prescribing: A titanic challenge. British Journal of Clinical Pharmacology 74: 676-684.

Rudic RD, Brinster D, Cheng Y, Fries S, Song WL, Austin S, *et al.* (2005). COX-2-derived prostacyclin modulates vascular remodeling. Circulation Research 96: 1240-1247.

Saeki M, Saito Y, Jinno H, Sai K, Kaniwa N, Ozawa S, *et al.* (2005). Genetic polymorphisms of UGT1A6 in a Japanese population. Drug Metabolism and Pharmacokinetics 20**:** 85-90.

Saely CH, Muendlein A, Vonbank A, Sonderegger G, Aczel S, Rein P, *et al.* (2008). Type 2 diabetes significantly modulates the cardiovascular risk conferred by the PAI-1 - 675 4G/5G polymorphism in angiographied coronary patients. Clinica Chimica Acta 396**:** 18-22.

Santos PG (2014). The combinations of statins and fibrates: Pharmacokinetic and clinical implications. Clinica e Investigacion en Arteriosclerosis 26**:** 7-11.

Scheiman JM (1996). NSAIDS, gastrointestinal injury, and cytoprotection. Gastroenterology Clinics of North America 25**:** 279-298.

Schleef R, Higgins DL, Pillemer E, & Levitt LJ (1989). Bleeding diathesis due to decreased functional activity of type 1 plasminogen activator inhibitor. Journal of Clinical Investigation 83**:** 1747-1752.

Schmassmann A, Zoidl G, Peskar BM, Waser B, Schmassmann-Suhijar D, Gebbers JO, *et al.* (2006). Role of the different isoforms of cyclooxygenase and nitric oxide synthase during gastric ulcer healing in cyclooxygenase-1 and -2 knockout mice. American Journal of Physiology - Gastrointestinal and Liver Physiology 290**:** G747-G756.

Schoen RT, & Vender RJ (1989). Mechanisms of nonsteroidal anti-inflammatory drug-induced gastric damage. The American Journal of Medicine 86**:** 449-458.

Schönberger J, Wang L, Shin JT, Sang DK, Depreux FFS, Zhu H, *et al.* (2005). Mutation in the transcriptional coactivator EYA4 causes dilated cardiomyopathy and sensorineural hearing loss. Nature Genetics 37**:** 418-422.

Scott SA, Sangkuhl K, Stein CM, Hulot JS, Mega JL, Roden DM, *et al.* (2013). Clinical pharmacogenetics implementation consortium guidelines for CYP2C19 genotype and clopidogrel therapy: 2013 update. Clinical Pharmacology and Therapeutics 94**:** 317-323.

Seifried A, Schultz J, & Gohla A (2013). Human HAD phosphatases: Structure, mechanism, and roles in health and disease. FEBS Journal 280**:** 549-571.

Serebruany VL, Steinhubl SR, Berger PB, Malinin AI, Baggish JS, Bhatt DL, *et al.* (2005). Analysis of risk of bleeding complications after different doses of aspirin in 192,036 patients enrolled in 31 randomized controlled trials. American Journal of Cardiology 95**:** 1218-1222.

Serrano P, Lanas A, Arroyo MT, & Ferreira IJ (2002). Risk of upper gastrointestinal bleeding in patients taking low-dose aspirin for the prevention of cardiovascular diseases. Alimentary Pharmacology and Therapeutics 16**:** 1945-1953.

Settipane RA, Constantine HP, & Settipane GA (1980). Aspirin intolerance and recurrent urticaria in normal adults and children. Epidemilogy and review. Allergy: European Journal of Allergy and Clinical Immunology 35**:** 149-154.

Shaheen NJ, Hansen RA, Morgan DR, Gangarosa LM, Ringel Y, Thiny MT*, et al.* (2006). The burden of gastrointestinal and liver diseases, 2006. American Journal of Gastroenterology 101**:** 2128-2138.

Shimasaki N, Watanabe K, Hara M, & Kosaki K (2004). EYA1 mutation in a newborn female presenting with cardiofacial syndrome. Pediatric Cardiology 25**:** 411-413.

Shimozawa N, Okajima K, & Harada N (2007). Estrogen and isoflavone attenuate stress-induced gastric mucosal injury by inhibiting decreases in gastric tissue levels of CGRP in ovariectomized rats. American Journal of Physiology - Gastrointestinal and Liver Physiology 292**:** G615-G619.

Shimozawa N, Okajima K, Harada N, Arai M, Ishida Y, Shimada S*, et al.* (2006). Contribution of Sensory Neurons to Sex Difference in the Development of Stress-Induced Gastric Mucosal Injury in Mice. Gastroenterology 131**:** 1826-1834.

Shiotani A, Sakakibara T, Takami M, Yamanaka Y, Imamura H, Tarumi K-i*, et al.* (2008). Upper gastrointestinal ulcer in Japanese patients taking low dose aspirin. Gastroenterology 134**:** A736-A736.

Shiotani A, Sakakibara T, Yamanaka Y, Nishi R, Imamura H, Fujita M*, et al.* (2009). The preventive factors for aspirin-induced peptic ulcer: Aspirin ulcer and corpus atrophy. Journal of Gastroenterology 44**:** 717-725.

Sica DA, Gehr TWB, & Ghosh S (2005). Clinical pharmacokinetics of losartan. Clinical Pharmacokinetics 44**:** 797-814.

Silver SJ, Davies EL, Doyon L, & Rebay I (2003). Functional dissection of eyes absent reveals new modes of regulation within the retinal determination gene network. Molecular and Cellular Biology 23**:** 5989-5999.

Sim E, Lack N, Wang C-J, Long H, Westwood I, Fullam E, *et al.* (2008). Arylamine N-acetyltransferases: Structural and functional implications of polymorphisms. Toxicology 254**:** 170-183.

Singh BK, Haque SE, & Pillai KK (2014). Assessment of nonsteroidal anti-inflammatory drug-induced cardiotoxicity. Expert Opinion on Drug Metabolism and Toxicology 10**:** 143-156.

Singh G, & Triadafilopoulos G (1999). Epidemiology of NSAID induced gastrointestinal complications. Journal of Rheumatology 26**:** 18-24.

Sirois C, Moisan J, Poirier P, & Grégoire JP (2014). Myocardial infarction and gastro-intestinal bleeding risks associated with aspirin use among elderly individuals with type 2 diabetes. Annals of Medicine 46**:** 335-340.

Smecuol E, Pinto Sanchez MI, Suarez A, Argonz JE, Sugai E, Vazquez H, *et al.* (2009). Low-Dose Aspirin Affects the Small Bowel Mucosa: Results of a Pilot Study With a Multidimensional Assessment. Clinical Gastroenterology and Hepatology 7**:** 524-529.

Soriano LC, & García Rodríguez LA (2010). Risk of upper gastrointestinal bleeding in a cohort of new users of low-dose ASA for secondary prevention of cardiovascular outcomes. Frontiers in Pharmacology OCT.

Sostres C, & Gargallo CJ (2012). Gastrointestinal lesions and complications of low-dose aspirin in the gastrointestinal tract. Best Practice and Research: Clinical Gastroenterology 26**:** 141-151.

Sostres C, Gargallo CJ, Arroyo MT, & Lanas A (2010). Adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and coxibs) on upper gastrointestinal tract. Best Practice & Research Clinical Gastroenterology 24**:** 121-132.

Souza MHLP, Paula Lemos H, Oliveira RB, & Cunha FQ (2004). Gastric damage and granulocyte infiltration induced by indomethacin in tumour factor 1 (TNF-R1) or inducible nitric oxide synthase (iNOS) deficient mice. Gut 53**:** 791-796.

Srinivasan AF, Rice L, Bartholomew JR, Rangaswamy C, La Perna L, Thompson JE, *et al.* (2004). Warfarin-Induced Skin Necrosis and Venous Limb Gangrene in the Setting of Heparin-Induced Thrombocytopenia. Archives of Internal Medicine 164**:** 66-70.

Starodub OT, Demitrack ES, Baumgartner HK, & Montrose MH (2008). Disruption of the Cox-1 gene slows repair of microscopic lesions in the mouse gastric epithelium. American Journal of Physiology - Cell Physiology 294**:** C223-C232.

Stefansson S, McMahon GA, Petitclerc E, & Lawrence DA (2003). Plasminogen activator inhibitor-1 in tumor growth, angiogenesis and vascular remodeling. Current Pharmaceutical Design 9**:** 1545-1564.

Steingoetter A, Sauter M, Curcic J, Liu D, Menne D, Fried M*, et al.* (2015). Volume, distribution and acidity of gastric secretion on and off proton pump inhibitor treatment: A randomized double-blind controlled study in patients with gastro-esophageal reflux disease (GERD) and healthy subjects. BMC Gastroenterology 15.

Stokes MP, Rush J, Macneill J, Ren JM, Sprott K, Nardone J*, et al.* (2007). Profiling of UV-induced ATM/ATR signaling pathways. Proc Natl Acad Sci U S A 104**:** 19855-19860.

Strassburg CP, Nguyen N, Manns MP, & Tukey RH (1998). Polymorphic expression of the UDP-glucuronosyltransferase UGT1A gene locus in human gastric epithelium. Molecular pharmacology 54**:** 647-654.

Stucki M, Clapperton JA, Mohammad D, Yaffe MB, Smerdon SJ, & Jackson SP (2005). MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell 123**:** 1213-1226.

Sun L, Lv H, Wei W, Zhang D, & Guan Y (2010). Angiotensin-converting enzyme D/I and plasminogen activator inhibitor-1 4G/5G gene polymorphisms are associated with increased risk of spontaneous abortions in polycystic ovarian syndrome. Journal of Endocrinological Investigation 33**:** 77-82.

Swarnakar S, Ganguly K, Kundu P, Banerjee A, Maity P, & Sharma AV (2005). Erratum: Curcumin regulates expression and activity of matrix metalloproteinases 9 and 2 during prevention and healing of indomethacin-induced gastric ulcer (Journal of Biological Chemistry (2005) 280 (9409-9415)). Journal of Biological Chemistry 280**:** 29988.

Szabo S, & Vincze A (2000). Growth factors in ulcer healing: Lessons from recent studies. Journal of Physiology Paris 94**:** 77-81.

Tadjuidje E, Wang TS, Pandey RN, Sumanas S, Lang RA, & Hegde RS (2012). The eya tyrosine phosphatase activity is pro-angiogenic and is inhibited by benzbromarone. PLoS One 7.

Taira T, Watanabe T, Tanigawa T, & et a (2009). Roles of syclooxygenase-2 and prostaglandin E receptors in gastric mucosal defence in *Helicobacter pylori*-infected mice. *Inflammopharmacol* 15**:** 132-138.

Takeda Y, Hatano S, Sentoku N, & Matsuoka M (1999). Homologs of animal eyes absent (eya) genes are found in higher plants. Molecular and General Genetics 262**:** 131-138.

Takeuchi K, Aihara E, Sasaki Y, Nomura Y, & Ise F (2006). Involvement of cyclooxygenase-1, prostaglandin E2 and EP1 receptors in acid-induced HCO3 - secretion in stomach. Journal of Physiology and Pharmacology 57**:** 661-676.

Takeuchi K, Araki H, Umeda M, Komoike Y, & Suzuki K (2001). Adaptive gastric cytoprotection is mediated by prostaglandin EP1 receptors: A study using rats and knockout mice. Journal of Pharmacology and Experimental Therapeutics 297**:** 1160-1165.

Takeuchi K, Kato S, Takeeda M, Ogawa Y, Nakashima M, & Matsumoto M (2003). Facilitation by endogenous prostaglandins of capsaicin-induced gastric protection in rodents through EP2 and IP receptors. Journal of Pharmacology and Experimental Therapeutics 304**:** 1055-1062.

Takezono Y, Joh T, Oshima T, Suzuki H, Seno K, Yokoyama Y*, et al.* (2004). Role of prostaglandins in maintaining gastric mucuscell permeability against acid exposure. Journal of Laboratory and Clinical Medicine 143**:** 52-58.

Tanaka A, Hase S, Miyazawa T, & Takeuchi K (2002). Up-regulation of cyclooxygenase-2 by inhibition of cyclooxygenase-1: A key to nonsteroidal anti-inflammatory drug-induced intestinal damage. Journal of Pharmacology and Experimental Therapeutics 300**:** 754-761.

Tanaka A, Matsumoto M, Hayashi Y, & Takeuchi K (2005). Functional mechanism underlying cyclooxygenase-2 expression in rat small intestine following administration of indomethacin: Relation to intestinal hypermotility. Journal of Gastroenterology and Hepatology (Australia) 20**:** 38-45.

Temple R (2006). HY's law: Predicting serious hepatotoxicity. Pharmacoepidemiology and Drug Safety 15**:** 241-243.

Terano A, Sakata-Horie K, Shimada T, Hiraishi H, Yoshiura K, Yoneda M*, et al.* (2001). The role of cellular migration in the repair process of gastric epithelial cells. Life Sciences 69**:** 3083-3089.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR*, et al.* (1995). Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81**:** 801-809.

Tootle TL, Silver SJ, Davies EL, Newman V, Latek RR, Mills I*, et al.* (2003). The transcription factor Eyes absent is a protein tyrosine phosphatase. Nature 426**:** 299-302.

Tschugguel W, Blaicher A, Kramer L, Felfernig M, Jüngling G, Kurz M*, et al.* (1995). Platelet disorders in uraemia before and after haemodialysis under the influence of low dose aspirin. Thrombosis Research 80**:** 225-233.

Tukey RH, & Strassburg CP (2000). Human UDP-glucuronosyltransferases: metabolism, expression, and disease. Annual Review of Pharmacology and Toxicology 40**:** 581-616.

Valkhoff VE, Sturkenboom MCJM, & Kuipers EJ (2012). Risk factors for gastrointestinal bleeding associated with low-dose aspirin. Best Practice & Research Clinical Gastroenterology 26**:** 125-140.

van der Ven AJA, Koopmans PP, van der Meer JWM, & Vree TB (1991). Adverse reactions to co-trimoxazole in HIV infection. The Lancet 338**:** 431-433.

Van Oijen MGH, Barthélémy C, Janssen MJR, Joiris E, Peters WHM, Laheij RJF*, et al.* (2009a). Effect of Genetic Polymorphisms in UDP-Glucuronosyltransferase 1A6 (UGT1A6) on Acetylsalicylic Acid Metabolism in Healthy Female Volunteers. Pharmacology 83**:** 237-242.

Van Oijen MGH, Huybers S, Peters WHM, Drenth JPH, Laheij RJF, Verheugt FWA*, et al.* (2005a). Polymorphisms in genes encoding acetylsalicylic acid metabolizing enzymes are unrelated to upper gastrointestinal health in cardiovascular patients on acetylsalicylic acid. British journal of clinical pharmacology 60**:** 623-628.

Van Oijen MGH, Huybers S, Peters WHM, Drenth JPH, Laheij RJF, Verheugt FWA*, et al.* (2005b). Polymorphisms in genes encoding acetylsalicylic acid metabolizing enzymes are unrelated to upper gastrointestinal health in cardiovascular patients on acetylsalicylic acid. British Journal of Clinical Pharmacology 60**:** 623-628.

Van Oijen MGH, Koetsier MIA, Laheij RJF, Roelofs HMJ, Te Morsche RHM, Peters WHM*, et al.* (2009b). Genetic polymorphisms in UDP-Glucuronosyltransferase 1A6 are not associated with NSAIDs-related peptic ulcer haemorrhage. Drug Metabolism Letters 3**:** 199-204.

Reference section

Vane J, & Booting R (1998). Anti-inflammatory drugs and their mechanism of action. . Inflamm res 47**:** S78-S87.

Virdis A, Colucci R, Fornai M, Blandizzi C, Duranti E, Pinto S*, et al.* (2005). Cyclooxygenase-2 inhibition improves vascular endothelial dysfunction in a rat model of endotoxic shock: Role of inducible nitric-oxide synthase and oxidative stress. Journal of Pharmacology and Experimental Therapeutics 312**:** 945-953.

Vore M, Montgomery C, & Meyers M (1983). Steroid d-ring glucuronides: Characterization of a new class of cholestatic agents. Drug Metabolism Reviews 14**:** 1005-1019.

Vore M, & Slikker W (1985). Steroid D-ring glucuronides: a new class of cholestatic agents. Trends in Pharmacological Sciences 6**:** 256-259.

Voutilainen M, Mantynen T, Farkkila M, Juhola M, & Sipponen P (2001). Impact of non-steroidal anti-inflammatory drug and aspirin use on the prevalence of dyspepsia and uncomplicated peptic ulcer disease. Scand J Gastroenterol 36**:** 817-821.

Vreeburg EM, Levi M, Rauws EAJ, Deventer SJHV, Snel P, Bartelsman JWFM*, et al.* (2001). Enhanced mucosal fibrinolytic activity in gastroduodenal ulcer haemorrhage and the beneficial effect of acid suppression. Alimentary Pharmacology and Therapeutics 15**:** 639-646.

Wallace JL, Caliendo G, Santagada V, Cirino G, & Fiorucci S (2007). Gastrointestinal Safety and Anti-Inflammatory Effects of a Hydrogen Sulfide-Releasing Diclofenac Derivative in the Rat. Gastroenterology 132**:** 261-271.

Wallace JL, McKnight W, Reuter BK, & Vergnolle N (2000). NSAID-Induced gastric damage in rats: Requirement for inhibition of both cyclooxygenase 1 and 2. Gastroenterology 119**:** 706-714.

Wang R (2002). Two's company, three's a crowd: Can H2S be the third endogenous gaseous transmitter? FASEB Journal 16**:** 1792-1798.

Wasse H, Gillen DL, Ball AM, Kestenbaum BR, Seliger SL, Sherrard D*, et al.* (2003). Risk factors for upper gastrointestinal bleeding among end-stage renal disease patients. Kidney International 64**:** 1455-1461.

Watanabe T, Higuchi K, Hamaguchi M, Shiba M, Tominaga K, Fujiwara Y*, et al.* (2004). Monocyte chemotactic protein-1 regulates leukocyte recruitment during gastric ulcer

recurrence induced by tumor necrosis factor-α. American Journal of Physiology - Gastrointestinal and Liver Physiology 287**:** G919-G928.

Wehling M (2014). Non-steroidal anti-inflammatory drug use in chronic pain conditions with special emphasis on the elderly and patients with relevant comorbidities: management and mitigation of risks and adverse effects. European Journal of Clinical Pharmacology.

Wester K, Jönsson AK, Spigset O, Druid H, & Hägg S (2008). Incidence of fatal adverse drug reactions: A population based study. British Journal of Clinical Pharmacology 65**:** 573-579.

White TJ, Arakelian A, & Rho JP (1999). Counting the costs of drug-related adverse events. PharmacoEconomics 15**:** 445-458.

WHO (1972). International drug monitoring: the role ofnational centres. Tech Rep Ser WHO 498.

Wiffen P, Gill M, Edwards J, & Moore A (2002). Adverse drug reactions in hospital

patients. A systematic review of the prospective and retrospective studies. Bandolier Extra pp 1–16.

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC*, et al.* (2004). <div xmlns="http://www.w3.org/1999/xhtml">DRUG-DRUG INTERACTIONS FOR UDP-GLUCURONOSYLTRANSFERASE SUBSTRATES: A PHARMACOKINETIC EXPLANATION FOR TYPICALLY OBSERVED LOW EXPOSURE (AUC<sub>I</sub>/AUC) RATIOS</div&gt. Drug Metabolism and Disposition 32**:** 1201.

Wiman B, & Hamsten A (1990). The fibrinolytic enzyme system and its role in the etiology of thromboembolic disease. Seminars in Thrombosis and Hemostasis 16**:** 207-216.

Wodzinski MA, Bardhan KD, Reilly JT, Cooper P, & Preston FE (1993). Reduced tissue type plasminogen activator activity of the gastroduodenal mucosa in peptic ulcer disease. Gut 34**:** 1310-1314.

Wolfe MM, Lichtenstein DR, & Singh G (1999). Gastrointestinal toxicity of non-steroidal anti-inflammatory drugs. N Engl J Med 340**:** 1888-1899.

Wu HC, Tuo BG, Wu WM, Gao Y, Xu QQ, & Zhao K (2008). Prevalence of peptic ulcer in dyspeptic patients and the influence of age, sex, and Helicobacter pylori infection. Digestive Diseases and Sciences 53**:** 2650-2656.

Wu K, Li Z, Cai S, Tian L, Chen K, Wang J*, et al.* (2013). EYA1 Phosphatase Function Is Essential To Drive Breast Cancer Cell Proliferation Through Cyclin D1. Cancer research 73**:** 4488-4499.

Wypasek E, Ciesla M, Suder B, Janik L, Sadowski J, & Undas A (2015). CYP2C9 polymorphism and unstable anticoagulation with warfarin in patients within the first 3 months following heart valve replacement. Advances in Clinical and Experimental Medicine 24**:** 607-614.

Xu P-X (2013). The EYA-SO/SIX complex in development and disease. Pediatric Nephrology 28**:** 843-854.

Xu PX, Adams J, Peters H, Brown MC, Heaney S, & Maas R (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. Nature Genetics 23**:** 113-117.

Yeomans N, Lanas A, Labenz J, Van Zanten SV, Van Rensburg C, Rácz I*, et al.* (2008). Efficacy of esomeprazole (20 mg once daily) for reducing the risk of gastroduodenal ulcers associated with continuous use of low-dose aspirin. American Journal of Gastroenterology 103**:** 2465-2473.

Yeomans ND (2011). Aspirin: Old drug, new uses and challenges. Journal of Gastroenterology and Hepatology (Australia) 26**:** 426-431.

Zanardo RCO, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, & Wallace JL (2006). Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB Journal 20**:** E1411-E1418.

Zeng N, Wu J, Zhu WC, Ma L, Jia ZL, & Shi B (2013). Associations between EYA1 single-nucleotide polymorphisms and non-syndromic orofacial clefts in Western Han Chinese. J Oral Pathol Med 42**:** 711-715.

Zhang L, Yang N, Huang J, Buckanovich RJ, Liang S, Barchetti A*, et al.* (2005). Transcriptional coactivator Drosophila Eyes Absent Homologue 2 is up-regulated in epithelial ovarian cancer and promotes tumor growth. Cancer Research 65**:** 925-932.

Zhou XM, Wong BCY, Fan XM, Zhang HB, Lin MCM, Kung HF*, et al.* (2001). Non-steroidal anti-inflammatory drugs induce apoptosis in gastric cancer cells through up-regulation of bax and bak. Carcinogenesis 22**:** 1393-1397.

Zhu GH, Wong BCY, Eggo MC, Ching CK, Yuen ST, Chan EYT*, et al.* (1999). Non-steroidal anti-inflammatory drug-induced apoptosis in gastric cancer cells is blocked by protein kinase C activation through inhibition of c-myc. British Journal of Cancer 79**:** 393-400.

Zimmermann KC, Waterhouse NJ, Goldstein JC, Schuler M, & Green DR (2000). Aspirin induces apoptosis through release of cytochrome c from mitochondria. Neoplasia 2**:** 505-513.