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

Thesis submitted in accordance with the requirements of the University of Liverpool degree of Doctor in Philosophy

By

Celestine Esume

July 2016
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.

Celestine Esume

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

To God Almighty, who gives bread to the eater and seed to the sower and make all things possible, may your name be praised!

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!
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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).
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General introduction
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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)

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

<table>
<thead>
<tr>
<th>Severity level</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>An ADR occurred but no change in treatment with suspected drug required</td>
</tr>
<tr>
<td>2</td>
<td>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</td>
</tr>
<tr>
<td>3</td>
<td>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.</td>
</tr>
<tr>
<td>4</td>
<td>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</td>
</tr>
<tr>
<td>5</td>
<td>Any level 4 ADR that requires intensive medical care</td>
</tr>
<tr>
<td>6</td>
<td>The ADR caused permanent harm to patient</td>
</tr>
<tr>
<td>7a</td>
<td>The ADR was indirectly linked to death of patient</td>
</tr>
<tr>
<td>7b</td>
<td>The ADR was directly linked to death of patient</td>
</tr>
</tbody>
</table>
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

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

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 H₁-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 of 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

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

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

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

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 NSAID 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

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>N</th>
<th>Outcome measures</th>
<th>Gene</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Negovan et al., 2015)</td>
<td>Prospective</td>
<td>211</td>
<td>Endoscopic UGIB</td>
<td>AGT</td>
<td>AGT A-20C variant allele increases risk</td>
</tr>
<tr>
<td>(Martínez et al., 2004)</td>
<td>Prospective</td>
<td>218</td>
<td>Endoscopic UGIB</td>
<td>CYP2C9</td>
<td>CYP2C9 *2 alleles ↑ risk</td>
</tr>
<tr>
<td>(Hayashi et al., 2012)</td>
<td>Case-control</td>
<td>548</td>
<td>PUD</td>
<td>IL-17</td>
<td>IL-17 rs2275913 A allele increases risk</td>
</tr>
<tr>
<td>(Pilotto et al., 2007)</td>
<td>Case-control</td>
<td>78</td>
<td>Endoscopic UGIB</td>
<td>CYP2C9</td>
<td>CYP2C9*1/<em>3, CYP2C9</em>1/<em>2 ↑ risk. Higher risk among CYP2C9</em>3 carriers</td>
</tr>
<tr>
<td>(Van Oijen et al., 2005a)</td>
<td>Prospective</td>
<td>160</td>
<td>Symptomatology</td>
<td>UGT1A6</td>
<td>No association</td>
</tr>
<tr>
<td>(Musumba et al., 2013)</td>
<td>Case-control</td>
<td>1239</td>
<td>Endoscopic UGIB</td>
<td>CYP2C family</td>
<td>CYP2C19*17 associated with PUD</td>
</tr>
<tr>
<td>(Martin et al., 2001)</td>
<td>Retrospective</td>
<td>54</td>
<td>GU or GU scar</td>
<td>CYP2C9</td>
<td>No association found</td>
</tr>
<tr>
<td>(Arisawa et al., 2007)</td>
<td>Retrospective</td>
<td>480</td>
<td>PUD</td>
<td>COX-1</td>
<td>1676T allele increases risk; c.-842A&gt;G/c.50G&gt;T no association seen</td>
</tr>
<tr>
<td>(Van Oijen et al., 2005a)</td>
<td>Retrospective</td>
<td>194</td>
<td>PUD</td>
<td>COX-1</td>
<td>c.-842A&gt;G/c.50G&gt;T showed inverse association</td>
</tr>
<tr>
<td>(Halushka et al., 2003)</td>
<td>Prospective</td>
<td>38</td>
<td>Products of AA pathway (PGF$<em>{a2}$, TXA$</em>{a2}$)</td>
<td>COX-1</td>
<td>c.-842A&gt;G/c.50G&gt;T haplotype had higher inhibition of PG formation by aspirin</td>
</tr>
<tr>
<td>(Piazuelo et al., 2008)</td>
<td>Case-control</td>
<td>354</td>
<td>UGIB</td>
<td>eNOS gene</td>
<td>The ‘a’ allele associated with lower risk</td>
</tr>
<tr>
<td>(López-Rodríguez et al., 2008)</td>
<td>Prospective</td>
<td>69</td>
<td>Ibuprofen safety pharmacokinetics pharmacodynamics</td>
<td>CYP2C9</td>
<td>CYP2C9<em>3 linked to ↓ metabolism and clearance of R−ibuprofen, CYP2C8</em>3=↑clearance of R−ibuprofen, ↓iNOS expression, CYP2C8*3 and *4 linked to ↓ adverse effect</td>
</tr>
</tbody>
</table>

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$_{a2}$, thromboxane A$_{a2}$; 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 known 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 antimycobacterial agents such as dapsone, para-aminosalicylic acid and the sulphones. 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

<table>
<thead>
<tr>
<th>COX-selectivity</th>
<th>Chemical classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non selective COX-inhibitors</td>
<td></td>
</tr>
<tr>
<td>• Aspirin</td>
<td><strong>Carboxylic acids</strong></td>
</tr>
<tr>
<td>• Aceclofenac, diclofenac, indomethacin, ketorol, sulindac, tolmetin</td>
<td>• Salicylic Acids: Aspirin, diflunisal, salsalate, trisalicylate</td>
</tr>
<tr>
<td>• Dexketoprofen, dexibuprofen, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, loxoprofen, naproxen, oxaprozin</td>
<td>• Acetic Acids: Aceclofenac, indomethacin, ketorolac, sulindac, tolmetin</td>
</tr>
<tr>
<td>• Flufenamic acid, meclofemanic acid, mefenamic acid, Tolfenamic acid</td>
<td>• Propionic Acids: Dexketoprofen, exibuprofen, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, loxoprofen, naproxen, oxaprozin</td>
</tr>
<tr>
<td>• Droxican, isoxicam, lornoxicam, piroxicam, tenoxicam</td>
<td>• Fenamic Acids: Flufenamic acid, meclofemanic acid, mefenamic acid, tolfenamic acid</td>
</tr>
<tr>
<td>• Phenazone, phenylbutazone, propyphenazonene</td>
<td><strong>Enolic Acids</strong></td>
</tr>
<tr>
<td>COX 2 selective inhibitors</td>
<td>• Oxicams: Droxican, isoxicam, lornoxicam, piroxicam, tenoxicam</td>
</tr>
<tr>
<td>• Celecoxib, etoricoxib, parecoxib, parvocoxib, rofecoxib</td>
<td>• Pyrazolones: Phenazine, phenylbutazone, propyphenazonene</td>
</tr>
<tr>
<td>• At low dose: etodolac, meloxicam, nabumetone, nimesulide</td>
<td><strong>Sulfinilides</strong></td>
</tr>
<tr>
<td></td>
<td>• Nimesulide</td>
</tr>
</tbody>
</table>

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).
Chapter 1

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).

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

Chapter 1

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 worldwide 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.

<table>
<thead>
<tr>
<th>NSAID-related renal complications</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Acute tubular necrosis</td>
<td>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 &amp; Shirali, 2014).</td>
</tr>
<tr>
<td>• Acute kidney injury</td>
<td></td>
</tr>
<tr>
<td>• Pre-renal azotaemia</td>
<td></td>
</tr>
<tr>
<td>• Acute papillary necrosis</td>
<td></td>
</tr>
<tr>
<td>• Hypertension</td>
<td></td>
</tr>
<tr>
<td>• Chronic tubule-interstitial nephritis</td>
<td></td>
</tr>
<tr>
<td>• Acute interstitial nephritis</td>
<td></td>
</tr>
<tr>
<td>• Membranous nephropathy</td>
<td></td>
</tr>
<tr>
<td>• Hyponatremia</td>
<td></td>
</tr>
<tr>
<td>• Minimal change disease</td>
<td></td>
</tr>
<tr>
<td>• Hyperkalaemia and minimal change disease</td>
<td></td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>NSAID-related cardiovascular complication</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid and electrolyte retention</td>
<td>Interference with the renin-angiotensin system (RAS) via PG inhibition (Camin et al., 2015).</td>
</tr>
<tr>
<td>Hypertension</td>
<td>NSAIDs cause fluid and electrolyte retention, and, long term cause an increase in blood pressure (Plouin, 2006).</td>
</tr>
<tr>
<td>Increased risk of cardiovascular events</td>
<td>A number of non-clinical studies have reported that inhibition of prostacyclin (PGI₂) 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).</td>
</tr>
<tr>
<td>Worse prognosis for ischemic reperfusion injury</td>
<td>In ischemic reperfusion injury that could occur following heart attack, COX-2 has been shown to be cardioprotective and is a major source of PGI₂ (Bolli et al., 2002).</td>
</tr>
<tr>
<td>Increased thrombotic events</td>
<td>Reduction in the level of antithrombotic PGI₂, an enhanced synthesis of leukotrienes and generation of reactive oxygen species and consumption of antithrombotic nitric oxide (Antman et al., 2005).</td>
</tr>
<tr>
<td>Aggravation of doxorubicin cardiotoxicity</td>
<td>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).</td>
</tr>
<tr>
<td>Degradation of aortic elastin protein</td>
<td>Blockage of cross linkage formation (Oitate et al., 2007a; Oitate et al., 2007b)</td>
</tr>
<tr>
<td>Endothelial dysfunction</td>
<td>Reduction in available nitric oxide due to COX-2 inhibition (Virdis et al., 2005).</td>
</tr>
</tbody>
</table>

NSAID-related cardiovascular complications occurring via various mechanisms such as modulation of the RAS, fluid and electrolyte retention, inhibition of PGI₂ 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
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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 videoendoscopy 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

Endoscopically 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)

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

*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.

![Diagram of NSAID-induced gastrointestinal injury](image)

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 \( \text{Ca}^{2+} \), release of cytochrome C and generation of reactive oxygen species culminating in cell death.
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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 suppress 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 (PGI2) and prostaglandin E2 (PG E2). 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).
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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)

<table>
<thead>
<tr>
<th>Gastroprotective Effect</th>
<th>Receptors (Species)</th>
<th>COX-isoform</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibit acid secretion</td>
<td>EP&lt;sub&gt;3&lt;/sub&gt; (Mouse, rat) IP (mouse)</td>
<td>COX 1</td>
<td>(Kato et al., 2005; Nishio et al., 2007)</td>
</tr>
<tr>
<td>Stimulate acid secretion</td>
<td>EP&lt;sub&gt;4&lt;/sub&gt; (rat)</td>
<td></td>
<td>(Kato et al., 2005)</td>
</tr>
<tr>
<td>Stimulate mucous secretion in stomach</td>
<td>EP&lt;sub&gt;4&lt;/sub&gt; (rat)</td>
<td></td>
<td>(Araki et al., 2000)</td>
</tr>
<tr>
<td>Stimulate HCO&lt;sub&gt;3&lt;/sub&gt; secretion in stomach</td>
<td>EP&lt;sub&gt;1&lt;/sub&gt; (rat, mouse)</td>
<td>COX 1</td>
<td>(Baumgartner et al., 2004; Takeuchi et al., 2006)</td>
</tr>
<tr>
<td>Stimulate HCO&lt;sub&gt;3&lt;/sub&gt; in duodenum</td>
<td>EP&lt;sub&gt;4&lt;/sub&gt; (rat, human)</td>
<td></td>
<td>(Aoi et al., 2004; Larsen et al., 2005)</td>
</tr>
<tr>
<td>Maintain mucosal hydrophobicity</td>
<td>? (mouse)</td>
<td>COX 1</td>
<td>(Darling et al., 2004)</td>
</tr>
<tr>
<td>Supress ↑TNFα suppression</td>
<td>EP&lt;sub&gt;2&lt;/sub&gt;, EP&lt;sub&gt;4&lt;/sub&gt; (mouse)</td>
<td>COX 2</td>
<td>(Taira et al., 2009)</td>
</tr>
<tr>
<td>Inhibit neutrophil adherence and activation</td>
<td>EP&lt;sub&gt;2&lt;/sub&gt;, EP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>COX 2</td>
<td>(Taira et al., 2009)</td>
</tr>
<tr>
<td>Inhibit epithelial cell apoptosis</td>
<td>EP&lt;sub&gt;2&lt;/sub&gt;, EP&lt;sub&gt;4&lt;/sub&gt; (guinea pig)</td>
<td></td>
<td>(Hoshino et al., 2003)</td>
</tr>
<tr>
<td>↓ gastric hypermotility</td>
<td>EP&lt;sub&gt;1&lt;/sub&gt; (rat)</td>
<td>COX 1</td>
<td>(Araki et al., 2000; Tanaka et al., 2005)</td>
</tr>
<tr>
<td>↓epithelial paracellular permeability</td>
<td>? (rat)</td>
<td>COX 1</td>
<td>(Takezono et al., 2004)</td>
</tr>
<tr>
<td>Adaptive gastric cytoprotection</td>
<td>EP&lt;sub&gt;1&lt;/sub&gt; (rat, mouse)</td>
<td></td>
<td>(Takeuchi et al., 2001)</td>
</tr>
<tr>
<td>Accelerate restitution</td>
<td>? (rat, mouse)</td>
<td>COX 1/2</td>
<td>(Starodub et al., 2008; Terano et al., 2001)</td>
</tr>
<tr>
<td>Accelerate ulcer healing</td>
<td>EP&lt;sub&gt;4&lt;/sub&gt; (mouse)</td>
<td>COX 2</td>
<td>(Hatazawa et al., 2007)</td>
</tr>
<tr>
<td>Resist ischemia and reperfusion induced injury</td>
<td>IP</td>
<td>COX 2</td>
<td>(Kotani et al., 2006)</td>
</tr>
</tbody>
</table>

GMBF: gastric mucosal blood flow; CGRP: calcitonin gene related peptide; COX: cyclooxygenase; EP: Prostaglandin E<sub>2</sub> 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\textsubscript{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\textsubscript{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$_2$S) 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$_2$S. These two enzymes, cystathione-β-synthetase (CBS) and cystathione-Υ-lyase (CSE), involved in the synthesis of H$_2$S are expressed in the gastric mucosal cells. H$_2$S 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$_2$S has been demonstrated to possess anti-inflammatory properties following the observation that H$_2$S-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$_2$S 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$_2$S after the use of NSAID, demonstrated to be mediated by the suppression of the expression of a key enzyme, cystathione-$\gamma$-lyase necessary for the conversion of L-cysteine to H$_2$S (Fiorucci et al., 2007). Hence, the reduction of mucosal H$_2$S 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$_2$S-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, \textit{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 \textit{EYA1} in NSAID-related gastrointestinal injuries.

The aim of this study was to elucidate the biological plausibility and functional effects of \textit{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 \textit{UGT1A6} gene (Shiotani et al., 2009; Van Oijen et al., 2009a; Van Oijen et al., 2009b), and a plausible role for \textit{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 \textit{UGT1A6} and low-dose aspirin related upper gastrointestinal complications in a Caucasian population.
Chapter 2

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Chapter 2

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). In 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-glucuronosyltransferases (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 (Radomsinska-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-naphtol (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
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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

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</table>

^High enzyme activity *in vitro* (Krishnaswamy et al., 2005b; Nagar et al., 2004); ^βlow 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; ^Δsilent effect and renamed from UGT1A6*1b; ^γrenamed from UGT1A6*1c; ^αRenamed from UGT1A6*1d; ^€renamed from UGT1A6*5.
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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 -80ºc.

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).
Figure 2.2: Map of England showing the 15 sites from which the patient cohorts were 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
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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.
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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

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

### 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
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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 \textit{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

<table>
<thead>
<tr>
<th>Endoscopy indication, n %</th>
<th>Patients with PUD (n=800)</th>
<th>Patients without PUD (n=397)</th>
<th>Univariate analysis using Chi square</th>
<th>95%CI</th>
<th>LRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematemesis/melena</td>
<td>317 (39.6%)</td>
<td>24 (6.0%)</td>
<td>4.396-9.670</td>
<td>6.491</td>
<td></td>
</tr>
<tr>
<td>Dyspepsia/abdominal pain</td>
<td>228 (28.5%)</td>
<td>189 (46.9%)</td>
<td>0.510-0.691</td>
<td>0.593</td>
<td></td>
</tr>
<tr>
<td>Anaemia</td>
<td>109 (13.6%)</td>
<td>68 (16.9%)</td>
<td>0.598-0.691</td>
<td>0.788</td>
<td></td>
</tr>
<tr>
<td>Reflux symptoms</td>
<td>20 (2.5%)</td>
<td>8 (2.0%)</td>
<td>0.559-2.713</td>
<td>1.229</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>146 (17.8%)</td>
<td>114 (28.3%)</td>
<td>0.509-0.780</td>
<td>0.629</td>
<td></td>
</tr>
<tr>
<td><strong>NSAID used</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose aspirin</td>
<td>318 (38.1%)</td>
<td>94 (23.3%)</td>
<td>42.6</td>
<td>43.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>High dose aspirin</td>
<td>18 (2.2%)</td>
<td>1 (0.2%)</td>
<td>42.6</td>
<td>43.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>Drug</td>
<td>Never (n, %)</td>
<td>Current (n, %)</td>
<td>Former (n, %)</td>
<td>95% CI</td>
<td>LRT</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
<td>----------------</td>
<td>--------------</td>
<td>----------------</td>
<td>------</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>104 (12.5%)</td>
<td>18 (4.5%)</td>
<td>26.9</td>
<td>30.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>77 (9.2%)</td>
<td>15 (3.7%)</td>
<td>15.4</td>
<td>17.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>4 (0.5%)</td>
<td>1 (0.2%)</td>
<td>7.4</td>
<td>8.4</td>
<td>0.007</td>
</tr>
<tr>
<td>Naproxen</td>
<td>30 (3.6%)</td>
<td>6 (1.5%)</td>
<td>7.4</td>
<td>8.4</td>
<td>0.004</td>
</tr>
<tr>
<td>Oxicams</td>
<td>15 (1.8%)</td>
<td>0 (0%)</td>
<td>15.4</td>
<td>17.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>Coxibs</td>
<td>13 (1.6%)</td>
<td>2 (0.5%)</td>
<td>7.4</td>
<td>8.4</td>
<td>0.007</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>4 (0.5%)</td>
<td>1 (0.2%)</td>
<td>7.4</td>
<td>8.4</td>
<td>0.007</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3 (0.4%)</td>
<td>0 (0%)</td>
<td>26.9</td>
<td>30.8</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Concomitant medications, n (%)**

<table>
<thead>
<tr>
<th>Medication</th>
<th>Never (n, %)</th>
<th>Current (n, %)</th>
<th>Former (n, %)</th>
<th>95% CI</th>
<th>LRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proton pump inhibitors</td>
<td>299 (37.5%)</td>
<td>228 (57.4%)</td>
<td>44.6</td>
<td>44.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Antisecretory medications</td>
<td>32 (4.0%)</td>
<td>13 (3.3%)</td>
<td>0.493</td>
<td>0.505</td>
<td>0.524</td>
</tr>
<tr>
<td>Antiplatelets</td>
<td>44 (5.5%)</td>
<td>19 (4.8%)</td>
<td>0.188</td>
<td>0.190</td>
<td>0.786</td>
</tr>
<tr>
<td>Anticoagulants</td>
<td>51 (6.4%)</td>
<td>20 (5.0%)</td>
<td>1.05</td>
<td>1.08</td>
<td>0.365</td>
</tr>
<tr>
<td>Steroids</td>
<td>32 (4.0%)</td>
<td>14 (3.5%)</td>
<td>7.8</td>
<td>9.4</td>
<td>0.001</td>
</tr>
<tr>
<td>SSRIs</td>
<td>54 (6.8%)</td>
<td>52 (13.1%)</td>
<td>13.2</td>
<td>12.5</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Smoking*, n (%)**

<table>
<thead>
<tr>
<th>Smoking Status</th>
<th>Never (n, %)</th>
<th>Current (n, %)</th>
<th>Former (n, %)</th>
<th>95% CI</th>
<th>LRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never</td>
<td>276 (34.5%)</td>
<td>185 (46.6%)</td>
<td>0.627-0.835</td>
<td>0.723</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>247 (30.9%)</td>
<td>106 (26.7%)</td>
<td>0.932-1.374</td>
<td>1.129</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>311 (38.9%)</td>
<td>113 (28.5%)</td>
<td>1.118-1.60</td>
<td>1.333</td>
<td></td>
</tr>
</tbody>
</table>

**Alcohol intake*, n (%)**

<table>
<thead>
<tr>
<th>Alcohol Intake</th>
<th>Never (n, %)</th>
<th>Current (n, %)</th>
<th>Former (n, %)</th>
<th>95% CI</th>
<th>LRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Audit score &lt;8</td>
<td>672 (84%)</td>
<td>327 (82.4%)</td>
<td>0.940-1.056</td>
<td>0.994</td>
<td></td>
</tr>
<tr>
<td>Audit score 8-15</td>
<td>126 (15.8%)</td>
<td>57 (14.5%)</td>
<td>0.803-1.430</td>
<td>1.070</td>
<td></td>
</tr>
<tr>
<td>Audit score &gt;15</td>
<td>37 (4.6%)</td>
<td>20 (5.0%)</td>
<td>0.531-1.516</td>
<td>0.531</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Status</th>
<th>n</th>
<th>NSAIDs Users (n=588)</th>
<th>Non-users (n=609)</th>
<th>NSAIDs users (n=588)</th>
<th>Non-users (n=609)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>AC</td>
<td>CC</td>
<td>AA</td>
</tr>
<tr>
<td>Ulcer</td>
<td>800</td>
<td>0.48</td>
<td>0.41</td>
<td>0.10</td>
<td>0.41</td>
</tr>
<tr>
<td>No ulcer</td>
<td>397</td>
<td>0.52</td>
<td>0.39</td>
<td>0.08</td>
<td>0.46</td>
</tr>
<tr>
<td>*No UGIB</td>
<td>155</td>
<td>0.50</td>
<td>0.36</td>
<td>0.13</td>
<td>0.46</td>
</tr>
<tr>
<td>Ulcer with UGIB</td>
<td>155</td>
<td>0.50</td>
<td>0.36</td>
<td>0.13</td>
<td>0.46</td>
</tr>
<tr>
<td>Ulcer without UGIB</td>
<td>645</td>
<td>0.48</td>
<td>0.42</td>
<td>0.09</td>
<td>0.40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Status</th>
<th>n</th>
<th>NSAIDs Users (n=426)</th>
<th>Non-users (n=771)</th>
<th>NSAIDs Users (n=426)</th>
<th>Non-users (n=771)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>AC</td>
<td>CC</td>
<td>AA</td>
</tr>
<tr>
<td>Ulcer</td>
<td>800</td>
<td>0.47</td>
<td>0.42</td>
<td>0.11</td>
<td>0.44</td>
</tr>
<tr>
<td>No ulcer</td>
<td>397</td>
<td>0.51</td>
<td>0.42</td>
<td>0.07</td>
<td>0.47</td>
</tr>
<tr>
<td>*UGIB</td>
<td>155</td>
<td>0.49</td>
<td>0.37</td>
<td>0.14</td>
<td>0.49</td>
</tr>
<tr>
<td>*No UGIB</td>
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<td>0.43</td>
<td>0.09</td>
<td>0.45</td>
</tr>
<tr>
<td>Ulcer with UGIB</td>
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<td>0.37</td>
<td>0.14</td>
<td>0.49</td>
</tr>
<tr>
<td>Ulcer without UGIB</td>
<td>645</td>
<td>0.47</td>
<td>0.43</td>
<td>0.10</td>
<td>0.43</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Covariates tested against UGT1A6 SNP</th>
<th>n</th>
<th>Test of association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binary ulcer status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>800</td>
<td>397</td>
</tr>
<tr>
<td>Control</td>
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<td></td>
</tr>
<tr>
<td>Upper GI bleed status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>155</td>
<td>1042</td>
</tr>
<tr>
<td>Control</td>
<td>397</td>
<td></td>
</tr>
<tr>
<td>Ulcer and GI bleed status*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GU</td>
<td>407</td>
<td>397</td>
</tr>
<tr>
<td>DU</td>
<td>313</td>
<td>397</td>
</tr>
<tr>
<td>Ulcer location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender - female</td>
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<td>224</td>
</tr>
<tr>
<td>Gender - male</td>
<td>470</td>
<td>170</td>
</tr>
<tr>
<td>Use of NSAIDs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>588</td>
<td>609</td>
</tr>
<tr>
<td>Control</td>
<td>609</td>
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</tr>
<tr>
<td>Use of PPI</td>
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<td></td>
</tr>
<tr>
<td>Cases</td>
<td>515</td>
<td>670</td>
</tr>
<tr>
<td>Control</td>
<td>670</td>
<td></td>
</tr>
</tbody>
</table>

\(^\text{a}\)excluding non-aspirin NSAIDs; \(^\text{b}\)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 20\textsuperscript{th} 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 ($I^2 = 0$) 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.

![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.](image-url)
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, UGT1A6 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 \textit{H. pylori} positive patients were included in the analysis could be as a result of the confounding effect of \textit{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 \textit{UGT1A6} gene in NSAID-related ulcer. Secondly, we did not genotype other \textit{UGT1A6} SNP which are in close LD with rs1105879, particularly, rs2070959 and rs6759892 which are thought to be the most relevant as far as \textit{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 \textit{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 \textit{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

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

3.1.3 Genetic Variation of PAI-1

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

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3.2.2 Genomic Deoxyribonucleic acid extraction

3.2.3 Allelic discrimination for PAI-1 SNP rs1799889

3.2.4 Statistical analysis

3.3 Results

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3.3.2 Genotype and allele frequency of study patients

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

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Table 3.5: Association of NSAID and ulceration status with PAI-1 SNP
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 α2-plasmin inhibitor (α2-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 α2-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).
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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’AGACAAGGTTGTGACAAGAGA’, while the reverse primer sequence was 5’GGCCGCCTCCGATGATAC’. 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

<table>
<thead>
<tr>
<th>Clinical Variable</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ulc</strong>er <strong>Ca</strong>ses</td>
<td></td>
</tr>
<tr>
<td><strong>NSAID</strong></td>
<td></td>
</tr>
<tr>
<td>Non-aspirin NSAIDs</td>
<td>80 (30.8)</td>
</tr>
<tr>
<td>Aspirin NSAIDs</td>
<td>148 (57.1)</td>
</tr>
<tr>
<td>Both aspirin and Non-aspirin NSAIDs</td>
<td>31 (11.9)</td>
</tr>
<tr>
<td>Number of cases NSAID positive</td>
<td>259 (50.4)</td>
</tr>
<tr>
<td>Number of cases NSAID negative</td>
<td>255 (49.6)</td>
</tr>
<tr>
<td><strong>Total number of cases</strong></td>
<td>514</td>
</tr>
<tr>
<td><strong>Ulc</strong>er <strong>si</strong>te</td>
<td></td>
</tr>
<tr>
<td>Gastric ulcer</td>
<td>237 (46.1)</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>228 (44.4)</td>
</tr>
<tr>
<td>Both</td>
<td>35 (6.8)</td>
</tr>
<tr>
<td>Pyloric</td>
<td>12 (2.3)</td>
</tr>
<tr>
<td>Missing</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>514</td>
</tr>
<tr>
<td><strong>Non-ulc</strong>er <strong>co</strong>nt<strong>ro</strong>ls</td>
<td></td>
</tr>
<tr>
<td>Non-aspirin NSAID exposed</td>
<td>11 (15.7)</td>
</tr>
<tr>
<td>Aspirin-NSAID exposed</td>
<td>59 (84.3)</td>
</tr>
<tr>
<td>Both aspirin and non-aspirin NSAID exposed</td>
<td>0 (0)</td>
</tr>
<tr>
<td>No ulcer, with exposure to NSAID</td>
<td>70 (28.9)</td>
</tr>
<tr>
<td>No ulcer, and no exposure to NSAID</td>
<td>172 (71.1)</td>
</tr>
<tr>
<td><strong>Total number of controls</strong></td>
<td>242</td>
</tr>
<tr>
<td><strong>Total number of participants</strong></td>
<td>756</td>
</tr>
</tbody>
</table>
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

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

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

<table>
<thead>
<tr>
<th>Status</th>
<th>n</th>
<th>NSAIDs Users (n=332)</th>
<th>Non-users (n=424)</th>
<th>NSAIDs Users (n=332)</th>
<th>Non-users (n=424)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5G/5G</td>
<td>4G/5G</td>
<td>4G/4G</td>
<td>5G/5G</td>
</tr>
<tr>
<td>Ulcer</td>
<td>514</td>
<td>0.22</td>
<td>0.52</td>
<td>0.26</td>
<td>0.24</td>
</tr>
<tr>
<td>No ulcer</td>
<td>242</td>
<td>0.25</td>
<td>0.55</td>
<td>0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>UGIB</td>
<td>94</td>
<td>0.18</td>
<td>0.50</td>
<td>0.32</td>
<td>0.17</td>
</tr>
<tr>
<td>No UGIB</td>
<td>662</td>
<td>0.24</td>
<td>0.49</td>
<td>0.28</td>
<td>0.20</td>
</tr>
<tr>
<td>Ulcer with UGIB</td>
<td>94</td>
<td>0.18</td>
<td>0.50</td>
<td>0.32</td>
<td>0.17</td>
</tr>
<tr>
<td>Ulcer no UGIB</td>
<td>420</td>
<td>0.24</td>
<td>0.52</td>
<td>0.24</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Status</th>
<th>n</th>
<th>Aspirin Users (n=238)</th>
<th>Non-aspirin users (n=518)</th>
<th>Aspirin-users (n=238)</th>
<th>Non users (n=518)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5G/5G</td>
<td>4G/5G</td>
<td>4G/4G</td>
<td>5G/5G</td>
</tr>
<tr>
<td>Ulcer</td>
<td>514</td>
<td>0.21</td>
<td>0.52</td>
<td>0.27</td>
<td>0.24</td>
</tr>
<tr>
<td>No ulcer</td>
<td>242</td>
<td>0.27</td>
<td>0.42</td>
<td>0.31</td>
<td>0.21</td>
</tr>
<tr>
<td>UGI bleed</td>
<td>94</td>
<td>0.18</td>
<td>0.50</td>
<td>0.32</td>
<td>0.20</td>
</tr>
<tr>
<td>No UGIB</td>
<td>662</td>
<td>0.24</td>
<td>0.48</td>
<td>0.28</td>
<td>0.23</td>
</tr>
<tr>
<td>Ulcer with UGIB</td>
<td>94</td>
<td>0.17</td>
<td>0.49</td>
<td>0.34</td>
<td>0.20</td>
</tr>
<tr>
<td>Ulcer without UGIB</td>
<td>420</td>
<td>0.22</td>
<td>0.50</td>
<td>0.28</td>
<td>0.22</td>
</tr>
</tbody>
</table>

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 \textit{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 \textit{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, \textit{H. pylori} status, PPI use, gender, and \textit{PAI-1} was 26\%. A sensitivity analysis was done, including only cases without any evidence of \textit{H. pylori} as cases (n=398); there was no association between SNP and binary ulcer status (p=0.124). However, an analysis including only \textit{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 \textit{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 \textit{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

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

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

<table>
<thead>
<tr>
<th>Covariates tested against PAI-1 SNP rs1799889</th>
<th>n</th>
<th>Cases</th>
<th>Control</th>
<th>p</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binary ulcer status</td>
<td></td>
<td>259</td>
<td>255</td>
<td>0.516</td>
<td>0.871; 95% CI 0.56, 1.32</td>
</tr>
<tr>
<td>Upper GI bleed status</td>
<td></td>
<td>94</td>
<td>662</td>
<td>0.198</td>
<td>0.631; 95% CI 0.39, 1.13</td>
</tr>
<tr>
<td>Ulcer and GI bleed status*</td>
<td></td>
<td>94</td>
<td>420</td>
<td>0.644</td>
<td>0.831; 95% CI 0.42, 1.85</td>
</tr>
<tr>
<td>Ulcer status, expanded cohort</td>
<td></td>
<td>514</td>
<td>242</td>
<td>0.742</td>
<td>0.930; 95% CI 0.58, 1.47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ulcer location:</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GU</td>
<td></td>
<td>237</td>
<td>242</td>
<td>0.144</td>
<td>0.980; 95% CI 0.48, 1.60</td>
</tr>
<tr>
<td>DU</td>
<td></td>
<td>228</td>
<td>242</td>
<td>0.170</td>
<td>0.857; 95% CI 0.54, 1.58</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>514</td>
<td>242</td>
<td>0.0001</td>
<td>1.790, 95% CI 1.30, 2.45</td>
</tr>
<tr>
<td>PPI use</td>
<td></td>
<td>311</td>
<td>355</td>
<td>0.001</td>
<td>3.560; 95% CI 1.95, 6.53</td>
</tr>
</tbody>
</table>

*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, PAI-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

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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).

![Manhattan plot of GWAS study on NSAID-related GI complications](image)

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).
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.

Figure 4.2: A: The Rossmann-like fold of HAD phosphatases formed by repeating β-α units (Seifried et al., 2012). 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.
Chapter 4

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

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

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 drosophila 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

EYA1 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 EYA4 with associated heart failure and sensorineural loss has been reported. Biochemical analysis has demonstrated that following this deletion mutation, EYA4’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 *EYA4*, produced by the deletion mutation, to bind *EYA4* and SIX proteins. In a study of the role of *EYA4* 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 EYA1-4. 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 foregoing, 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°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 from 100 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: $2^{-\Delta C_T} (EYA1 \text{ gene})/ 2^{-\Delta C_T} (\text{Beta actin gene})$. 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°C 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°C and the protein supernatant transferred into a new eppendorf tube and stored frozen at -80°C.

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 dH₂O, 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 radish 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**

<table>
<thead>
<tr>
<th>Covariates tested against EYA1 SNP rs12678747</th>
<th>n</th>
<th>Test of association</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSAIDs ulcer vs No ulcer</td>
<td>466</td>
<td>0.0003</td>
<td>1.52; 95% CI 1.21, 1.91</td>
</tr>
<tr>
<td>H. pylori + NSAIDs ulcer</td>
<td>230</td>
<td>0.0004</td>
<td>1.63; 95% CI 1.24, 2.13</td>
</tr>
<tr>
<td>NSAIDs ulcer vs no ulcer in H. pylori positive patients^</td>
<td>230</td>
<td>0.0001</td>
<td>2.79; 95% CI 1.57, 4.95</td>
</tr>
<tr>
<td>GU</td>
<td>407</td>
<td>0.787</td>
<td></td>
</tr>
<tr>
<td>DU</td>
<td>313</td>
<td>0.192</td>
<td></td>
</tr>
<tr>
<td>Binary ulcer status</td>
<td>800</td>
<td>0.970</td>
<td>1.00; 95% CI 0.82, 1.23</td>
</tr>
<tr>
<td>Upper GI bleed status</td>
<td>155</td>
<td>0.514</td>
<td></td>
</tr>
<tr>
<td>Ulcer and GI bleed status*</td>
<td>155</td>
<td>0.297</td>
<td></td>
</tr>
</tbody>
</table>

^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

<table>
<thead>
<tr>
<th>Status</th>
<th>n</th>
<th>NSAIDs Users (n=588)</th>
<th>Non-users (n=609)</th>
<th>NSAIDs Users (n=588)</th>
<th>Non-users (n=609)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>AT</td>
<td>TT</td>
<td>AA</td>
</tr>
<tr>
<td>Ulcer</td>
<td>800</td>
<td>0.38</td>
<td>0.50</td>
<td>0.12</td>
<td>0.57</td>
</tr>
<tr>
<td>No ulcer</td>
<td>397</td>
<td>0.56</td>
<td>0.32</td>
<td>0.12</td>
<td>0.51</td>
</tr>
<tr>
<td>*UGIB</td>
<td>155</td>
<td>0.43</td>
<td>0.45</td>
<td>0.12</td>
<td>0.63</td>
</tr>
<tr>
<td>*No UGIB</td>
<td>1042</td>
<td>0.42</td>
<td>0.47</td>
<td>0.11</td>
<td>0.54</td>
</tr>
<tr>
<td>Ulcer with UGIB</td>
<td>155</td>
<td>0.43</td>
<td>0.45</td>
<td>0.12</td>
<td>0.63</td>
</tr>
<tr>
<td>Ulcer no UGIB</td>
<td>645</td>
<td>0.38</td>
<td>0.52</td>
<td>0.10</td>
<td>0.57</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Status</th>
<th>n</th>
<th>Aspirin Users (n=426)</th>
<th>Non-aspirin users (n=771)</th>
<th>Aspirin-users (n=426)</th>
<th>Non users (n=771)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>AT</td>
<td>TT</td>
<td>AA</td>
</tr>
<tr>
<td>Ulcer</td>
<td>800</td>
<td>0.35</td>
<td>0.52</td>
<td>0.13</td>
<td>0.54</td>
</tr>
<tr>
<td>No ulcer</td>
<td>397</td>
<td>0.58</td>
<td>0.32</td>
<td>0.10</td>
<td>0.51</td>
</tr>
<tr>
<td>*UGI bleed</td>
<td>155</td>
<td>0.42</td>
<td>0.46</td>
<td>0.12</td>
<td>0.56</td>
</tr>
<tr>
<td>*No UGIB</td>
<td>1042</td>
<td>0.40</td>
<td>0.48</td>
<td>0.12</td>
<td>0.52</td>
</tr>
<tr>
<td>Ulcer with UGIB</td>
<td>155</td>
<td>0.42</td>
<td>0.45</td>
<td>0.13</td>
<td>0.55</td>
</tr>
<tr>
<td>Ulcer without UGIB</td>
<td>645</td>
<td>0.33</td>
<td>0.54</td>
<td>0.13</td>
<td>0.54</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Covariates tested against EYA1 SNP rs12678747</th>
<th>n</th>
<th>Test of association</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^5$Binary ulcer status</td>
<td>Cases</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>397</td>
</tr>
<tr>
<td>$^5$Upper GI bleed status</td>
<td>155</td>
<td>1042</td>
</tr>
<tr>
<td>$^5$Ulcer and GI bleed status*</td>
<td>155</td>
<td>645</td>
</tr>
</tbody>
</table>

*Ulcer and bleed=case, ulcer no bleed=control; $^5$H. pylori positive patients only in both case and controls. GU=gastric ulcer; DU=duodenal ulcer. $^5$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

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

*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 $4.86 \times 10^{-6}$, $6.7 \times 10^{-4}$ and $1.0 \times 10^{-3}$ 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

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>EYA1</th>
<th>β-Actin</th>
<th>ΔC&lt;sub&gt;T&lt;/sub&gt;</th>
<th>Relative expression (2^ΔC&lt;sub&gt;T&lt;/sub&gt;)</th>
<th>^P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS</td>
<td>36.94</td>
<td>19.29</td>
<td>17.65</td>
<td>4.86X10^-6</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>HEK</td>
<td>36.88</td>
<td>26.33</td>
<td>10.55</td>
<td>6.7X10^-4</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>Healthy HGB</td>
<td>38.22</td>
<td>26.63</td>
<td>11.59</td>
<td>1.0X10^-3</td>
<td>*P&lt;0.05</td>
</tr>
</tbody>
</table>

*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 (4.86X10^-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

<table>
<thead>
<tr>
<th></th>
<th>EYA1 protein density analysis</th>
<th>β-actin protein density analysis</th>
<th>Relative expression (EYA1/β-actin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Density (CNT/MM²)</td>
<td>Mean density ± SE</td>
<td>Density (CNT/MM²)</td>
</tr>
<tr>
<td>5µg sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10µg sample</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>20µg sample</td>
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</table>

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 than 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

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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

<table>
<thead>
<tr>
<th>Author</th>
<th>Title</th>
<th>Aspirin conc.</th>
<th>Treatment duration</th>
<th>High through-put screening method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leung et al., 2009</td>
<td>Aspirin-induced mucosal cell death in Human gastric cells: Role of caspase-independent mechanism</td>
<td>40mM</td>
<td>2-4 hrs</td>
<td>Acridine orange-ethidium bromide(EtBr) assay</td>
</tr>
<tr>
<td>Redlak et al., 2005</td>
<td>Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells</td>
<td>40mM</td>
<td>1-4 hrs</td>
<td>Cell death ELISA kit</td>
</tr>
<tr>
<td>Power et al., 2004</td>
<td>Aspirin-induced mucosal cell death in Human Gastric cells: Evidence supporting an apoptotic mechanism</td>
<td>3-50mM</td>
<td>24 hrs</td>
<td>Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) assay</td>
</tr>
<tr>
<td>Redlak et al., 2008</td>
<td>Prevention of deoxycholate-induced gastric apoptosis by aspirin: Roles of NF-kB and PKC signalling</td>
<td>40mM</td>
<td>1 hr</td>
<td>Acridine orange-ethidium bromide (EtBr) assay</td>
</tr>
<tr>
<td>Redlak et al., 2007</td>
<td>Aspirin-Induced Apoptosis in Human Gastric Cancer Epithelial Cells: Relationship with Protein Kinase C Signaling</td>
<td>40mM</td>
<td>1-4 hrs</td>
<td>Cell death ELISA Kit</td>
</tr>
<tr>
<td>Gu et al., 2005</td>
<td>Activation of the caspase-8/Bid and Bax pathways in aspirin-induced apoptosis in gastric cancer</td>
<td>1-4mM</td>
<td>1-3 days</td>
<td>MTT, acridine orange staining</td>
</tr>
<tr>
<td>Zhou et al., 2001</td>
<td>Non-steroidal anti-inflammatory drugs induce apoptosis in gastric cancer cells through up-regulation of bax and bak</td>
<td>1mM</td>
<td>0, 12, 24, 36, 48, 60</td>
<td>MTT, acridine orange staining</td>
</tr>
<tr>
<td>Zhu et al., 1999</td>
<td>Non-steroidal anti-inflammatory drug-induced apoptosis in gastric cancer cells is blocked by protein kinase C activation through inhibition of c-myc</td>
<td>0.1-10mM</td>
<td>1-3 days</td>
<td>Measurement of DNA content of cells by PI staining and FACS analysis to detect the preG1 peak; 2) Agarose gel electrophoresis of genomic DNA to detect fragment; 3) AO staining to detect the typical morphological changes under fluorescence microscopy</td>
</tr>
</tbody>
</table>
5.1.3 Benzbromarone and benzaron 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 benzaron 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 \textit{in vitro}, \textit{in vivo} and \textit{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\textsubscript{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 benzaron 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 benzaron, 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 benzaron and an IC\textsubscript{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 \textit{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 S\textsubscript{139} 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 S\textsubscript{139} 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 1-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 trypsinized 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°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.
Chapter 5

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

Molecular weight of Aspirin = 180 g/mol (C₉H₈O₄)

75mg of Aspirin = 0.075 g

No. of moles in 0.075g of aspirin = \( \frac{\text{weight}}{\text{Mol weight}} = \frac{0.075}{180} = 4.166 \times 10^{-4} \text{ M} = 0.416 \text{ mM} \)

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

For 100mls gastric juice, \( C = \frac{0.416}{0.1} = 4.2 \text{ mM/L} \)
For 300mg of aspirin:

Number of moles = \( \frac{\text{weight}}{\text{Mol weight}} = \frac{0.3}{180} = 1.66 \times 10^{-3} = 1.66\text{mM} \)

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

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

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

<table>
<thead>
<tr>
<th>Aspirin concentrations (mM) in media</th>
<th>pH of media at each aspirin concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.30</td>
</tr>
<tr>
<td>0.2</td>
<td>8.20</td>
</tr>
<tr>
<td>1</td>
<td>8.20</td>
</tr>
<tr>
<td>2</td>
<td>8.08</td>
</tr>
<tr>
<td>10</td>
<td>7.79</td>
</tr>
<tr>
<td>20</td>
<td>7.37</td>
</tr>
<tr>
<td>50</td>
<td>6.40</td>
</tr>
<tr>
<td>100</td>
<td>3.98</td>
</tr>
</tbody>
</table>

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.
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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 Zvd 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.
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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.
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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 (15,000 cells/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 15,000 cells/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.](image-url)
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

<table>
<thead>
<tr>
<th>Aspirin treatment for 30 minutes, 3 and 12 hours</th>
<th>Viability (%) of treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>3O min ± SE</td>
</tr>
<tr>
<td>0mM</td>
<td>100±0</td>
</tr>
<tr>
<td>0.2mM</td>
<td>94.1±4.5</td>
</tr>
<tr>
<td>1mM</td>
<td>92.1±3.0</td>
</tr>
<tr>
<td>2mM</td>
<td>86.7±5.5</td>
</tr>
<tr>
<td>10mM</td>
<td>74.9±4.3</td>
</tr>
<tr>
<td>20mM</td>
<td>72.3±3.1</td>
</tr>
<tr>
<td>50mM</td>
<td>54.4±2.7</td>
</tr>
<tr>
<td>100mM</td>
<td>6.0±4</td>
</tr>
<tr>
<td>Staurosporine (1µM)</td>
<td>79.3±3.2***</td>
</tr>
</tbody>
</table>

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= 30minutes; 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 (>4µM) (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

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

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 12 hours 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 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 benz bromarone, no Zvad, 12 hours assay; narrow horizontal bars= aspirin+5μM benz bromarone 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 50 mM. 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.
Chapter 5

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 benzbrormarone 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
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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 (>65 years) 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, lifestyle, 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.
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