# Genetic factors affecting antimicrobial-induced liver injury

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## Abstract

Flucloxacillin and co-amoxiclav are both associated with drug-induced liver injury (DILI). HLA genotype is an important predictor of DILI susceptibility but it is likely that non-HLA risk factors also contribute. This study aimed to characterise non-HLA risk factors in larger cohorts (155 flucloxacillin and 165 co-amoxiclav adjudicated cases) than previously. Drug causality of the cases was assessed using the RUCAM method which showed 88.1% of the cases were either highly probable or probable but 11.9% of them indicated a possible causality for the drug. Variants showing associations in previous candidate gene, genome-wide association and exome sequencing studies were genotyped to extend these findings.

A SNP (rs2476601) in *PTPN22*, which encodes a protein involved in T-cell-receptor signalling had already been shown to be a risk factor for co-amoxiclav DILI. This was confirmed by genotyping co-amoxiclav DILI cases (n=99) (OR=2.74, 95% CI=1.58–4.77; P=$4.1 \times 10^{-4}$). There was also a significant effect for flucloxacillin DILI (OR=1.9, 95% CI=1.1–3.1; P=0.02).

Exome sequencing performed previously on 66 UK co-amoxiclav DILI cases reported significant associations for several variants, including rs117511121 in *IL12RB1* and rs145855109 in *TPH1*. Additional cases (n=99) were genotyped for rs117511121, confirming the association (OR 6.5, 95% CI=1.5-27.8; P=0.012). No association with *IL12RB1* genotype was seen for flucloxacillin DILI. Functional analysis of *IL12RB1* using reporter gene constructs revealed significantly lower luciferase activity for the variant constructs. The *TPH1* variant was confirmed to be associated with co-amoxiclav DILI (n=99) (OR=14.73, 95% CI=2.94–73.92; P=0.013). Polymorphisms in the following genes showed no significant association with DILI due to either drug: *FMO5, GPX1, GSTM1, GSTT1, HFE, KCNJ1, SHMT1, SLCO1B1, SOD2, ST6GAL1* and *UGT1A1*.

The findings for *PTPN22* and *IL12RB1* confirm the relevance of T cell responses to co-amoxiclav DILI. Odds ratios of 17 for DILI risk can be calculated for individuals with the at risk HLA alleles (*A*02:01* and *DRB1*15:01*) and the *PTPN22* and *IL12RB1* variants, assuming an additive model. *PTPN22* is also relevant to flucloxacillin DILI but, though biologically plausible as a risk factor, appears minor compared with *HLA-B*57:01*.

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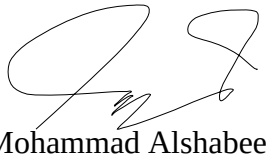
## Declaration of Originality

I hereby certify that the work described in this thesis is entirely my own except the work specifically stated in the text. No part of this thesis has been published or submitted for a previous degree.



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| **ABCB11** | ATP-binding cassette, sub-family B member 11 |
| **ABCB4** | ATP-binding cassette, sub-family B member 4 |
| **ABCC2** | ATP-binding cassette, sub-family C member 2 |
| **Acryl/Bis** | Acrylamide/ bis-acrylamide |
| **AD** | Aithal and Day |
| **ALP** | Alkaline phosphatase |
| **ALT** | Alanine transaminase |
| **AOR** | Adjusted odds ratio |
| **APC** | Antigen presenting cells |
| **AST** | Aspartate aminotransferase |
| **ATP** | Adenosine triphosphate |
| **BMI** | Body mass index |
| **BP** | Blood pressure |
| **BSEP** | Bile Salt Export Pump |
| **CARM** | Centre for Adverse Reactions Monitoring |
| **CDS** | Clinical diagnostic scale |
| **CI** | Confidence interval |
| **CIOMS** | Council for International Organizations of Medical Sciences |
| **Co-amoxiclav** | Amoxicillin-clavulanic acid |
| **COMT** | Catechol-O-methyl transferase |
| **COX-2** | Cyclooxygenase 2 |
| **CYP** | Cytochrome |
| **DEPC** | Diethyl pyrocarbonate |
| **DILI** | Drug-induced liver injury |
| **DILIGEN** | Idiosyncratic Drug-induced Liver Injury Study |
| **DILIN** | Drug Induced Liver Injury Network |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dNTPs** | Deoxyribonucleotide triphosphates |
| **ECG** | Electrocardiograms |
| **FAM** | Fluorescein amidite |
| **FDA** | Food and Drug Administration |
| **FIC1** | Familial intrahepatic cholestasis type 1 |
| **FMO** | Flavin-containing monooxygenase |
| **GADD45G** | Growth arrest and DNA-damage-inducible protein GADD45 gamma |
| **GPRD** | General practice research database |
| **CPRD** | Clinical practice research datalink |
| **GPX1** | Glutathione peroxidase 1 |
| **GRID** | Genetic Resource Investigating Diabetes study |
| **GSTs** | Glutathione S-transferases |
| **GWAS** | Genome wide association study |
| **H2O2** | Hydrogen peroxide |
| **HAART** | Highly active antiretroviral therapy |
| **HBV** | Hepatitis B virus |
| **HCV** | Hepatitis C virus |

| | |
|---|---|
| **HDL** | High-density lipoprotein |
| **HER2** | Human Epidermal Growth Factor Receptor 2 |
| **HFE** | Hemochromatosis gene |
| **HIV** | Human immunodeficiency virus |
| **HLA** | Human leukocyte antigen |
| **HMG-CoA** | 3-hydroxy-3-methyl-glutaryl-CoA |
| **5-HT** | 5-hydroxytryptamine |
| **IBD** | Irritable bowel disorder |
| **iDILIC** | International Drug-induced Liver Injury Consortium |
| **IL** | Interleukin |
| **IL12RB1** | Interleukin 12 receptor, beta 1 |
| **JIA** | Juvenile idiopathic arthritis |
| **KASP** | KBiosciences Competitive Allele-Specific PCR |
| **KC** | Kupffer cells |
| **KCNJ1** | Potassium inwardly-rectifying channel, subfamily J, member 1 |
| **LB** | Lysogeny broth |
| **LD** | Linkage disequilibrium |
| **LYP** | Lymphoid-specific phosphatase |
| **MAF** | Minimum allele frequency |
| **MDR3** | Multidrug resistance protein 3 |
| **MHC** | Major histocompatibility complex |
| **MnSOD** | Manganese Superoxide Dismutase |
| **MRP2** | Multidrug resistance-associated protein 2 |
| **MV** | Maria and Victorino |
| **NAFLD** | Non-alcoholic fatty liver disease |
| **NAT2** | N-acetyltransferase 2 |
| **NEB** | New England Biolabs |
| **NIH** | National Institutes of Health |
| **NK** | Natural killer |
| **NKT** | Natural killer T cells |
| **NSAIDs** | Nonsteroidal anti-inflammatory drugs |
| **OATP1B1** | Organic anion-transporting polypeptide 1B1 |
| **OR** | Odds ratio |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBS** | Phosphate-buffered solution |
| **PCR** | Polymerase chain reaction |
| **POPRES** | Population Reference sample |
| **PRP** | Penicillinase resistant penicillins |
| **PTPN22** | Protein tyrosine phosphatase, non-receptor type 22 |
| **PXR** | Pregnane X receptor |
| **RA** | Rheumatoid arthritis |
| **RFLP** | Restriction Fragment Length Polymorphism |
| **RNA** | Ribonucleic acid |
| **ROMK** | Renal outer medullary potassium channel |
| **ROS** | Reactive oxygen species |
| **RR** | Relative risk |

| | |
|---|---|
| **RUCAM** | Roussel Uclaf Causality Assessment Method |
| **SARS** | Severe acute respiratory syndrome |
| **SH3** | Src homology 3 |
| **SHMT1** | Serine hydroxymethyltransferase 1 |
| **SLCO1B1** | Solute carrier organic anion transporting polypeptide 1B1 |
| **SLE** | Systemic lupus erythematosus |
| **SLS** | Scientific laboratory supplies |
| **SMS** | Smith-Magenis syndrome |
| **SNP** | Single-nucleotide polymorphism |
| **SOB** | Super Optimal Broth medium |
| **SOD2** | Superoxide dismutase 2 |
| **SOLiD** | Sequencing by Oligonucleotide Ligation and Detection |
| **SPSS** | Statistical Package for the Social Sciences |
| **ST6GAL1** | ST6 beta-galactosamide alpha-2,6-sialyltransferase 1 |
| **STAT4** | Signal Transduction And Transcription |
| **T1D** | Type 1 diabetes mellitus |
| **T2D** | Type 2 diabetes mellitus |
| **TAE** | Tris/ Acetic acid /EDTA buffer |
| **TB** | Tuberculosis |
| **TBE** | Tris/Borate/EDTA buffer |
| **TCR** | T-cell-receptor |
| **TDM** | Therapeutic drug monitoring |
| **TEMED** | Tetramethylethylenediamine |
| **Th1** | T helper 1 |
| **Th2** | T helper 2 |
| **TNF** | Tumor necrosis factor |
| **TPH1** | Tryptophan hydroxylase 1 |
| **UGT1A** | UDP glucuronosyltransferase 1 family, polypeptide A |
| **ULN** | Upper limit of normal |
| **USPHS** | U. S. Public Health Service |
| **UTR** | Untranslated region |
| **UV** | Ultraviolet |
| **VKORC1** | Vitamin K epoxide reductase complex subunit 1 |

# Chapter 1. Introduction

# 1. Introduction

## 1.1 Drug-induced liver injury (DILI)

The liver is particularly susceptible to drug toxicity due to its vital role in xenobiotic metabolism and elimination. Drug-induced liver injury (DILI) involves a widely variable range of toxicity comprising alteration in liver biochemical tests and different levels of liver damage; for example, hepatitis, necrosis, steatosis, cirrhosis, fulminant liver failure, and blood clots of the veins within the liver which probably leads to significant patient morbidity and mortality (Farmer and Brind, 2011). The toxicity could occur within days of drug exposure or may possibly delay in onset for up to several months (Dall'Olio et al., 2004) (Hussaini and Farrington, 2007). The majority of DILI cases, particularly mild and moderate, return to normal condition upon cessation of the causative hepatotoxic agent though cholestatic reactions tend to be prolonged compared to hepatocellular (Manmeet et al., 2011). However, in some cases there may be persistent liver damage as has been proven in the study conducted in Newcastle by Aithal *et al.* (1999) where they found that 39% of DILI patients still showed positive evidence of liver disease on an average of five years post DILI incidence. In contrast, elevated levels of liver enzymes may resolve in some patients even if they continue with their drug treatment. This is known as the phenomenon of adaptation (Watkins et al., 2008). At present, no predicting factors can differentiate between individuals who may develop reversible DILI from those who suffer prolonged liver dysfunction (Senior, 2009). Despite the rarity of idiosyncratic hepatotoxicity (approx 1 case in 1000 to 100,000 patients on certain medications), it is considered a serious medical problem and may be the cause of up to 17% of acute liver failure cases (Hussaini and Farrington, 2007). In a prospective French population-based study (Sgro et al., 2002), about 14 cases per 100,000 patient prescriptions were reported with an estimated incidence of 8000 DILI cases and approximate 500 mortality rate per year. Higher DILI incidence rate was reported by Björnsson and colleagues (2013) who prospectively reviewed prescription databases of 4209 patients in Iceland over 2-year period (2010-2011) and the DILI rate observed reached up to 19.1 cases per 100,000 inhabitants. In general, DILI occurrence accounts for an average of 3% to 9% of all worldwide reported adverse drug reactions (Food and Drug Administration, 2008).

## 1.2 DILI classifications, phenotypes and symptoms

Liver injury can be classified based on histological features and the rise in liver enzymes into mild, moderate or acute severe type. The Food and Drug Administration (FDA) in the United States has defined severe types of DILI as those cases with elevated "serum alanine transaminase (ALT) > 3 times the upper limit of normal with a serum bilirubin > 2 times the upper limit of normal in the absence of biliary obstruction" (Davidson et al., 1978). The most common patterns of DILI are a cholestatic phenotype, due to bile duct obstruction and disturbances in bile secretion, a hepatocellular phenotype that is characterized by the rise of liver enzymes particularly ALT, or a mixed presentation, however, hepatocellular toxicity is the more frequent DILI form (Jackson et al., 2009). At early stages of DILI, cholestatic patients are asymptomatic with only increases in serum alkaline phosphatase (ALP) but later characterized by yellowish appearance of skin and eyes as a consequence of abnormally high blood levels of bilirubin (jaundice, the "sign of increased risk of severe liver failure") (European Association for the Study of the Liver, 2009; Rochling and Zetterman, 2008). Cholestasis may be accompanied by pruritus, dark urine, clay-coloured or white stools and nausea or vomiting. On the other hand, hepatocellular patients are mainly asymptomatic or have a range of fairly mild symptoms such as general malaise. Also, hepatotoxicity can be categorized into predictable (intrinsic, developed secondary to direct chemical reactions within hepatocyte cells) or unpredictable reactions (idiosyncratic) which account for the majority of liver injury due to drug use (Fingerote, 2008). Predictable toxicity is often detectable during the process of drug development in preclinical studies using suitable animal models. However, idiosyncratic toxicity, believed to be related more to the genetic characteristics of susceptible individuals, may only be discovered after widespread use of the medication in the post marketing phase (Andrews and Daly, 2008). Therefore, drug manufactures and health practitioners are requested to continue drug postmarketing surveillance and risk assessment of larger numbers of patients to detect adverse reactions which were not discovered previously.

## 1.3 Hepatotoxic drugs

A range of prescribed and over the counter medications, vitamins, hormones as well as herbal products and dietary supplements can induce hepatic toxicity in susceptible

individuals. Antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs) and anticonvulsants are believed to be the most common hepatotoxic agents (Chang and Schiano, 2007) but over one thousand medications of various classes, (Table 1.1), are known to induce liver injury including antidepressants, statins, antiretroviral therapy, immunomodulatory agents, antihypertensives and antineoplastic agents. The best known hepatotoxic drug is paracetamol which is responsible for almost 40% of the current acute liver failure cases in the United States (Fingerote, 2008) though these are generally due to deliberate overdose not idiosyncratic toxicity. Different hepatotoxic medications may induce different toxic reactions; for example, NSAIDs, statins, paracetamol and ketoconazole, tend to cause hepatocellular injury, while tricyclic antidepressants, anabolic steroids, estrogens (oral contraceptives), flucloxacillin and clopidogrel more likely induce cholestatic reactions. However, many other therapeutic agents such as sulfonamides, cyclosporine, amitriptylene, carbamazepine and captopril are associated with a mixed liver injury phenotype (Fingerote, 2008). Over the past decades, several regulatory actions were released by FDA against many new drugs including product approval rejections, use warnings, withdrawal from the market and restrictions on drug use; idiosyncratic injury was considered the most common single cause of such decisions (Food and Drug Administration, 2008). DILI is the leading cause of drug withdrawals and hence considered one of the main obstacles facing drug discovery and development process. A list of some drug regulatory actions made by FDA between 1995 and 2007 are provided in Table 1.2.

### *1.3.1 Antimicrobials*

Antimicrobials are the leading hepatotoxic drug group and are responsible for up to 70% of all documented DILI cases (Hussaini and Farrington, 2007). One recent study (Chalasani et al., 2008) in the United States on 300 DILI patients found that 45.5% of the enrolled cases were due to antimicrobials. Numerous reports and clinical trials have shown that large numbers of antibiotics have the ability to injure the liver (Table 1.3). Larger DILI epidemiology study conducted in Switzerland has followed 4209 newly admitted patients to screen the incidence of DILI during patients' hospitalization and found that 1.4% of medical inpatients developed liver injury related mainly to antibiotics and antituberculosis drugs (Meier et al., 2005).

**Table 1.1: Drugs and compounds predominantly associated with hepatocellular or cholestatic damage.**

| Hepatocelullar Damage | Cholestatic Damage |
|---|---|
| Acarbose | Amoxicillin-clavulanic acid |
| Allopurinol | Azathioprine |
| Amiodarone | Captopril, enalapril, fosinopril |
| Amoxicillin, Ampicillin | Benoxaprofen |
| Anti-HIV:(didanosine, zidovudine, protease inhibitors) | Bupropion |
| Non-steroidal antiinflammatory drugs: | Carbamazepine |
| (ibuprofen, diclofenac, piroxicam, indometacin) | Carbimazole |
| Asparaginase | Cloxacillin, dicloxacillin |
| Bentazepam | Clindamycin |
| Bromfenac | Ciprofloxacin, norfloxacin |
| Chlormethiazole | Contraceptive steroids |
| Cocaine, Ecstasy and amphetamine derivatives | Cyproheptadine |
| Diphenytoin | Diazepam, Nitrazepam |
| Disulfiram | Estrogens |
| Ebrotidine | Gold compounds,penicillamine |
| Fluoxetine, paroxetine | Herbal remedies: |
| Flutamide | [Chaparral leaf (*Larrea tridentate*), Glycyrrhizin, |
| Halothane | Greater celandine (*Chelidonium majus*)] |
| Herbal remedies: | Irbesartan |
| [(Chaso and Onshido, Herbalife®, Germander (*Teucrium chamaedrys*), senna Pennyroyal oil, kava-kava (*Piper Methysticum*), *Camellia sinnensis,* (green tea), Chinese herbal medicines)] | Lipid lowering drugs: (atorvastatin, fluvastatin) |
| | Macrolide antibiotics: (Erythromycin) |
| | Mirtazapine |
| | Penicillin G |
| Leflunomide | Phenotiazines (chlorpromazine) |
| Lipid lowering drugs: (lovastatin, pravastatin) | Raloxifen |
| Isoniazid | Rofecoxib, Celecoxib |
| Ketoconazole, Mebendazole, albendazole, pentamidine | Rosiglitazone, pioglitazone |
| Mesalazine | Sulfamethoxazole-trimethoprim |
| Methotrexate | Sulfonylureas: (Glibenclamide,Chlorpropamide) |
| Minocycline | Sulindac |
| Nitrofurantoin | Terbinafine |
| Nefazodone | Tamoxifen |
| Omeprazole | Tetracycline |
| Pemoline | Ticlopidine, Clopidogrel |
| Pyrazinamide | Thiabendazole |
| Risperidone | Tricyclic antidepressants: (Amitriptyline, Imipramine) |
| Ritodrine | |
| Sulfasalazine | |
| Telithromycin | |
| Terbinafine | |
| Tetracycline | |
| Tolcapone | |
| Topiramate | |
| Trazodone | |
| Troglitazone | |
| Trovafloxacin | |
| Valproic acid | |
| Venlafaxine | |
| Verapamil | |
| Vitamin A | |
| Ximelagatran | |

Adapted fromAndrade et al. (2009).

**Table 1.2: Examples of regulatory actions issued for some drugs due to DILI association (1995-2007).**

| Withdrawals | Second line | Warnings |
|---|---|---|
| Bromfenac | Felbamate | Paracetamol |
| Troglitazone | Tolcapone | Leflunomide |
| Pemoline | Trovafloxacin | Nefazodone |
| Alpidem | | Nevirapine |
| Chlormezanon | | Pyrazinamide/rifampin |
| Tolrestat | | Terbinafine |
| Tolcapone | | Valproic acid |
| Amineptine | | Zifirlukast |
| Trovafloxacin | | Atomoxetine |
| Ximelagatran | | interferon 1b –1b and 1a |
| Lumiracoxib | | Saquinavir |
| Aprotinin | | Infliximab |
| Thioridazine | | Bosentan |
| Rofecoxib | | Telithromycin |
| Rapacuronium | | kava |

Adapted from Food and Drug Administration (2008).

**Table 1.3: Main antimicrobials linked to DILI (Kaplowitz and Deleve, 2003).**

| |
|---|
| Amoxicillin-clavulanic acid (co-amoxiclav) |
| Antituberculosis drugs, particularly isoniazid and pyrazinamide |
| Antifungals particularly azole group containing |
| Flucloxacillin |
| Macrolides (erythromycin, clarithromycin, azithromycin) |
| Nitrofurantoin |
| Quinolones (e.g. ciprofloxacin) |
| Tetracyclines (e.g. minocycline, doxycycline) |
| Trimethoprim- sulfamethoxazole |

Adapted from Kaplowitz and Deleve (2003).

#### *1.3.1.1 Co-amoxiclav*

Co-amoxiclav is a broad spectrum antibiotic containing the semisynthetic aminopenicillin amoxicillin trihydrate and a β-lactamase inhibitor (clavulanic acid) indicated in a wide range of infectious diseases including anaerobic infections (Easton et al., 2003). Analysis of antibiotics prescribing pattern in 26 European countries over 5 years between 1997 and 2002 showed that co-amoxiclav was among the top prescribed agents with a remarkable yearly increase in the product sale (Goossens et al., 2005).

Despite the possibility of an adverse drug reaction occurrence like nausea, vomiting, diarrhea and skin rashes which are common to most antibiotics, co-amoxiclav is a well tolerated drug with acceptable safety records. The first incident reported about co-amoxiclav hepatotoxicity was in 1988 (Garcia Rodriguez et al., 1996); thereafter, various trials have confirmed the cholestatic nature of co-amoxiclav toxicity, though other phenotypes may occur (O'Donohue et al., 2000). A number of studies have revealed variable incidence of DILI, between 1and 17 cases/ 100,000 prescriptions associated with co-amoxiclav use (Hussaini and Farrington, 2007). The highest reported incidence was seen in the United Kingdom in the study conducted by Garcia Rodriguez et al. (1996). This result was later supported by the findings of Hussaini et al. (2007) who described co-amoxiclav as the most common cause of jaundice. Co-amoxiclav was also reported as the leading drug causing DILI in the largest two prospective registries conducted in Spain, among 505 drugs involved (Andrade et al., 2005), and in the USA Drug Induced Liver Injury Network (DILIN) study. Among more than 100 different DILI drugs excluding paracetamol reported in DILIN project, co-amoxiclav was the reported cause in 23 DILI cases and then trimethoprim–sulfamethaxazole, nitrofurantoin and isoniazid came second with 13 cases each (Chalasani et al., 2008).

#### *1.3.1.2 Flucloxacillin*

Flucloxacillin is a semisynthetic narrow spectrum beta-lactam antibiotic, first developed in 1964, used in the management of infections caused by gram positive micro-organisms in particular penicillinase producing staphylococci. Flucloxacillin and other penicillinase resistant penicillins (PRP) including nafcillin, methicillin, oxacillin, cloxacillin and dicloxacillin are considered second generation penicillins

consisted of isoxazolyl ring connected to a beta-lactam ring but with a different side chain (Figure 1.1). Flucloxacillin's antibacterial activity is due to its beta-lactam ring whereas the side chain determines the antibacterial spectrum and pharmacologic properties. All penicillins, including both flucloxacillin and amoxicillin, inhibit bacterial cell wall synthesis leading to bacterial cell lysis. The resultant inhibition occurred as a consequence of specific binding of penicillins with penicillin-binding proteins (transpeptidase enzymes) located inside the bacterial cell wall (Dave et al., 2014). As discussed in Section 1.3.1.1, amoxicillin is a broad spectrum penicillin whose aminopenicillin side chain allows entry into gram negative as well as gram positive bacterial cells. In the case of flucloxacillin, the presence of the isoxazolyl group plays a major role of the class resistance to bacterial strains producing beta-lactamases but this compound is not able to cross gram negative cell envelopes. Flucloxacillin is a 3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolylpenicillin, which differs from dicloxacillin by substitution of chlorine with flourine atom at position 6 of the phenyl ring; the halogen atom in this position is deleted in cloxacillin. Within this drug class, flucloxacillin is the only marketed agent in the UK while dicloxacillin is the only isoxazolyl penicillin available in the US. Unlike nafcillin and methicillin, flucloxacillin is acid stable and can be administered orally as well as parenterally in a range of 0.25-1 gram every 6 hours with a maximum dose of 8 grams per day in cases of severe infections like osteomyelitis and endocarditis (Turnidge and Grayson, 1993).

*In vitro* cytotoxicity studies conducted by Lakehal et al. (2001) did not show toxicity signals from human hepatocytes or biliary epithelial cells when both cell lines exposed to high flucloxacillin concentration margins up to 500 mg/L. However, clinical trials performed retrospectively and prospectively have confirmed hepatotoxicity of flucloxacillin with its phenotype characteristics recognized predominantly as cholestatic, and the risk of toxicity varies between 3.6 and 8.5 cases per 100,000 prescriptions (Li et al., 2009; Russmann et al., 2005). It was reported among the commonest drugs causing jaundice (Hussaini et al., 2007). This fact of drug toxicity propelled the Australian Department of Human Services and Health to stop manufacturer advertisement of flucloxacillin in 1994 and to restrict its use to severe infections only; this action plan resulted in reduction of flucloxacillin prescription by 30% thereafter in Australia (Roughead et al., 1999).

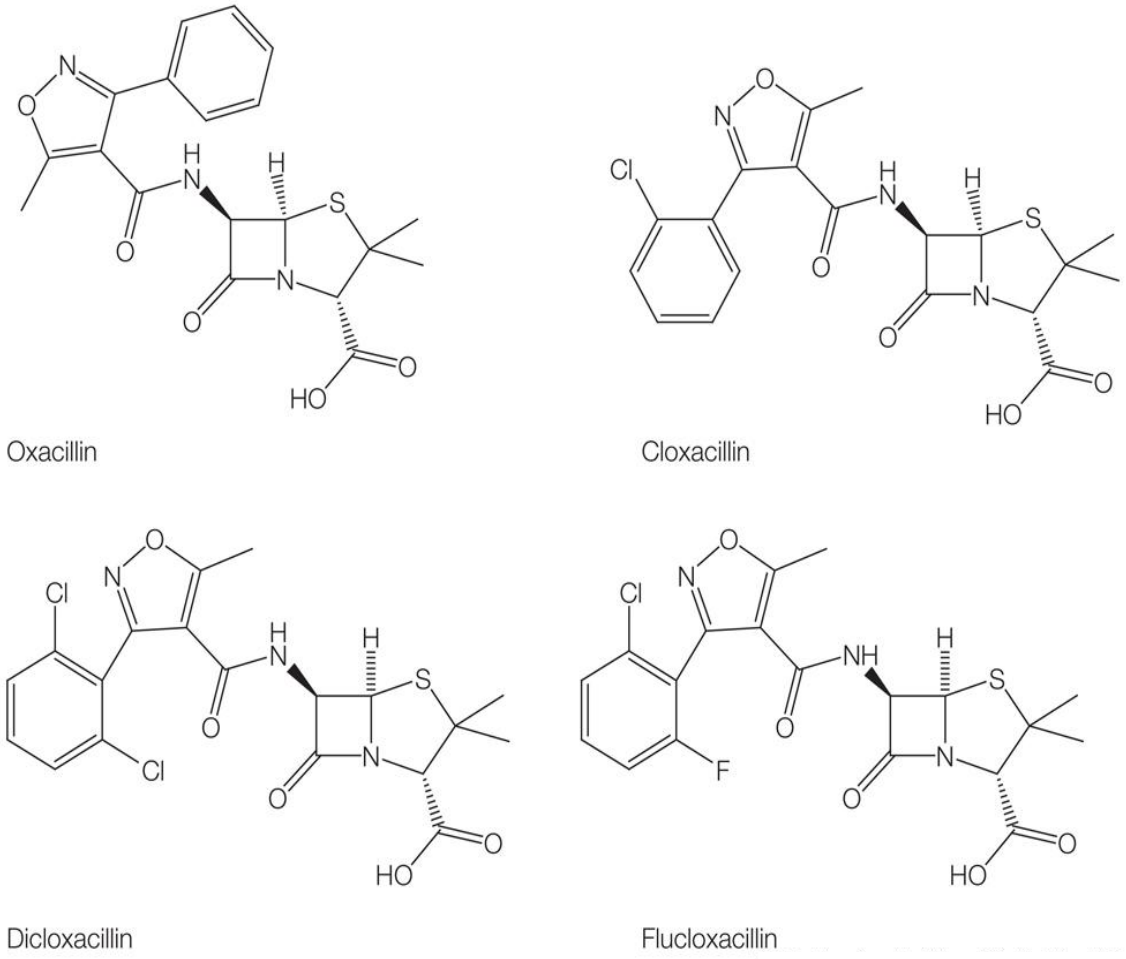


**Figure 1.1: Schematic diagrams of penicillinase resistant penicillins.** Adapted from (Sutherland et al., 1970).

On the other hand, flucloxacillin is still heavily used in hospitals and primary care settings in the UK as a first line antibiotic in the treatment of staphylococcal infections (e.g. cellulitis) despite publications of several UK warning reports including the concerns from the UK Medicines Controls Agency that illustrated the serious idiosyncratic hepatotoxicity associated with flucloxacillin use (Russmann et al., 2005).

The reason why flucloxacillin is associated with liver toxicity in some patients whereas the rate of this toxicity with other beta lactam antibiotics such as amoxicillin is much lower is still fairly unclear. However, female gender, age over 55, duration of therapy above 14 days and higher doses are the major factors that increase the risk of flucloxacillin hepatotoxicity in addition to being positive for HLA-B*57:01 (Andrews and Daly, 2008; Daly et al., 2009; Hussaini and Farrington, 2007; Kaplowitz and Deleve, 2003).

#### *1.3.1.3 Other antimicrobials and DILI*

Other than co-amoxiclav and flucloxacillin, a range of antimicrobials listed in Table 1.3 are known to cause liver injury. Antituberculosis drugs, particularly isoniazid and pyrazinamide, are considered the most common antimicrobial class related to DILI incidence (Bell and Chalasani, 2009; Chalasani and Bjornsson, 2010; Chalasani et al., 2008; Cho et al., 2007; Fernandez-Villar et al., 2004; Huang et al., 2003; Kopanoff et al., 1978; Lang et al., 2007; Vuilleumier et al., 2006). In a recent surveillance study of DILI cases throughout 2002 to 2006 based in Thailand, 85% of the recorded antimicrobial DILI (n=80) were caused by antituberculosis agents while the remaining 12 cases (15%) were reported to different antibiotics (Treeprasertsuk et al., 2010). The bacteriostatic macrolides (erythromycin, clarithromycin and azithromycin) have a high potential to induce cholestatic liver damage as proved by multiple reports (Kaplowitz and Deleve, 2003) (Andrade et al., 2009). Macrolides came usually third after co-amoxiclav and flucloxacillin among non-tuberculosis antimicrobials in term of their association significance to DILI and their high percentage of total reported cases. According to the UK-based General Practice Research Database of DILI reported between January 1994 to December 1999, the strongest non-tuberculosis antimicrobial associations observed were belongs to co-amoxiclav (adjusted odds ratio (AOR) = 94.8; 95% CI=27.8-32), flucloxacillin

(AOR=17.7; 95% CI=4.4-71.0) then macrolides (AOR=6.9; 95% CI=2.3-21.0) (de Abajo et al., 2004). Among the 360 non-tuberculosis antimicrobial DILI cases registered by The New Zealand Centre for Adverse Reactions Monitoring (CARM) between the period of 2000 to 2012, macrolides contributed to 22% of the of DILI cases compared to 47% that were related to co-amoxiclav and flucloxacillin (Medsafe, 2012). Tetracyclines (4%), cotrimoxazole (4%) and quinolones (3%) were also reported to DILI but came down the list. In the Swedish antimicrobial DILI surveillance, DILI cases caused by quinolone agents (ciprofloxacin and moxifloxacin) were more frequently (22%) than cases related to macrolide group (13%). The differences in DILI incidence rate of particular drug or class of antimicrobials from one country to another may be affected by differences in the pattern of antibiotic use restriction guidelines towards specific agents according to the regular resistance figures reported by infection control committees in each country. Apart from two fatal incidents of liver toxicity which occurred to elderly individuals upon administration of ciprofloxacin (Fuchs et al., 1994; Grassmick et al., 1992), its use is considered generally safe. The new generation long acting quinolone trovafloxacin was associated with serious hepatic injury which may end up with hepatocellular vacuolar degeneration and necrosis, therefore its use is currently very limited (Owens and Ambrose, 2005). Hepatic toxicity induced by tetracyclines was first reported several decades ago in 1964 and 1965 (1964; Wruble et al., 1965) and then many other DILI cases were routinely recorded worldwide with a low incidence rate of 1.5 cases per 100,000 prescriptions (Andrade and Tulkens, 2011). Fewer incidents of liver toxicity were documented for the newer generation tetracyclines (doxycyline and minocycline). They are considered less hepatotoxic (mainly cholestatic) than tetracyline with an expected incidence rate of one case per 18 million prescriptions (Polson, 2007). Biliary sludge caused by ceftriaxone (third generation cephalosporine) –calcium precipitate is very common in adults (25%) and pediatric patients (40%) (Kaur and Singh, 2011). Despite the small number of DILI cases reported as a result of drug intake of the combination trimethoprim/sulfamethoxazole known as cotrimoxazole, few cases developed fulminant liver failure (Ransohoff and Jacobs, 1981). Other beta-lactam penicillins rarely cause hepatotoxic reactions. Antifungal agents can also cause liver injury (Andrade and Tulkens, 2011).

## 1.4 Pathogenesis of DILI

Drug-related adverse hepatic reactions are thought to evolve either due to a direct exposure of hepatocytes to toxic parent drugs or their metabolites (non-immune pathway) or as a result of inflammatory mediators activation (immune mediated), though the exact mechanisms by which drugs enhance liver toxicity is not clear yet (Kaplowitz, 2004). In contrast to non-immune pathway, the immune mediated DILI is commonly accompanied by the classical allergic reactions including eosinophilia, fever and rash, although absence of such reactions does not necessarily exclude immune response contribution to DILI as in the case of drug-induced autoimmunity (Uetrecht, 2008).

### *1.4.1 Metabolic hepatotoxicity*

Non-immune-related drug induced liver injury appears to be triggered by ingestion of toxic dose of certain drugs or through the production of toxic drug metabolites, generated during phase I and phase II of drug metabolism, which could affect the biochemistry of liver cells including hepatocytes, sinusoidal endothelial cells or bile duct epithelium and lead to cell death (Kaplowitz, 2002). Numerous environmental and genetic factors, discussed in detail in Sections 1.5 and 1.6, particularly polymorphisms affecting drug metabolising enzymes, have been suggested to play a role in induction of metabolic-related DILI.

### *1.4.2 Immunological pathway*

As shown in Figure 1.2, two immune hypotheses, the hapten and the danger, have been suggested to be involved in drug-induced immune-mediated hepatotoxicity (Uetrecht, 2008). In the hapten hypothesis, the reactive drug or reactive metabolite, the hapten, may initiate an immune response through their covalent binding to liver proteins such as cytochrome P450 enzymes as a large carrier. Neither the drug or its metabolite nor the carrier protein is able by itself to elicit the first signal of immune response; however, formulation of drug-protein-peptide complex molecule acts as the signal (Kaplowitz and DeLeve, 2013). This peptide complex, known as antigen, will need to be presented to HLA molecules by antigen presenting cells (APC) before the interaction step with helper T cell receptors located on CD4+ cells takes place. This interaction stimulates downstream activation of both cytotoxic T cells (CD8+) and B cells which in turn enhance production of hapten-specific antibodies and

autoantibodies. This is considered the first signal among immune response process which requires a further signal known as the danger signal. External factors (e.g. drugs) or host elements like released cytokines, viruses or bacteria have the potential to act as a danger signal that enhances an oxidative stress response in liver cells causing cell damage. Consequently, the enhanced expression of these costimulatory factors stimulates helper T cell to interact with HLA molecules to initiate immune response which may ultimately cause effects such as mitochondrial impairment and disruption of adenosine triphosphate (ATP) synthesis leading to cellular apoptosis or necrosis (Au et al., 2011).

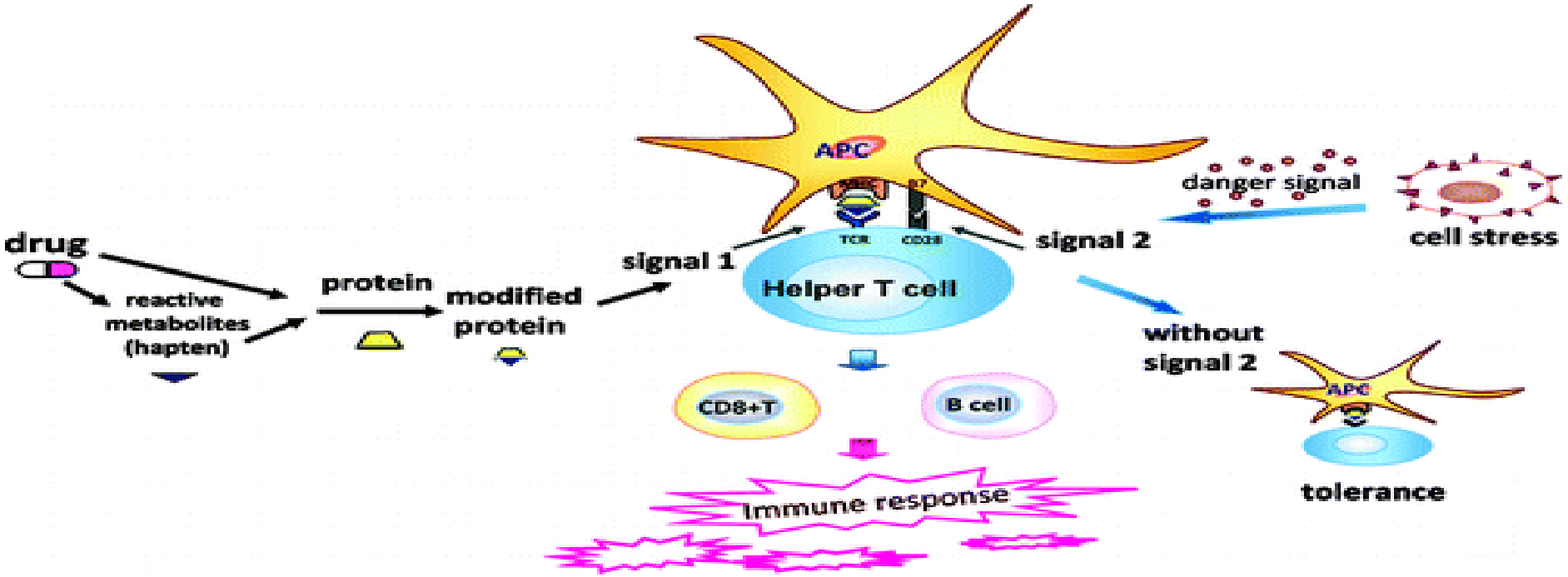


**Figure 1.2: Hapten and danger hypotheses.** The hapten hypothesis involves a chemically reactive drug or reactive metabolite acting as a hapten by binding to protein, which is then taken up by an APC and processed. The processed antigen is presented in the context of MHC to a helper T cell; this represents signal 1. The danger hypothesis involves cell damage or stress (possibly caused by the drug or reactive metabolite) causing the release of danger signals that lead to upregulation of costimulatory factors; this is signal 2. Without signal 2, the result is immune tolerance. Adopted from (Uetrecht, 2008).

## 1.5 DILI non-genetic susceptibility factors

Limited risk factors are known to enhance DILI development including genetic characteristics and environmental factors (non-genetic) (see Table 1.4); therefore, it is difficult to predict an individual's susceptibility to DILI from a certain drug except in the case of known previous history of DILI development due to exposure to that particular drug. The chance of liver injury recurrence is significantly high upon drug rechallenge, though certain patients may not experience repeated episodes of DILI when same drug is given again several weeks or months later. This phenomenon confirms that genetic predisposition is not the only contributing factor to hepatic adverse drug reactions (Watkins et al., 2008). The individual's age, sex, co-morbid diseases and polypharmacy appear to be the most common non-genetic factors affecting host susceptibility. In addition, drug dose, tendency of ingested drug to interact with other drugs, drug metabolism by cytochrome P450 and other enzymes may play a major role in DILI susceptibility as well. A recent Spanish study investigated the incidence of antituberculosis DILI in two different groups, a risk factor group (n=231) and non-risk factor group (n=240). Risk factors were age above 50 years, history of chronic liver disease, hepatitis C virus (HCV) or hepatitis B virus (HBV) infection, using other drugs, baseline elevated AST/ALT, chronic alcoholism and malnutrition. DILI incidence was 3-4 times higher in the risk group than the non-risk factor group (Fernandez-Villar et al., 2004).

### *1.5.1 Age*

Increased age was reported as a risk to develop hepatic injury in antituberculosis drug-exposed patients (Gronhagen-Riska et al., 1978). However, other researchers observed that only cholestatic/mixed toxicity type was more frequent in older patients while the younger population tend to develop hepatocellular DILI reactions (Ghabril et al., 2010). In a prospective Spanish study (Lucena et al., 2006) which recruited 69 co-amoxiclav DILI cases over a 10 year-period (1994-2004), 65% of the study population were over 55 years old. This finding is consistent with the typical pattern of cholestatic toxicity seen with co-amoxiclav. Further evidence of an age contribution to DILI was obtained by a consortium of several centres in the United States, the DILIN study, which enrolled 300 DILI cases over 3 years (2004-2007).

**Table 1.4: Factors that cause predisposition to idiosyncratic DILI.**

| **Non-genetic factors** | **Genetic variability** |
|---|---|
| Age | **Phase 1 enzymes** |
| | CYP2B6 |
| Sex | CYP2E1 |
| | CYP2C8 |
| Daily dose | |
| | **Phase 2 and detoxifying Enzymes** |
| Metabolism profile | NAT2 |
| | GSTM1 and T1 |
| Drug interactions | UGT1A, UGT2B7 |
| | **Drug transporters** |
| Alcohol | BSEP (ABCB11) |
| | MRP2 (ABCC2) |
| **Underlying comorbidities** | MDR3 (ABCB4) |
| (pre-existing liver disease, HIV infection, diabetes) | **Immunologic** |
| | HLA allele |
| | Cytokines ( IL-4, IL-6, IL-10, TNF -α) |
| | **Mitochondrial enzymes** |
| | MnSOD (SOD2) |
| | GPX1 |

Adapted from (Chalasani and Bjornsson, 2010).

The majority of patients with cholestatic and mixed phenotype were older (mean age of 54 years) than the hepatocellular group (mean age of 44 years) (Chalasani et al., 2008). Likewise, Lucena et al. (2009) found that 91 out of 149 (61%) of cholestatic patients recruited between April 1994 to August 2007 were over 60 years old and reported that relative risk of cholestatic type of injury may increase by 1.024 (odds ratio for an age interval) for each year.

### *1.5.2 Sex*

A considerable amount of literature has been published on the influence of individual's gender on DILI development. Women have been shown to have a higher risk of developing hepatotoxicity, particularly hepatocellular injury, caused by drugs in multiple studies relating to different drug products (chlorpromazine, erythromycin, isoniazid, flucloxacillin, methyldopa and nitrofurantoin) (Bell and Chalasani, 2009; de Abajo et al., 2004). In a 3 year retrospective study conducted by the FDA (1988-1991), females accounted for 79% of the reported 180 diclofenac-associated hepatotoxicity cases (Banks et al., 1995). Similarly, statisticaly significant gender deference was obtained (P=0.007) towards female sex which represented 63% of the hepatocellular DILI cases registered by Swedish University hospital during the period 1995 to 2005 (De Valle et al., 2006). Furthermore, the DILIN prospective trial found that 65% of hepatocellular DILI cases were women. Also, a recent Spanish study involving 603 DILI cases (310 males and 293 females) determined that 82% of the cases who experienced fulminant liver failure and/or needed liver transplantation were females (21 females versus 4 males) (Lucena et al., 2009). This finding supports previous results obtained by Russo et al. (2004) who noticed that 76% of patients who experienced acute hepatic necrosis due to non-paracetamol drugs and needed liver transplantation were women. These figures indicate a strong relationship between female sex and DILI severity. On the contrary though, males have shown greater DILI susceptibility with a smaller range of drugs like azathioprine and co-amoxiclav (Bell and Chalasani, 2009; Farmer and Brind, 2011).

### *1.5.3 Race*

Racial differences among populations could affect drug responsiveness and toxicity as a consequence of their genetic variability (Wood, 1998). Polymorphisms common

in certain ethnic groups may be found rarely in other ethnicities; for example, the NAT2*5B haplotype, which is formed by the three variants 341C, 481T, and 803G, is found more commonly among white Europeans and very rare in East Asians and Africans. Instead, high frequencies of NAT2*7 in East Asians and NAT2*12A in African populations are seen (Kang et al., 2009; Sabbagh et al., 2008). Similar differences are found with regard to allele frequencies for CYP2C9*2 and CYP2C9*3 between white Europeans (11-13% and 6-8%, respectively) and individuals from East Asia (zero and 2-4%, respectively) (Buzoianu et al., 2012; DeLozier et al., 2005; Garcia-Martin et al., 2006; Xie et al., 2002). In a U. S. Public Health Service (USPHS) cooperative surveillance study involving 13,838 individuals on isoniazid (Kopanoff et al., 1978), statistics have shown higher incidence of DILI in white males than African-Americans. On the other hand, the risk of isoniazid related DILI was higher in Asian males (twice the risk in whites and 14 times the risk in African Americans). No significant differences were seen between women of different races included in the study.

### *1.5.4 Co-morbidities*

Few co-morbidities are known to increase the risk for DILI among individuals. Patients with human immunodeficiency virus (HIV) and/or pre-existing liver diseases including HBV, HCV and non-alcoholic fatty liver disease (NAFLD) were found at increased risk of DILI due to specific drugs. In patients co-infected with HCV or HIV, development of antituberculosis drugs-induced hepatotoxicity increased by 5 fold and 4 fold, respectively, compared to the healthy population and the risk increased to 14.4 fold in those positive for both viruses (Ungo et al., 1998). Similar results were obtained when protease inhibitor-containing highly active antiretroviral therapy (HAART) used for 82 HIV-1 patients co-infected with HBV (n=27) or HCV (n=55) in comparison to 312 patients infected with HIV-1 only. Higher relative risk (RR) of HAART induced hepatotoxicity seen in co-infected groups (RR= 2.78 and 2.46 in HBV and HCV groups, respectively) (den Brinker et al., 2000). However, no associations with DILI detected in hepatitis C positive patients when various medications to treat additional diseases were used (e.g. statins) (Khorashadi et al., 2006). On the other hand, NAFLD patients have shown higher propensity to develop liver injury when exposed to tamoxifen to manage breast cancer (Elefsiniotis et al., 2004). Use of tamoxifen as an adjuvant therapy in 60

women with breast cancer and liver steatosis induced hepatotoxicity in 43.3% of them, however, clinical and laboratory data of the affected group were found significantly different in body mass index (BMI), baseline fasting glucose, cholesterol and triglyceride levels than non-affected group ($P<0.001$). Type-2 diabetics as well as obese patients were found more susceptible to hepatic injury when treated with drugs that induce mitochondrial oxidative stress like methotrexate (Boelsterli and Lim, 2007). In a small size study (n=78), all diabetic patients who administered methotrexate for treatment of psoriasis developed liver fibrosis compared to 52% of non-diabetics (Rosenberg et al., 2007).

### *1.5.5 Polypharmacy and drug interactions*

Polypharmacy, which refers to usage of combination of several medications by a patient, is commonly seen in older populations as a result of their multiple diseases. This concomitant administration of various drug products at a time may lead to drug interactions and could exacerbate adverse drug reactions, reduce or synergize their efficacy. Hepatotoxicity of some drug combinations could occasionally be augmented and the risk becomes greater than the toxicity risk of each drug used separately. The synergistic hepatotoxic effect of the antituberculosis 4-drug regimen (rifampicin, isoniazid, pyrazinamide and ethambutol) is a well recognised example though use of such regimens is vital in treating this disease. A retrospective study involved 128 acute and clinically relevant DILI patients registered between 1994 and 1999 in UK-based General Practice Research Database (GPRD, currently known as Clinical Practice Research Datalink (CPRD)) found that DILI was 6 times higher among patients receiving two or more hepatotoxic drugs (de Abajo et al., 2004). Interactions between drugs that are metabolized by cytochrome P450 may lead to enzyme induction (e.g. rifampicin and carbamazepine) or reduction (e.g. amiodarone, ciprofloxacin and erythromycin) which in turn increase or decrease drug concentration, toxicity or efficacy. Therefore, patients who receive 2 or more hepatotoxic medications with a risk of interaction may require frequent therapeutic drug monitoring (TDM) to ensure their safety and drug efficacy.

### *1.5.6 Alcohol*

Alcohol consumption may also increase risk of liver injury through its competitive mechanism with other drugs for enzyme metabolism in the liver, particularly

CYP2E1. It could alter clinical drug responses of large numbers of drugs from many therapeutic classes including analgesics, antihistamines, antihypertensive, anti-arrhythmics, inotropic tranquilizers, anticoagulants, and antidiabetic drugs which ultimately induce severe interaction and toxicity (Oneta, 2000). It is well established that alcohol abuse increases the risk of DILI susceptibility of certain drugs comprising methotrexate, halothane, isoniazid and other antituberculosis drugs (Chalasani and Bjornsson, 2010). Fernandez-Villar et al. (2003) reported that the risk of isoniazid hepatotoxicity increased by 4 fold with weekly excessive alcohol intake of >280 g of ethanol for men and >140 g of ethanol for women for ⩾1 year (P= 0.002, 95% CI=1.6–10.8). Alcohol was also found to increase tetracycline hepatotoxicity in a population based case–control study conducted in the USA (P<0.001, OR=3.51), the mechanism suggested was through depletion of the antioxidant glutathione. This risk is exacerbated during fasting and pregnancy (Heaton et al., 2007).

### *1.5.7 Drug dose*

In the intrinsic type of drug hepatotoxicity, there is a classical dose-response relationship as in the case of paracetamol where the majority of DILI tends to develop when deliberate overdose occurs. In contrast, idiosyncratic hepatotoxicity due to drug exposure is believed to be dose independent, though certain medications have shown higher incidence of DILI toxicity when higher doses were administered (Ghabril et al., 2010). Diclofenac, co-amoxiclav, flucloxacillin, bosentan, mianserin and duloxetine were all reported to show dose dependent hepatoxicity (Kenyon and Nappi, 2003; Lammert et al., 2008). Uetrecht (2001) has suggested several years ago that daily doses equal to 10 mg or less are expected to show lower incidence of idiosyncratic drug reactions. This novel assumption was later tested by Lammert and colleagues (2008) who investigated the relationship between daily dose of oral medications and idiosyncratic DILI reported in two publicly available pharmaceutical databases of the United States (2005) and the Swedish Adverse Drug Reactions Advisory Committee (1970-2004). Two hundred and thirty commonly prescribed medications were stratified based on their administered doses into three groups (≤10 mg/day, 11-49 mg/day, and ≥50 mg/day) and their association with DILI was evaluated. Liver failure, liver transplantation, or liver-related death were more frequent with drugs given at higher doses (32%, 13% and 28%, respectively for

≥50 mg/day group) compared to lower and intermediate groups of the American population (17%, 0% and 11% for ≤10 mg/day group and 12%, 2% and 11% for 11-49 mg/day group, respectively). Similarly, results obtained from Swedish cohort indicated that 77% of reported DILI cases belong to the higher doses group while apparent fewer numbers of DILI developed due to other groups (9% of DILI related to ≤10 mg/day group and 14% of DILI related to 11-49 mg/day group). In addition, more frequent liver transplantations and deaths were recorded (13.2%) in the ≥50 mg/day group compared to lower (2%) and intermediate (9.4%) doses groups. These findings provided an efficient proposal for drug manufacturers to develop highly potent drugs which could act at low concentrations in order to reduce the risk of more severe DILI type (Ballet, 2010).

## 1.6 Predisposing genetic factors

Comparison of well defined and stratified DILI cases to a properly selected matched control group is an efficient approach to identify genetic risk factors contributed to DILI development. Unfortunately, despite the increased number of such studies to explore those factors, the exact genetic profile for propensity to DILI is not fully understood with some important exceptions. DILI as a complex disease is believed to develop due to interactions of genetic and environmental risk factors. Incidences of different patterns of liver toxicity caused by various DILI compounds make researchers facing more difficulty to determine genetic basis of general risk factor to DILI susceptibility (Daly and Day, 2012). A range of genetic associations with DILI have been reported, though for most the overall contribution to DILI susceptibility are low (Uetrecht, 2008) and a number of these reported associations have not been confirmed by others.

### *1.6.1 Polymorphisms in Phase 1 metabolizing enzymes: Cytochrome P450*

Most drugs undergo two main phases of metabolism within liver in order to be activated or inactivated; phase 1 metabolism (bioactivation or toxification) involves hydrolysis, oxidation or reduction reactions to produce more water-soluble molecules (hydrophilic) whereas drugs in phase 2 (detoxification) exposed to conjugation reactions which further enhance metabolite solubility (Corsini and Bortolini, 2013). Possible associations with DILI for variants in genes encoding enzymes from both

phases have been investigated in several studies (see Table 1.5). Cytochrome P450 enzymes are a superfamily responsible for most phase I metabolizing reactions and serve mainly as catalysts for xenobiotic oxidation. In humans, 10 out of 57 functional cytochrome P450 enzymes (CYP) belong to three families (CYP1, CYP2 and CYP3), are considered the most common metabolizers of currently used medications. CYPs 2C9, 3A4, 2C8, 2E1, and 1A2 are the CYP enzymes expressed highest in liver followed by 2A6, 2D6, 2B6 and 2C19. CYP3A4 appears to be responsible for the majority of drug oxidation reactions (Zanger and Schwab, 2013). Mutation of the genes encoding CYP450 enzymes may alter their metabolizing activity and could affect levels of reactive metabolites (Pachkoria et al., 2007a). Polymorphisms in the CYP3A genes, 3A4, 3A5 and 3A7, have not been so far demonstrated to show any associations with DILI whereas a few allelic variations in the CYP2 family, which represents approximately 18% of the entire CYP450 hepatic proteins in human, show associations of mild to moderate clinical significance (Ma and Lu, 2011). In the study conducted by Daly et al. (2007), a borderline association (P=0.04) was detected between CYP2C8 haplotype and diclofenac hepatotoxicity.

Among CYP2 family, CYP2C9 is known to metabolize a variety of widely used drugs like warfarin, hypoglycaemic agents (e.g. tolbutamide), angiotensin receptor antagonists (e.g. losartan), HMG-CoA reductase inhibitors (e.g. fluvastatin), phenytoin and the majority of NSAIDs group such as diclofenac, ibuprofen, indomethacin, naproxen and the selective Cox-2 inhibitors. One study evaluated the role of certain CYP2C9 variants (CYP2C9*2 and CYP2C9*3) in diclofenac DILI but failed to detect positive findings and a second study involving DILI due to a wider range of drugs reported similar findings; this could be because both the parent drug and the metabolite produced by CYP2C9 are not themselves hepatotoxic (Aithal et al., 2000a; Pachkoria et al., 2007b). However, more recently patients positive for CYP2C9*2 were found to be at increased risk to develop liver injury due to exposure to bosentan; the agent indicated for pulmonary arterial hypertension (Markova et al., 2013).

Association of polymorphisms affecting CYP2E1, the enzyme responsible for metabolism of several drugs e.g. paracetamol, chlorzoxazone, halothane, desflurane, enflurane and isoflurane, with DILI was assessed in two different studies conducted

in Taiwan and China (Huang et al., 2003; Wang et al., 2009). An increased isoniazid hepatotoxicity risk was noticed among individuals showing a homozygous wild-type genotype when genotyped for the CYP2E1*5 allele (Rsa1 c1/c1 genotype) in both studies (OR=2.38, P=0.017 for Taiwanese and OR=2.02, P= 0.039 for Chinese). A higher risk was also observed for a similar CYP2E1 genotype in a mixed population (OR=3.4, OR=0.02) (Vuilleumier et al., 2006) but no association was detected in Korean patients (Cho et al., 2007).

A recent study, conducted on 114 Japanese patients, investigated the gene interaction of HLA and CYP2B6 with susceptibility to idiosyncratic hepatotoxicity related to ticlopidine, an antiplatelet drug, and found that patients positive for HLA-A*33:03 and CYP2B6*1H or *1J showed an increased tendency to develop ticlopidine hepatic injury (OR=38.82, 95% CI=8.08-196.0; P<0.001) (Ariyoshi et al., 2010).

**Table 1.5: DILI association studies with genes encoding drug metabolizing enzymes.**

| Gene | Drug (s) | Effect | Reference |
|---|---|---|---|
| CYP2D6 | Perhexiline | Positive | (Shah et al., 1982) |
| CYP2E1 | Isoniazid | Positive | (Huang et al., 2003; Lang et al., 2007; Vuilleumier et al., 2006) |
| GSTM1/GSTT1 | Tacrine | Positive | (Simon et al., 2000) |
| | Troglitazone | Positive | (Watanabe et al., 2003) |
| GSTM1 | Anti-TB | Positive | (Huang et al., 2007; Roy et al., 2001) |
| | Carbamazepine | Positive | (Ueda et al., 2007) |
| | Diverse | Positive | (Lucena et al., 2008) |
| GSTT1 | Anti-TB | Positive | (Leiro et al., 2008) |
| | Diverse | Positive | (Lucena et al., 2008) |
| | Tacrine | Positive/Negative | (Becquemont et al., 1997)/ (De Sousa et al., 1998) |
| NAT2 | Anti-TB | Positive | (Bozok Cetintas et al., 2008; Higuchi et al., 2007; Huang et al., 2002; Kim et al., 2009; Lee et al., 2010; Ng et al., 2014; Ohno et al., 2000; Possuelo et al., 2008) |
| NAT2 | Isoniazid | Positive | (Cho et al., 2007) |
| UGT1A | Tolcapone | Positive | (Acuna et al., 2002) |
| UGT1A9 | COMT* inhibitors | Positive | (Martignoni et al., 2005) |
| UGT2B7 | diclofenac | Positive | (Daly et al., 2007) |
| CYP2C9 | Diclofenac | Negative | (Aithal et al., 2000a) |
| | Diverse | Negative | (Pachkoria et al., 2007b) |
| CYP2C19 | Diverse | Negative | (Pachkoria et al., 2007b) |
| | Anti-TB | Negative | (Kim et al., 2009) |
| CYP2D6 | Anti-TB | Negative | (Kim et al., 2009) |
| CYP2E1 | Anti-TB | Negative | (Cho et al., 2007; Kim et al., 2009; Yamada et al., 2009) |
| CYP3A4 | Nevirapine | Negative | (Haas et al., 2006) |
| | Voriconazole | Negative | (Levin et al., 2007) |
| CYP3A5 | Nevirapine | Negative | (Haas et al., 2006) |
| | Voriconazole | Negative | (Levin et al., 2007) |
| GSTM1 | Tacrine | Negative | (Green et al., 1995) |
| GSTT1 | Anti-TB | Negative | (Huang et al., 2007) |
| NAT2 | Anti-TB | Negative | (Roy et al., 2001) |
| | Isoniazid | Negative | (Vuilleumier et al., 2006) |
| UGT1A1 | Anti-TB | Negative | (Kim et al., 2009) |
| UGT1A3 | Anti-TB | Negative | (Kim et al., 2009) |

*COMT is Catechol-O-methyl transferase. (Andrade et al., 2009; Daly and Day, 2012).

### *1.6.2 Polymorphisms in Phase 2 metabolizing enzymes: NAT2 and UGT*

Metabolism of isoniazid involves N-acetylation. N-acetyltransferase 2 (NAT2), which is expressed in liver, conjugates a number of drugs by acetylation and the gene encoding this enzyme is subject to a well studied polymorphism. Based on NAT2 enzyme activity, people can be categorized as rapid, intermediate or slow acetylators (Zabost et al., 2013). Various polymorphisms seen in slow acetylators (e.g. NAT2*5, *6 and *7) were found to be associated with DILI due to isoniazid (Chalasani and Bjornsson, 2010). The risk of isoniazid hepatotoxicity was reported as 5.4 fold higher in Korean patients with slow acetylator genotypes (95% CI=1.76-16.59; P=0.005) (Cho et al., 2007). More recently, 22 % of slow acetylator Brazilian patients on antituberculosis drugs developed liver injury compared to 9% of the rapid acetylator (OR 2.86, 95% CI=1.06-7.68; P=0.04) (Teixeira et al., 2011). A meta-analysis published recently has reviewed the relationship between NAT2 polymorphisms and antituberculosis (anti-TB) DILI in 11 Asians and 3 Caucasians studies; NAT2 slow acetylators were found at increased risk of hepatotoxicity compared to rapid acetylators in both ethnic groups (OR=4.88, 95% CI=3.35–7.11; P< 0.001 in Asians and OR=3.72, 95% CI=1.32–10.47; P= 0.013 in Caucasians) (Wang et al., 2012). Similar findings were obtained in the study conducted by Ng et al. (2014) that involved a mixed-ethnicity patient group (n=26) on anti-TB including isonizide (OR=4.25, 95% CI=1.36-13.22; P=0.012).

Several studies have examined the role of UDP-glucuronosyltransferases as detoxification enzymes in the development of DILI. Multiple variants located in UGT1A locus, particularly UGT1A6, are believed associated with slow metabolism of tolcapone, a catechol-O-methyl transferase inhibitor (COMT) used in Parkinson disease, which resulted in severe elevation of transaminase levels (Acuna et al., 2002). Martignoni et al. (2005) has supported these findings when he reported 2 patients positive for a UGT1A9 variant experienced an abnormal increase of liver enzymes upon exposure to COMT inhibitor agents. Thereafter, another study tried to find associations for UGT1A with DILI due to a different drug category (antituberculosis agents) but failed to detect positive signals (Kim et al., 2009). An association of UGT2B7 and diclofenac hepatotoxicity is another example of the role of phase 2 metabolizing enzymes in DILI development. UGT2B7 is known to catalyze diclofenac glucuronidation to form an acyl glucuronide which further undergoes oxidation reaction by CYP2C8; therefore, mutations involving one or both

genes are expected to affect diclofenac detoxification process (Daly et al., 2007). Results obtained by Daly and colleagues (2007) involving 24 diclofenac DILI cases indicated a strong association of UGT2B7*2 (OR=8.5, P=0.03) and borderline significance of CYP2C8 (P=0.04) with diclofenac hepatotoxicity. The contribution by UGT2B7 genotype has been confirmed in a recent study involving an enlarged population (n=30) (Urban et al., 2013).

The glutathione S-transferases (GSTs) isoenzymes mu 1 and theta 1 (GSTM1 and GSTT1) are considered potential candidate genes related to DILI development due to their vital role in detoxification of xenobiotic, endobiotic compounds and oxidative stress products through conjugation with glutathione (Leiro Fernandez et al., 2009). Null mutations (gene deletions) of one or both genes were reported as a factor contributing to hepatic injury due to various medications. Risk of anti-tuberculosis-induced hepatotoxicity was found significantly related to possession of GSTM1 null allele with double risk in cases compared with controls in an Indian study involved 66 pulmonary tuberculosis patients (Roy et al., 2001) and also in 63 DILI cases from Taiwan (Huang et al., 2007) (RR=2.13 and RR=2.23, respectively), however, no association was observed with GSTT1 polymorphism. These findings were confirmed by a meta-analysis of 7 Chinese studies (Tang et al., 2013) involving 741 anti-TB DILI cases and 1337 controls (OR=1.82; P<0.001 for GSTM1 and OR=0.99; P=0.89 for GSTT1) Paradoxically, the GSTT1 homozygous null variant has shown more frequent distribution in Spanish anti-tuberculosis DILI cases (n=35) than in controls (OR=2.6; P=0.03) with similar genotype frequency of GSTM1 among the comparable matched samples (Leiro et al., 2008). Individuals who carried GSTM1/GSTT1-double null genotype were reported to be at increased risk of DILI due to exposure to different medications including troglitazone and co-amoxiclav (Lucena et al., 2008; Watanabe et al., 2003).

### *1.6.3 Drug transporters*

Drug transporters play an important role in drug absorption, extent of tissue penetration and excretion. Transporter's altered function as a result of genetic polymorphism may lead to idiosyncratic toxicity (DeGorter et al., 2012). An example of drug uptake transporter is the organic anion transporting polypeptide 1B1

(OATP1B1, SLCO1B1) which is known as a strong predictor to statin-induced myopathy (Link et al., 2008). Proteins encoded by SLCO1B1 gene are exclusively expressed on the liver sinusoidal membrane and function as mediators for uptake and clearance of bilirubin and drug conjugates. In a knockout mouse model, animals carrying a mutated SLCO1B2 gene, the closest orthologic gene of the human SLCO1B1 and SLCO1B3, show lower liver uptake of certain tested drugs (pravastatin, lovastatin, and rifampicin) compared to wild-type controls and hence lower hepatotoxicity (DeGorter et al., 2012).

An association of other drug transporters like ATP-binding cassette, sub-family B and C (ABCB4, ABCB11 and ABCC2) with DILI was assessed by several researchers. ABCB11 and ABCC2 receptors are involved in drug efflux from hepatocytes into bile ducts via canalicular transportation whereas ABCB4 transports phospholipids across extra- and intra-cellular membranes (Daly et al., 2007; Lang et al., 2006; Rosmorduc and Poupon, 2007). Both ABCB4 which encodes multidrug resistance protein 3 (MDR3) and ABCB11 (the bile salt export pump, BSEP) were reported as risk factors to DILI development related to several drugs including oral contraceptives, certain antibiotics, proton pump inhibitors, and psychotropic drugs. Four nonsynonymous mutations, 3 in ABCB11 (D676Y, G855R and V444A) and 1 in ABCB4 (I764L) were found strongly associated with cholestatic DILI pattern while different polymorphism (L1082Q) in ABCB4 was more common in individuals who developed drug induced hepatocellular injury (Lang et al., 2007). An upstream variant (C-24T) known to be associated with a lower functional expression of ABCC2 gene, coding for multidrug resistance-associated protein 2 (MRP2), was investigated in 24 diclofenac-induced hepatotoxicity patients and compared to 46 drug-exposed and 100 drug non-exposed healthy populations. Variant allele (T) was more frequent in hepatotoxicity patients than in drug-exposed (OR= 5.0; P=0.005) and drug non-exposed groups (OR= 6.3; P=0.0002) (Daly et al., 2007). Functional studies conducted on ABCC2 gene have shown 39% reduction of promoter activity in those possessing combination of C-24T and G-1549A (Choi et al., 2007) variants. These two variants are in linkage disequilibrium and found to be associated with hepatocellular toxicity caused by a variety of drugs, in addition, different promoter polymorphism (-1774) has shown higher frequency in patients experiencing cholestatic or mixed phenotypes. Another novel variant (rs17222723, V1188E) in

MRP2 showed a modest association in one study on flucloxacillin DILI; however, the results did not show statistical significance when corrected for multiple testing (Bhatnagar et al., 2008). Very recently, a significant association was observed between a particular haplotype of ABCC2 (C-24T_ G-1549A_ V417I) and an acute type of rash known as maculopapular eruption induced by antituberculosis (Kim et al., 2012). Investigation of the role of ABCB1 (C3435T), which encodes MDR1, in hepatotoxicity induced by nevirapine (an antiretroviral drug) in a European population (Yuan et al., 2011) failed to confirm associations previously detected in two smaller studies conducted in the US and South Africa (Haas et al., 2006; Ritchie et al., 2006).

### *1.6.4 Human Leukocyte Antigens*

Production of proinflammatory mediators as a consequence of innate (Kupffer cells (KC), natural killer (NK) cells, and NKT cells) and/or adaptive (T and B lymphocytes) immune cells activation upon exposure to hepatotoxic drugs or drug metabolites is considered a crucial pathway for drug induced hepatic toxicity (Holt and Ju, 2006). Table 1.6 shows a list of studies that showed significant associations between immunoregulatory genes and DILI (Pachkoria et al., 2007a). The HLA genes in the major histocompatibility complex (MHC) are an integral part of human immune system. HLA genes, located on the short arm of chromosome 6, encode cell-surface antigens which enable T cells to differentiate between peptides presented from self and non-self cells (e.g. microorganisms). HLA antigens are categorized according to their structure and function into 3 classes, HLA class I, class II and class III. HLA class I which are expressed on most cell types present antigens from inside the cell to killer T cells (CD8+, cytotoxic T cells) while class II antigens which are specifically expressed on APCs present antigens from outside of the cell to T helper cells (CD4+). HLA class III antigens encode several components of the complement system (e.g. C2, C4a, C4b, Bf), hormones and inflammatory cytokines including tumor necrosis factor (TNF) alpha and beta (Turner, 2004). Drug hepatotoxicity related to HLA polymorphisms is believed to develop due to either direct binding of drug to HLA molecule or as a result of a complex of drug with a peptide being formed and then presented to the groove located in the HLA antigen (Aithal and Daly, 2010). Traditionally, HLA typing is used to determine tissue compatibility

before organ or tissue transplantation, to investigate blood matching prior to blood transfusion and in investigation of couples with recurrent miscarriages. Currently, it is also used to detect a wide range of polymorphisms in HLA loci which have shown susceptibility or resistance associations in over 50 different diseases involving cancers, drug-induced hypersensitivity, autoimmune and infectious diseases (Norcross et al., 2012; Shankarkumar et al., 2007; Trachtenberg and Erlich, 2001).

Certain candidate genes have been suggested based on biological relevance and/or relying on the recent data given by GWAS studies. A number of reports demonstrated a strong influence of HLA genes on DILI susceptibility more than a decade ago (Berson et al., 1994; Hautekeete et al., 1999; O'Donohue et al., 2000; Sharma et al., 2002). Genetic associations of HLA with DILI were first reported by Otsuka et al. (1985) who observed significant differences in frequency distribution of DR2 between individuals who developed halothane hepatotoxicity (58.3%) and non-affected controls (33.6%) (P=0.025; RR= 2.77) whereas the B44 locus was found more common in controls than in cases (MAF=50% in controls vs 12.7% in cases, P=0.001; RR= 6.86). 23.6 % of halothane DILI cases were also found to carry a specific haplotype HLA-A*24-B*52-DR2 which was absent in controls (P=0.0042). Then Stricker et al. (1988) have noticed higher frequency distribution of HLA-DR2 (56%) and HLA-DR6 (56%) in 52 DILI cases exposed to nitrofurans than in controls (29%), though the difference was not significant. Few years later, association with HLA-DR6 was confirmed with hepatotoxicity induced by chlorpromazine, an antipsychotic, when larger numbers of cases (n=71) were involved (Berson et al., 1994). The findings of Otsuka et al. (1985) regarding HLA-DR2 were further supported by several studies using candidate gene and GWAS approaches detected significant association between HLA-DRB1*15:01 variant (the HLA class II allele that corresponds to the DR2 serotype) and various drugs including lumiracoxib (a selective COX-2 inhibitor NSAID) and co-amoxiclav (Andrade et al., 2004; Donaldson et al., 2010; Hautekeete et al., 1999; O'Donohue et al., 2000; Singer et al., 2010). These results may indicate a general role for HLA-DRB1*15:01 in DILI development for a range of hepatotoxic drugs. In the case of lumiracoxib, a particular HLA haplotype (HLA-DRB1*15:01-DQB1*06:02-DRB5*01:01-DQA1*01:02) was more common in 41 drug-treated patients than 176 matched tolerant-controls (OR=5.0, 95% CI=3.6–7.0; $P = 6.8 \times 10^{-25}$) (Singer et al., 2010). In the UK-wide

DILIGEN study, DRB1 alleles have shown significant associations with both co-amoxiclav and flucloxacillin DILI. The DRB1*15 allele was found to be a risk factor in co-amoxiclav cases, the result which supports two other smaller studies (Hautekeete et al., 1999; O'Donohue et al., 2000) (n=35 and 22, respectively), while, in the case of flucloxacillin, this allele has been recognized as a protective variant against liver toxicity (Donaldson et al., 2010). The previously reported findings by Hautekeete et al. (1999) involved the haplotype DRB1*15:01-DRB5*01:01-DQB1*06:02 that was found to be associated with cholestatic hepatitis in 35 co-amoxiclav DILI cases versus 300 healthy controls. Also, DRB1*07 was seen to be protective against co-amoxiclav injury (borderline significant effect) (Donaldson et al., 2010), however, this effect was not seen for the larger cohort described in the Lucena et al. (2011) study when HLA typing was done.

The MHC region was screened among 56 Indian DILI patients on antituberculosis treatment and compared to 290 drug tolerant controls. The study results indicated that risk to DILI development was 4 times higher in patients negative for HLA-DQA1*01:02 ($P<0.001$) whereas individuals positive for HLA-DQB1*02:01 showed a doubling of risk ($P<0.01$) (Sharma et al., 2002). One recent GWAS study has detected several novel HLA mutations that are significantly associated with ximelagatran (an anticoagulant**)** hepatotoxicity. HLA-DRB1*07 allele was the fourth most significant marker noted in the study recruited 74 ximelagatran DILI cases and 130 treated controls (OR=4.41, 95% CI=2.20-8.87; $P= 9.1x10^{-6}$) and the results obtained were successfully replicated using smaller number of different patients and healthy individuals (OR=15, 95% CI=1.40-161.05; $P=5.97x10^{-3}$) (Kindmark et al., 2008).

**Table 1.6: Selected immunological determinants of hepatotoxicity.**

| Gene | Allele | Drug | Effect | Reference |
|---|---|---|---|---|
| **MHC genes** | | | | |
| HLA-A | *33:03 | Ticlopidine | Increased DILI incidence | (Hirata et al., 2008) (Ariyoshi et al., 2010) |
| | *02:01 | Co-amoxiclav | Increased DILI incidence | (Lucena et al., 2011) |
| HLA-B | *57:01 | Flucloxacillin | Increased incidence of cholestatic injury | (Daly et al., 2009) |
| | *18:01 | Co-amoxiclav | Increased DILI incidence | (Lucena et al., 2011) |
| HLA-DRB1 | *15:01 | Co-amoxiclav | Increased DILI incidence | (Hautekeete et al., 1999) (O'Donohue et al., 2000) (Donaldson et al., 2008) |
| | *15:01 | Various | Increased incidence of cholestatic injury | (Andrade et al., 2004) |
| | *15:01 | Luxiracoxib | Increased DILI incidence | (Singer et al., 2010) |
| | *01:01 | Nevirapine | Increased hypersensitivity incidence | (Martin et al., 2005) |
| | *07:01 | Ximelagatran | Increased incidence of elevated ALT | (Kindmark et al., 2008) |
| HLA-DQA1 | *02:01 | Lapatinib | Increased DILI incidence | (Spraggs et al., 2011) |
| HLA-DQB1 | *02:01 | Anti-TB | Increased DILI incidence | (Sharma et al., 2002) |
| | *06:02 | Various | Increased incidence of cholestatic injury | (Andrade et al., 2004) |
| **Non-HLA genes** | | | | |
| PTPN22 | rs2476601 | Co-amoxiclav | Increased DILI incidence | (Lucena et al., 2011) |
| STAT4 | rs7574865 | Various | Increased incidence of hepatocellular injury | (Urban et al., 2012) |
| **Cytokines genes** | | | | |
| IL-4 | C-590A | Diclofenac | Increased DILI incidence | (Aithal et al., 2004) |
| IL-6 | Intron & 3' repeat sequence | Tacrine | Increased maximum ALT | (Carr et al., 2007) |
| IL-10 | C-627A | Diclofenac | Increased DILI incidence | (Aithal et al., 2004) |
| IL-10 | G-1117A | Various | Lower eosinophilia incidence and poorer outcome | (Pachkoria et al., 2008) |

Adapted from (Daly and Day, 2009) (Daly and Day, 2012).

More importantly, in the UK-wide multicentre study published by Daly and co-workers performed on 51 DILI cases has indicated that the class I HLA allele B*57:01 is the main predictor for flucloxacillin hepatotoxicity (Daly et al., 2009) (Figure 1.3). B*57:01 was previously reported as an allele which strongly predicted susceptibility to abacavir-induced hypersensitivity. Abacavir is a nucleoside analog reverse transcriptase inhibitor used to treat HIV and AIDS. The proposed mechanism of its severe hypersensitivity is that the B*57:01 gene product binds non-covalently to abacavir which leads to modification of shape and chemistry of the antigen-binding cleft. This alteration activates abacavir-specific T-cells that stimulate CD8-positive T cells proliferation and systemic hypersensitivity reactions (Illing et al., 2012). HLA association with DILI was recently investigated in 22 Japanese patients experienced hepatic injury due to ticlopidine and compared to 85 drug tolerant individuals (Hirata et al., 2008). The study revealed five HLA alleles including HLA-B*44:03, C*14:03, DRB1*13:02 and DQB1*06:04 as risk factors for ticlopidine induced hepatotoxicity with highest association seen at HLA-A*33:03 locus (OR= 13.04, 95% CI= 4.40-38.59; the corrected P-value (Pc) =1.24 x $10^{-5}$). The risk of HLA-A*33:03 allele was much higher in patients who developed cholestatic liver injury (OR=36.50, 95% CI=7.25-183.82; Pc=7.32 x $10^{-7}$). Also, odds ratio increased to 38 in patients carrying a haplotype of HLA-A*33:03 allele and CYP2B6*1H or *1J (Ariyoshi et al., 2010). Possession of A*02:01 allele (HLA class I) and/or DRB1*15:01 (class II) was identified recently as risk factor of developing co-amoxiclav DILI in a genome-wide association study involving cases collected in the UK, Spain and in the US (Lucena et al, 2011) (**Error! Reference source not found.**).

In a study involving 37 cases (78% with European ancestry) and 286 controls, class II variants HLA-DQA1*02:01, DRB1*07:01, and DQB1*02:02 were reported as predisposing factors for DILI hepatotoxicity due to administration of lapatinib, a dual tyrosine kinase inhibitor used in the treatment of HER2 (Human Epidermal Growth Factor Receptor 2, known as CD340) -positive metastatic breast cancer. These findings were replicated using different groups of cases (n=24) and controls (n=155) and for these cases the association between the HLA class II haplotype and lapatinib DILI was stronger (Spraggs et al., 2011). Further analysis showed that 73% of

involved cases were positive for both HLA-DQA1*02:01 and DRB1*07:01 alleles compared to 21% of controls (Spraggs et al., 2012).

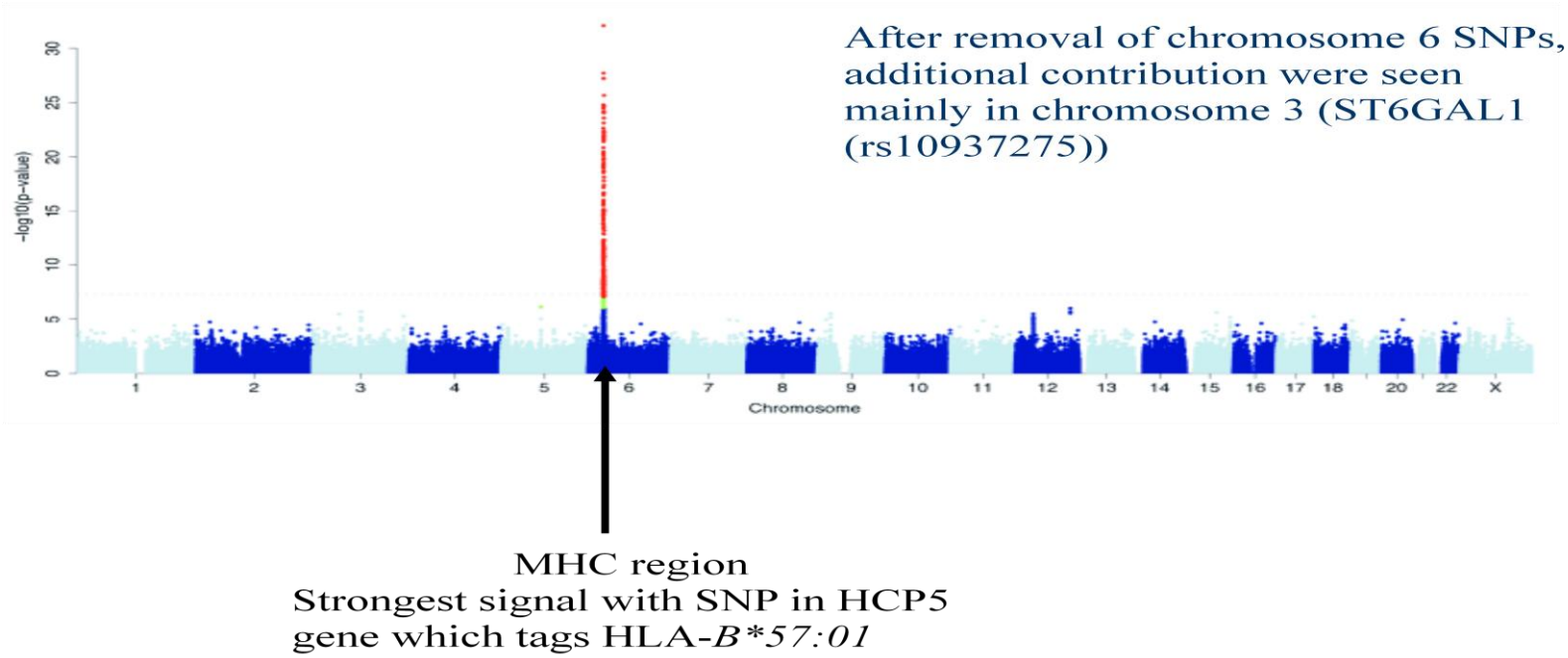


**Figure 1.3: Manhattan plot showing flucloxacillin DILI genome-wide association result. Each dot represents a SNP.** The x axis represents the position of the SNP on chromosomes. The y axis represents the $-\log_{10}$ of Cochran-Armitage trend P value of the SNP in the case-control association study. 51 DILI cases and 282 population controls were included in the study. SNPs with P values o$10^{-6}$ and $10^{-7}$ are highlighted in green and red, respectively. The strong signal in chromosome 6 lies in the MHC region (Daly et al., 2009).

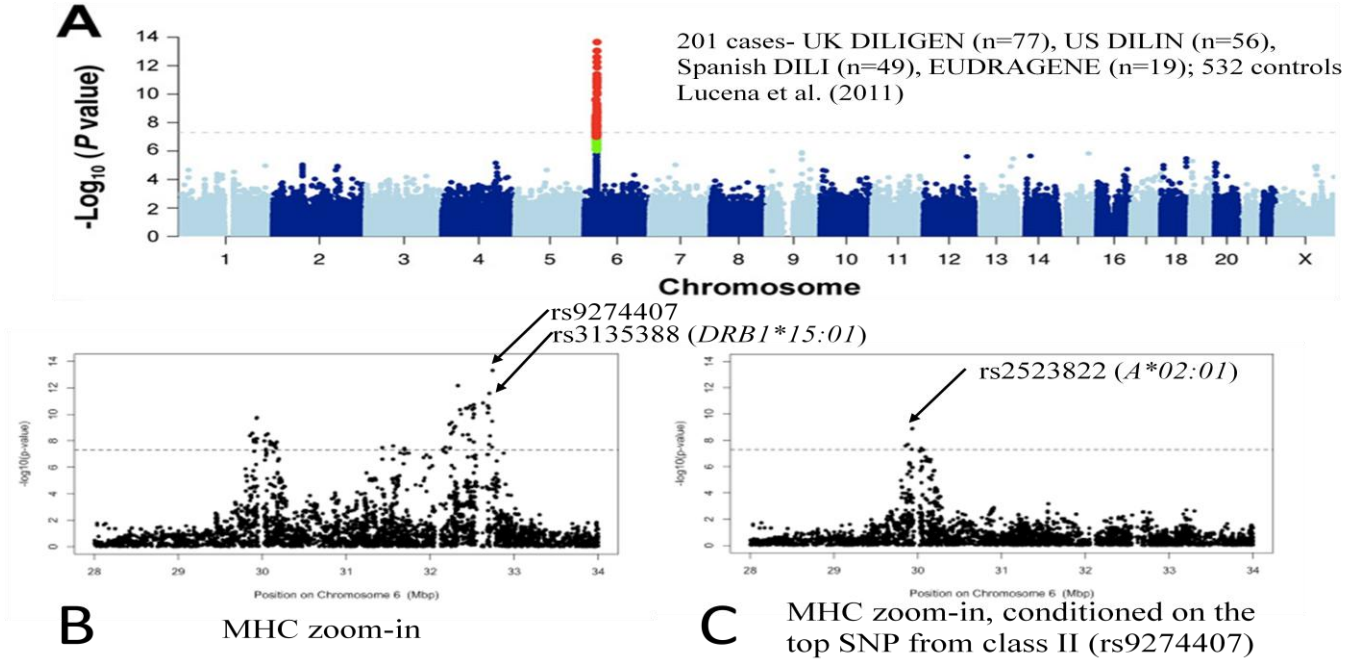


**Figure 1.4: Manhattan plot showing co-amoxiclav-DILI genome-wide association results (Lucena et al., 2011).** Each *point* represents association analysis results for a single SNP with chromosome position on the *x-axis* and -$\log_{10}$ *P*-value on the *y-axis*. 201 DILI cases and 532 population controls were included in the analysis. SNPs with *P* values smaller than $10^{-6}$ and $10^{-7}$ are highlighted in *green* and *red*, respectively. *Panel A* shows the results for the entire genome. *Panel B* is an enlargement of the results presented in *A*, *panel C* shows the analysis of each SNP in this chromosome region conditioned on the top class II SNP (rs9274407).

### *1.6.5 Cytokines: Interleukins (IL) and tumor necrosis factor (TNF)*

Cytokines have a crucial function in regulating immune system and inflammatory reactions. The IL-4 is an immunoregulatory gene that plays an important role in T cell proliferation, B cell activation and differentiation, whereas IL-10 gene has an anti-inflammatory effect that antagonizes IL-4 activity through direct function downregulation of type 1 and type 2 T helper cells (Th1 and Th2) (Asseman et al., 1999; Paul, 1997). Previously, certain promoter region variants of IL-4 gene were reported as risk factors for several autoimmune diseases. For example, in the UK, 138 Graves' disease cases and 77 autoimmune hypothyroidism affected individuals have shown significant reduction of -590T allele frequency compared to 101 healthy control group (Hunt et al., 2000), whereas, -589T polymorphism was found at higher frequency in subacute sclerosing panencephalitis and atopic dermatitis in Japanese patients than in controls (Inoue et al., 2002; Kawashima et al., 1998). More recent reports (Aithal and Day, 2007; Aithal et al., 2004) have suggested an association of IL-4 and IL-10 genotype with diclofenac-induced hepatotoxicity. Individuals positive for polymorphisms located in the upstream region of IL-10 (-627) or IL-4 (-590) have shown higher risk of diclofenac DILI susceptibility (OR=2.8 and 2.6, respectively) and the risk further increased to 5 times in cases possessing mutant alleles of both genes as a result of high IL-4 and lower IL-10 expressions, though these effects are considered minor compared to contribution of HLA genes to DILI.

The cytokine IL-6 is secreted during the acute phase of DILI inflammation and believed to activate cell surface signalling which leads to B cell maturation and proliferation. Genotyping of 69 Alzheimer's disease patients treated with tacrine, an anticholinesterase, has shown statistically significant association of an intronic SNP (rs1800795) and 3' repeat sequence located in IL-6 gene with tacrine transaminitis (Carr et al., 2007). Pachkoria et al. (2008) have evaluated genotype for immunoregulatory cytokine genes and their association with DILI due to several drugs. The study involved IL-4, IL-10 and TNF-alpha in 140 patients with predominant hepatocellular phenotype; twenty nine of them (21%) were due to co-amoxiclav. They concluded that all cases with serious DILI outcome carried a low or intermediate IL-10 producing haplotype. Later on, the DILIGEN study has also ascertained the significant association of two variant forms of TNF-alpha SNPs, G-

238A (rs361525) and T-1031C (rs1799964), with flucloxacillin hepatotoxicity and in reverse with co-amoxiclav DILI (Daly et al., 2009). However, this TNF association appears to relate to the location of the TNF genes on chromosome 6 adjacent to the HLA class I genes not to an additional risk relating to TNF.

### *1.6.6 Oxidative stress genes*

During inflammatory conditions, oxidative stress in cells or tissues can develop through production of reactive oxygen species (ROS). Failure of antioxidant defence systems to overcome this process, as a result of existence of variations in genes encoding the relevant detoxicating enzymes, is expected to produce severe cellular damage (Hensley et al., 2000). Superoxide dismutase 2 (SOD2) gene which encodes the mitochondrial manganese-dependent enzyme MnSOD is known to protect cells against ROS toxicities. The C allele of a non-synonymous polymorphism rs4880 in SOD2 (C47T) was considered a contributing risk factor (P=0.037) to hepatocellular DILI induced by various drugs including anti-TB in 115 Taiwanese patients (Huang et al., 2007). On the contrary, findings obtained by Lucena et al. (2010) showed a significant association of the T variant with cholestatic/mixed trait in a study involving 185 DILI Spanish patients (Pc= 0.0058, P=2.3). In addition to SOD2 findings, the Spanish cases have shown higher genotype frequency of a SNP rs1050450 in glutathione peroxidase 1 (GPX1) than tested controls (P=0.0112, OR=5.1).

### *1.6.7 Other non-HLA genetic factors*

Beside the strong HLA association reported in the GWAS study that involved 51 DILI cases (Daly et al., 2009), an additional contribution of the ST6GAL1 gene in chromosome 3 has been suggested for flucloxacillin DILI though this shows a smaller effect (OR=4.1, P=1.4 x $10^{-8}$) than those observed for HLA-B*57:01. A SNP (rs2476601) in PTPN22, which codes for a protein involved in T-cell-receptor signalling has already been shown to be a risk factor for DILI due to co-amoxiclav in a large (n=201) study (Lucena et al., 2011). Moreover, several other genes are candidates to have a potential effect on flucloxacillin-induced cholestasis, these include familial intrahepatic cholestasis type 1 gene (FIC1), farnesoid X receptor and nuclear pregnane X receptor (PXR) (Andrews and Daly, 2008). Genotyping of 51 flucloxacillin DILI cases, 64 tolerant patients and 90 healthy controls for the SNP

rs3814055 (C-25385T) in 5' UTR of PXR gene has determined a significant frequency differences between cases and both control groups (P=0.0023 for exposed controls, P=0.0079 for non-exposed controls). Functional studies have shown lower expression of PXR in patients carrying -25385CC genotype (Andrews et al., 2010). A GWAS study based in the US has compared 783 DILI white European patients due to various drugs to 3001 controls (Urban et al., 2012). Among 193 tested SNPs with known associations to autoimmune disease, a SNP rs7574865 located in STAT4 (signal transducer and activator of transcription 4) gene showed a trend towards significance ($P=4.5\times10^{-4}$) with the hepatocellular DILI cases (n=285); this finding was further reproduced in a different DILI cohort involving 168 hepatocellular injury patients (P=0.011).

## 1.7 Approaches to identify novel genetic markers

Several different approaches can be used to identify specific genes and variants that are risk factors for complex diseases. Among these methods which differ from each other in their sensitivity, specificity and cost, three main approaches, candidate gene, genome-wide association (GWAS) and exome sequencing, have been used to detect new genetic risk factors for DILI and other complex diseases. Significant associations obtained with newly discovered markers have to be confirmed in independent replication studies.

### *1.7.1 Candidate gene approach*

Candidate gene studies are the traditional molecular genetic methods used to pinpoint DILI associations with specific genes located on areas with known function, for example MHC region in chromosome 6. Other important targeted genes are those involved in pharmacokinetics process responsible for drug absorption, distribution, metabolism and excretion in addition to drug transporters and receptors that could affect pharmacodynamic properties of drugs (Shi et al., 2001). Studying variation located in such genes may explain the related drug toxicity or lack of efficacy. The candidate gene approach may also test polymorphisms in relation with functional regions located upstream or downstream of the true marker. Prior knowledge of genes functions and their involvement in drug biological activity could drive researchers to choose candidate gene analysis; otherwise different genetic testing

approaches would be preferable. The candidate gene approach is a relatively cheap technique and provided some valuable data on individual DILI susceptibility. However use of this approach is limited to known genes of functional polymorphisms and therefore unable to explore other potential regions of interest across the genome (Amos et al., 2010). Fortunately, development of haplotype-tagging system of human genome in 2005 (phase I) performed by US National Institutes of Health (NIH) (International HapMap project) allowed geneticists to examine non-functional variants which are in close distance to functional markers or in linkage disequilibrium with other true disease causing polymorphisms (Stram, 2004). Selection of a few tag SNPs to study can capture and predict a large number of other important SNPs of interest across the tested gene. HLA typing is more difficult, time consuming and more expensive than normal SNP genotyping assays, therefore, using tag SNPs in linkage with HLA loci is more practical and cost effective (Halperin et al., 2005). Many tag SNPs in multiple studies (e.g. rs2395029 tag for HLA-B*57:01, rs3135388 tag for DRB1*15:01 and rs2844821 tag for A*02:01) have been used effectively.

Candidate gene studies can be performed quite cheaply and may be of value particularly in developing countries where financial resources are limited. The vast majority of previously conducted genetic association studies have used the candidate gene approach which provided a number of successful genetic associations with DILI. As an example of such useful approach is the candidate study performed by Andrade et al. (2004), who detected HLA loci (HLA-DRB1*07 and DQB1*02) which were found less frequent among 65 cholestatic and mixed type cases (due to multiple drugs, 21 of them were due to co-amoxiclav) than in 635 matched controls (OR=0.37; P=0.003 for DRB1*07 allele and OR=0.39; P=0.0003 for DQB1*02), in contrast, HLA-DRB1*15 and DQB1*06 were more common in cases than in controls (OR= 2.31; P=0.002 for DRB1*15 allele and OR 2.32; P=0.001 for DQB1*06). These results supported previous significant associations between HLA-DRB1*15:01 and co-amoxiclav DILI obtained by (Hautekeete et al., 1999) who used a similar genetic testing technique. Recently, in a candidate gene study that compared 37 lapatinib DILI female patients to drug tolerant individuals (n=286), statistically significant frequency difference of the class II HLA-DQA1*02:01 genotype was determined (Spraggs et al., 2011). Associations with non-HLA genes

based using candidate gene methods were also reported including relationship between reduced IL-10 expression with lower incidence of eosinophilia and poorer outcome (Pachkoria et al., 2008), null genotype of GSTM1 and co-amoxiclav hepatotoxicity (Lucena et al., 2008), ABCB11 and cholestatic injury (Lang et al., 2007), ABCC2 and hepatocellular toxicity (Choi et al., 2007), and SOD2 and elevated level of transaminases (Huang et al., 2007).

### *1.7.2 GWAS approach*

In the post-genomic era, the new generation methods of GWAS and exome sequencing became the standard genetic association testing approaches. Major progress has been made in the genetics field since the advent of the GWAS approach, which allows researchers to examine genetic variation across the whole genome with improved resolution and to consider all genes as candidates and therefore considered as the best method to identify single nucleotide polymorphisms (SNPs) showing a highest level of association (Daly, 2009). The GWAS method depends on SNP microarray technology using Affymetrix GeneChips or Illumina BeadChips which allow reading millions of common genetic variants; this new technique has helped in identification of thousands of important novel disease loci with robust and replicable associations to several traits or disease phenotypes but the cost is high compared with the traditional candidate gene method. Both microarray methods are using same basic principle of genomic hybridization of DNA fragments to a fixed probe. The length of probe on the Affymetrix GeneChips is 25 nucleotides versus 50 nucleotides probe on the Illumina BeadChips which makes Illumina to provide higher specificity. In contrast, Affymetrix GeneChips have additional probes of each SNP which help to differentiate between true signal over noise (Barnes et al., 2005). The microarrays used for GWAS are expensive but the cost is decreasing over time, making the technology more accessible.

The major limitation in use of GWAS is the need to recruit thousands of cases and controls to be able to detect modest to strong associations; studies that involved limited sample size find it difficult to obtain sufficient statistical power (Witte, 2010). However, very strong associations such as that seen between HLA-B*57:01 and flucloxacillin DILI (Daly et al., 2009) and between simvastatin myopathy and SLCO1B1*15 (Link et al., 2008) were detected using small (approx. 50 to 100)

numbers of cases. In addition to testing allelic associations, GWAS data can provide information on most common haplotypes that are more frequently distributed in cases than controls with more details on variants interactions and whether associations detected are truly independent or not. Due to the fact that statistical significance reported from GWAS studies arisen from comparing large number of SNPs (up to one million) among individuals rather than direct head to head single SNP comparison, a P-value of $5\times10^{-8}$ was set as a strict significance cut point to determine risk loci (Witte et al., 1996).

### *1.7.3 Exome sequencing*

Developments in high-throughput technology has introduced whole exome sequencing tool using massive parallel sequencing of single DNA strand technique that can investigate the whole coding regions (exons; the functional units of genes) of the genome (Teer and Mullikin, 2010). Human genome contains 233,785 exons with an average of 9 exons per gene; length of exons' sequences are often below 200 base pairs (bp) (Sakharkar et al., 2004). A number of platforms are currently available to perform exome sequencing such as Illumina Genome Analyzer II, the Applied Biosystems SOLiD and the Ion Torrent. High efficiency of exome sequencing to identify rare as well as common variants makes it very useful to investigate a wide range of rare genetic disorders and complex diseases. Over the past two years, exome sequencing has presented novel causal mutations in several rare conditions (Mendelian disorders) such as congenital chloride-losing diarrhea, neonatal diabetes, hereditary motor and sensory neuropathy (Ku et al., 2012). These evidences encouraged clinical laboratories to use exome approach as a diagnostic tool beside its main indication to discover rare distinctive disease-related polymorphisms (Choi et al., 2009). Despite these benefits, exome sequencing is not recommended for routine screening due to its high cost (around $1000 per sample), which could limit its clinical validity unless prices continued to decline. Obtaining false-positive or false negative signals in exome sequencing is another limitation as a result of expected sequencing errors which necessitate having parallel testing using different method at the same time, also strong associations obtained require further replication tests to validate findings. Sequencing of exons, which represent only 1% of the whole genome, means that 99% of non-coding areas are not screened, this may lead to

missing of several important disease related intronic variants. Therefore, in the near future, once the cost of whole genome sequencing is reduced, it is expected to be the standard genetic testing method due to its ability to offer the advantages of both exome approach, by detecting rare variants, and GWAS through providing a wider screening of all portions of the genome (Majewski et al., 2011). The amount of sequencing data generated by exome approach is quite large detecting normally more than 10,000 polymorphisms which require careful data analysis and interpretation in order to translate these findings into an understanding of functional biology of genetic diseases (Ku et al., 2012). Alternatively, exome chip methods (e.g. Illumina Exome Beadchip) that can selectively genotype additional known rare variants in exomes can be used to refine GWAS data by genotyping for rare functional variants (Teer and Mullikin, 2010). Exome chip, however, "does not provide complete coverage of all functional variants at each locus" since they can only genotype for previously identified variants (Huyghe et al., 2013). Therefore, exome sequencing will still be needed to get deep coverage, though it is not expected to detect certain types of genetic disorders in which base pair change of DNA sequence is not the underlying cause as in the cases of copy number variations, structural variants (e.g. DNA translocations or inversions) and triplet repeat disorders (e.g. Huntington's disease and fragile X syndrome) (Majewski et al., 2011). This limitation can be overcomed by using whole genome sequencing technology. However, performing whole genome sequencing is technically more difficult than exome sequencing and analysis of data produced is still very challenging.

## 1.8 Aims of the study

The aim of the current project is to increase understanding of the genetic and mechanistic basis for drug-induced liver injury due to the antimicrobial agents flucloxacillin and co-amoxiclav by performing candidate-gene association studies and extending available data from genome-wide association and exome sequencing studies by genotyping additional cases.

This will involve:

(i) Data analysis on a panel of DILI cases due to flucloxacillin (n=155) and co-amoxiclav (n=165) from the DILIGEN and iDILIC studies to provide statistics on a variety of clinical and biochemical parameters such as age, gender, drug causal relationship (RUCAM scoring) and severity of hepatotoxicity. This will be compared with previous reports.

(ii) Studies on the relevance of a range of candidate genes which may have a role in DILI on the basis of either previous reports or because of biological plausibility. These genes include UGT1A1, SLCO1B1, HFE, SOD2, GPX1, GSTT1 and GSTM1.

(iii) Follow-up of gene associations suggested by previous GWAS and exome sequencing studies in the extended UK DILI population. This will involve particularly the genes ST6GAL1, PTPN22 and IL12RB1.

# Chapter 2. Materials and Methods

## 2.1 Materials

This chapter describes general materials and methods relevant to several different chapters. More specific methods sections are provided at the start of Chapters 3, 4, 5 and 6.

### *2.1.1 Suppliers of materials*

Contact details for suppliers of materials used are provided in Table 2.1. As far as possible, all chemicals were of analytical reagent grade. Routine chemicals were normally purchased from Sigma Aldrich or Fisher Scientific, oligonucleotides from Eurofins MWG Operon and Taq polymerase and restriction enzymes from New England Biolabs. Suppliers of other materials are listed in the text.

### *2.1.2 Preparation of solutions*

Reverse osmosis water, purified by Thermo Scientific Branstead Nanopure system, was used in the preparation of solutions. Further autoclaving at 120 °C, 15 pounds per inch (PSI) pressure for 20 min performed to sterilize prepared solutions where appropriate. Plastic ware was autoclaved unless purchased sterilized. Tissue culture solutions were filter-sterilized using 0.2 micron filters (Millipore), whereas readymade nuclease free water treated with diethyl pyrocarbonate (DEPC) (Fisher Scientific) was used for RNA work.

### *2.1.3 Commonly used stock solutions*

Compositions of commonly used buffers are described in Table 2.2. Readymade sterile water (Fresenius Kabi Limited, Cheshire, UK) was used for PCR.

## 2.2 DNA extraction

Extraction of DNA from peripheral blood leukocyte was performed by Julia Patch and Sally Coulthard using a perchlorate-chloroform extraction method as described in Daly et al. (1996). After extraction, working DNA stocks at concentration of 50 ng/ul were aliquoted and stored at 4 °C. DNA was quantitated using a Thermo Scientific nanodrop (2000) to measure the absorbance at 260 nm. Longterm storage of DNA was at -80°C.

**Table 2.1: List of suppliers and addresses.**

| Suppliers | Address |
|---|---|
| Applied Biosystems | California, USA |
| Bioline | London, UK |
| Bio-Rad | Hemel Hempstead, UK |
| BioWhittaker- Scientific laboratory supplies (SLS) | Yorkshire, UK |
| Eurofins MWG Operon | London, UK |
| European Tissue Collection | Porton Down, UK |
| Fisher scientific | Loughborough, UK |
| Fresenius Kabi Limited | Cheshire, UK |
| Greiner Bio-One | Stonehouse, UK |
| Invitrongen | Paisley, UK |
| Merck biosciences | Nottingham, UK |
| New England Biolabs (NEB) | Hitchin, UK |
| Promega | Southampton, UK |
| Qiagen | Crawley, UK |
| Sarstedt | Leicester, UK |
| Scientific Laboratories Supplies | Newcastle, UK |
| Sigma Aldrich | Gillingham, UK |
| VH Bio limited | Gateshead, UK |

**Table 2.2: Compositions of commonly used stock solutions.**

| Solution | Constituents |
|---|---|
| 10x TBE | 0.9 M Tris-base<br>0.9 M Boric Acid<br>20 mM EDTA pH 7.0 |
| 10xTAE | 10 mM Tris-base (48.4 gm/L)<br>17.4 M Glacial acetic acid (11.4 ml/L)<br>1 mM EDTA, pH 8.0 (3.7 gm/L) |
| DNA gel loading buffer | 0.25% (x/v) bromophenol blue<br>0.25% (w/v) xylene cyanol<br>30% glycerol |

## 2.3 Molecular biology methods

### *2.3.1 Primers*

Lyophilised oligonucleotide primers were resuspended at a concentration of 200 µM using sterile water and then part of the stock was further diluted to 25 µM. The diluted stock solutions were used to prepare the master mixture of PCR and stored at 4 °C while the remaining volume of stock concentration stored at -20 °C.

### *2.3.2 Polymerase Chain reaction Methodology*

Genomic DNA (typically 0.2 µg) was amplified in a final reaction volume of 25 µl containing 0.5 U Taq DNA polymerase (NEB), 0.25 µM forward primer, 0.25 µM reverse primer, 0.25 mM dNTPs (VH Bio), 1 X reaction buffer (50 mM potassium chloride, 10 mM Tris-HCl pH 9.0, 0.1% (V/V) Triton X-100 and 1.5 mM $MgCl_2$) (Promega). PCR mixture was made in 0.2 ml thin walled sterile tubes (Fisher Scientific). Thermal cycling was then performed in an Applied Biosystems 2720 Thermal Cycler. Denaturation and elongation thermal conditions were always fixed at 94°C and 72 °C, respectively, for 1 min each for 35 cycles, followed by a 7 min extension at 72ºC, while different annealing temperatures were applied according to the designed primers. To check for successful amplification, 6 µl of each sample was analyzed beside a 100 bp ladder molecular weight marker (NEB) on a 2% agarose gel.

### *2.3.3 Digestion of PCR products by using restriction enzymes (Restriction Fragment Length Polymorphism, PCR-RFLP) assays:*

Once amplification was confirmed, then 8 µl of PCR product were mixed with 2 µl 10 X appropriate restriction enzyme buffer and 2 units of restriction enzyme. Sterile water (8 µl) was added to give a final volume of 20 µl. The digest was incubated at the temperature recommended by the manufacturer for at least 3 hours but usually overnight. A similar procedure was used to digest plasmid DNA.

### *2.3.4 Electrophoresis and visualization of DNA*

#### *2.3.4.1 Agarose gel electrophoresis:*

For DNA optimization and analysis, 2% and 3% agarose gels were used. They were made from either nuclease free agarose tablets or powder (Bioline) dissolved in 1 X TBE buffer and after melting the gel in a microwave oven, the nucleic acid stain ethidium bromide (0.5 µg/ml) (Sigma Aldrich) was added. 6-7 µl of DNA was mixed with 2 µl DNA loading buffer and then applied to the wells of the gel. In addition, a 100 bp-1000 bp ladder molecular weight size marker (NEB) was added to one of the wells in order to measure the size of the PCR product. Electrophoresis was normally carried out at 65-70 V constant voltage for 15-30 minutes in 1 X TBE buffer.

#### *2.3.4.2 Polyacrylamide gel electrophoresis (PAGE):*

To visualize smaller-sized PCR (e.g. <100 bp) and to distinguish between digested PCR fragments with small size differences, 10% (W/V) polyacrylamide gels were prepared using 16 ml 30% (W/V) acrylamide solution (acrylamide/bis-acrylamide, 29:1, Fisher Scientific), 29 ml distilled water and 5 ml of 10 X TBE. After mixing the solution, 50 µl tetramethylethylenediamine (TEMED) and 500 µl of 10% (w/v) ammonium persulfate were added. The gel solution was poured between 200 mm X 200 mm sealed glass plates and left to set for 20 min. Thereafter, the gel was loaded onto the electrophoresis tank and 1 X TBE running buffer was added. Gel loading buffer (7 µl) was mixed with the 20 µl PCR digestion product and this solution was then applied to the wells. The gel was run at 150-200 V constant voltage for 3-4 hours in 1 x TBE buffer.

#### *2.3.4.3 Ethidium bromide and gel photography*

At the end of electrophoresis, the polyacrylamide gel was stained in 1 X TBE buffer and ethidium bromide (0.5µg/ ml) for 20-30 min. A TM36 ultraviolet (UV) transilluminator was used for visualising the gel. A photograph was taken by the Alpha imager 2200, version 5.1 (Alpha Innotech Corporation). Alternatively, gels were visualised on a BioRad gel documentation system and photographed by Flour S-Multi imager Quantity One software. Later, the GENi gel imager documentation system was used instead.

## 2.4 TaqMan SNP genotyping assays

TaqMan SNP assays, designed by Applied Biosystems, are delivered as 20X or 40X single tube mixtures (188 µl) of forward and reverse primers (900 µM) in addition to two reporter probes (200 µM). The 5′ end of each probe is linked to different fluorescent allele-specific dye; a Fluorescein amidite **(**FAM) is an allele 2 specific while VIC is the reporter for allele 1 (Figure 2.1). The 2X TaqMan universal PCR master mix (Applied Biosystems) used contains AmpliTaq Gold® DNA polymerase, dNTP and passive internal reference based on proprietary ROX dye.

### *2.4.1 TaqMan PCR procedure*

To prepare the reaction mix to amplify 48 samples in 48-well plate, 15 µl of 20X working stock of SNP Genotyping Assay (or 7.5 µl of 40X stock) were added to 285 µl of 2X universal master mix to be diluted with 200 µl of distilled water. After vortexing, 10 µl of the mixture were transferred into each well of 48 reaction plate before adding 20 ng of wet genomic DNA. The plate was sealed and inserted into the One Step Applied Biosystems real time PCR machine. PCR temperature was kept on hold for 10 min at 95 °C then reduced to 92 °C for 15 sec (denaturation) and further reduced in annealing and extension stages to 60 °C for 1 min each for 40 cycles.

### *2.4.2 Allelic discrimination of TaqMan assay*

The one step software is linked to the allelic fluorescence detection system which measures and plots different fluorescence signals on partitioning chart (X or Y axis). At the end of the experiment, discrete clusters with different colours usually showed wide separation based on their defined genotypes. A cluster which moved horizontally to the bottom side toward X axis represented homozygosity of one allele (XX); signals which moved upward vertically to the Y axis were considered homozygous for the other allele (YY) whereas those located in between X and Y axis indicated the heterozygous genotype (XY) (Figure 2.2).

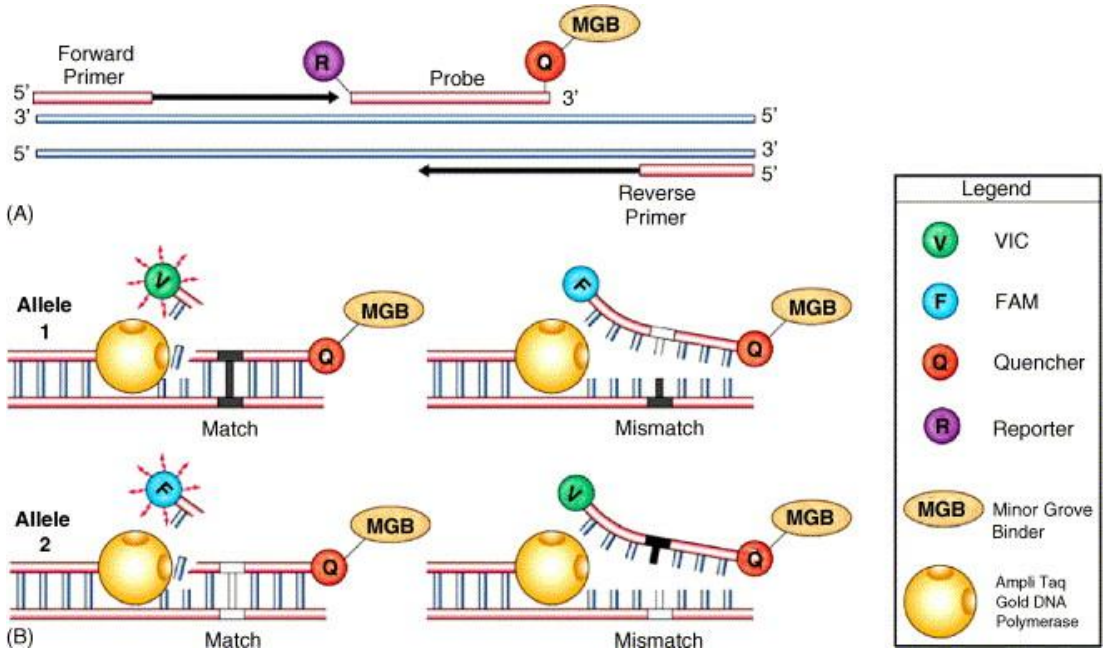


**Figure 2.1: TaqMan® SNP genotyping assay.** Each assay contains two specific primers targeting the region flanking the SNP site and two TaqMan fluorescent probes (FAM and VIC) with a Minor Groove Binder (MGB).

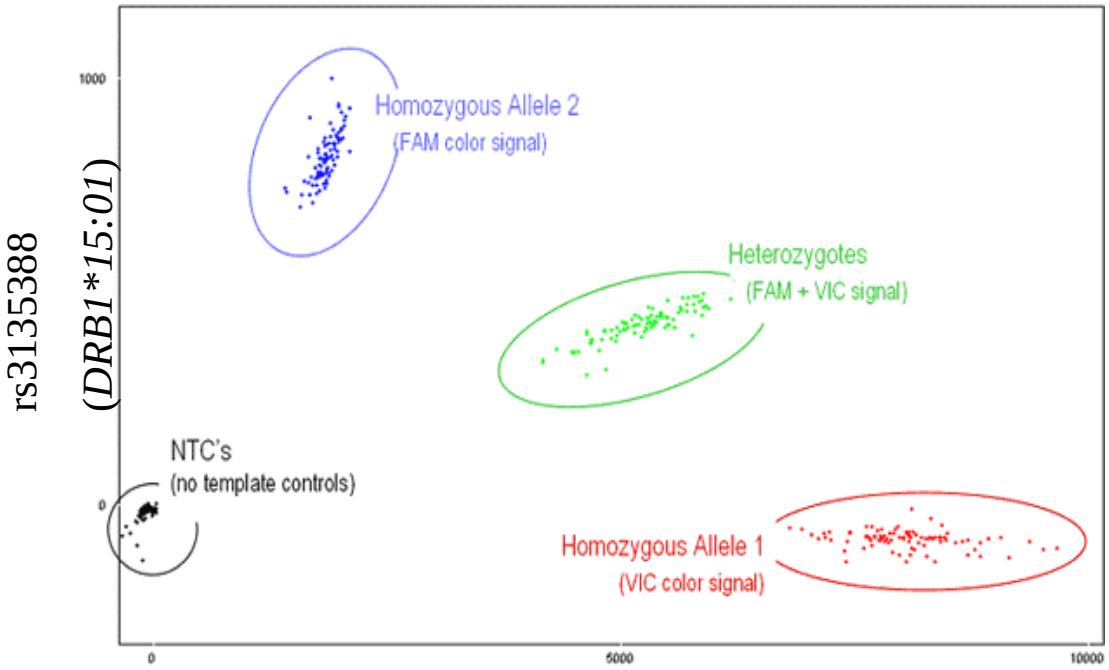


**Figure 2.2: Allelic discrimination plot of TaqMan SNP genotyping assay.** Red cluster is homozygous wild-type (XX), blue cluster is homozygous mutant (YY) and the green one is heterozygous (XY). Black cluster shows the no template controls and samples which failed to amplify.

## 2.5 KBiosciences genotyping

Genotyping for certain SNPs was conducted by KBiosciences. The KBiosciences Competitive Allele-Specific PCR (KASP) genotyping system is a homogenous, fluorescent, endpoint-genotyping technology using two allele-specific forward primers (one for each allele), one reverse primer and a universal reaction mixture. Each forward primer contains a unique unlabelled tail sequence at the 5' end. The master mix contains two different 5' fluor-labelled oligos; one labelled with FAM and one with VIC. Each fluor oligo is designed to interact with allele-specific primer (Figure 2.3). Quality control measures were carried out by including 2 negative controls (no-template vehicle, water only) and positive controls (DNA samples of known genotype) in every 96-well plate in addition to the genotyping replication of samples. Frozen samples were shipped to KBiosciences in 96 well plates on dry ice.

## 2.6 Statistical analysis

Genotyping results were analysed by SPSS 21.0 program; analysis included calculations of means and standard deviations of clinical parameters such as age, gender and pattern of liver damage. Binary logistic regression test was used to compare means of continuous data. Differences between cases and controls were examined using Fisher's exact test. P-value, odds ratio, 95% confidence interval and chi-square test for trend were calculated using Graphpad PRISM version 5.0. Compliance with Hardy-Weinberg equilibrium was also determined for control groups to confirm that they met standard quality criteria using a web-based calculator available at http://www.oege.org/software/hardy-weinberg.shtml. Haplotypes and their frequencies were assigned using Haploview version 4.1. Comparison of haplotypes frequencies between cases and controls was performed using UNPHASED software version 3.1.7 which determines the p values based on the likelihood ratio chi-square test (Dudbridge, 2013). Minitab 16 was used to calculate the statistical power and sample sizes needed.

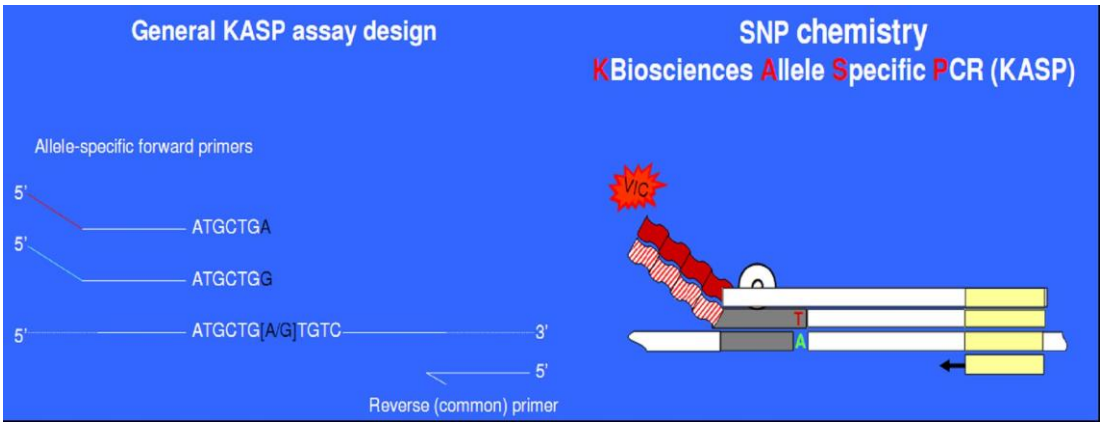


**Figure 2.3: KBiosciences (KASP) genotyping assay.** This is a fluorescent system contains two allele-specific forward primers, one reverse primer and a universal reaction mixture. Each forward primer contains a unique unlabelled tail sequence at the 5' end. The master mix contains two different 5' fluor-labelled oligos; one labelled with FAM and one with VIC.

# Chapter 3. Clinical Characterisation of the Flucloxacillin and Co-amoxiclav Drug-Induced Liver Injury Study Cohorts

## 3.1 Introduction

In genetic association studies, patients' characteristics including age, sex, race, concurrent use of medications and co-morbid diseases are considered common non-genetic factors that may affect the manifestations of a disease to be studied (Lucena et al., 2009). Other non-genetic determinants of propensity to DILI development which were extensively discussed in previous literature involve drug dose and duration of drug exposure (de Abajo et al., 2004; Kenyon and Nappi, 2003; Otani et al., 1989). An overview of the general effect of non-genetic factors on susceptibility to DILI was previously discussed in Section 1.5. Additionally, clinical and biochemical features of affected individuals are also considered important parameters that may show specific association with different patterns of diseases (Judge et al., 2013).

The large possible number of causes known to induce liver injury and lack of definite diagnostic or predictive tools make the assessment of causality a very difficult task. Therefore, correlation between a drug and a disease needs to be evaluated carefully in patients with suspected DILI using a standard adjudication method before starting looking for the risk factors contributed in the development of certain drug toxicities (Teschke et al., 2008). A number of standard structured scaling methods are currently available to measure the strength of association between the concerned drug and hepatotoxicity including Roussel Uclaf Causality Assessment Method (RUCAM), qualitative Council for International Organizations of Medical Sciences (CIOMS), expert opinion, the MV scale (performed by Maria and Victorino), AD scale (introduced by Aithal and Day) as well as the clinical diagnostic scale (CDS) (by Aithal, Rawlins and Day) (Aithal et al., 2000b; Aithal and Day, 1999; Danan and Benichou, 1993).

The RUCAM method was the first structured scaling process introduced in 1987 as an alternative to the previous traditional intuition methods (Danan et al., 1987). Causality assessment by RUCAM is based on the evaluation of a range of clinical and biochemical factors to identify whether a particular drug is truly implicated in the induction of liver injury. RUCAM system gives each of those factors illustrated below a specific score among the range of scores shown in the parentheses:

- Latency period (+1 or +2).
- Course of illness which is interpreted as the time needed for reduction ALT and ALP by 50% after drug discontinuation (-2, 0, +1, +2 or +3).
- Exclusion of other possible diseases (e.g. biliary obstruction, viral hepatitis) or non-drug causes (-3, -2, 0, +1 or +2).
- Use of concomitant drugs (0, -1, -2 or -3).
- Previous history of liver injury induced by the implicated drug (0, +1 or +2).
- Existence of risk factors for example older age above 55, pregnancy, alcohol consumption or genetic factors (2 scores: 0 or +1 each).
- Response to re-exposure to the same drug (-2, 0, +1 or +3).

The terms highly probable (score above 8), probable (score of 6-8), possible (score of 3-5), unlikely (score of 1-2) and unrelated (zero score) are the five levels used by RUCAM method to describe the rate of causality between the administered drug and the developed hepatic injury (Rockey et al., 2010). The first step to assess RUCAM score is to determine the pattern of liver injury whether classified as hepatocellular, mixed or cholestatic based on *R* ratio calculation (*R*= (ALT value/ALT ULN) / (ALP value / ALP ULN) (Table 3.1). $R > 5$ indicates hepatocellular reactions, $R$=2-5 indicates a mixed pattern whereas $R < 2$ is defined as cholestatic type of liver injury. DILI cases of either cholestatic or mixed phenotype are considered the same by RUCAM system and therefore similar scores are given to both patterns. R ratio is one of the criteria used to determine the scores related to latency period, course of illness and risk factors. RUCAM scores may vary between is -9 to +14, however, the range may drop to -7 to +11 when the step of rechallenge using the concerned agent did not take place. Patients without risk factors may have lower RUCAM scores of -7 to +9 and similarly the range of RUCAM score may widen or narrowed based on the existence or lack of other related factors (Benichou et al., 1993).

The aim of the studies described in this chapter is to examine various clinical and laboratory parameters of co-amoxiclav and flucloxacillin cases recruited, mainly retrospectively, for a genetic study to see if they are similar to those reported previously by epidemiological studies. The parameters proposed to study include pattern of liver injury, drug-disease causality assessment, elevation levels of liver enzymes, duration and percentage of reduction of liver enzyme levels post-drug discontinuation.

**Table 3.1: Classification of liver injury based on *R* ratio**

| *R** ratio | Type of hepatotoxicity |
|---|---|
| *R* > 5 | Hepatocellular |
| *R* = 2-5 | Mixed |
| *R* < 2 | Cholestatic |

* *R*= (ALT value/ALT ULN) / (ALP value / ALP ULN

## 3.2 Methods

### *3.2.1 DILI cases*

One hundred and fifty five cases of flucloxacillin DILI and 165 co-amoxiclav DILI cases were collected retrospectively and prospectively from collaborators in several European countries (Netherlands (1 case), Iceland (10 cases), Sweden (30 cases)) but mainly from centres throughout the United Kingdom (279 cases) based on known criteria of DILI detailed below. All cases were of white European ethnicity. Anonymised information on clinical features was obtained from the patient medical records and supplied by the hospital staff who collected the blood sample. The data was later extracted from two databases (DILIGEN and iDILIC) for the analysis described in this chapter. Compared to previous studies, this is the largest number of cases to be analyzed for both these types of DILI up to the present. Some of the cases (51 flucloxacillin cases and 77 co-amoxiclav) were previously described in Daly et al. (2009) and Lucena et al. (2011). A few of the co-amoxiclav cases were also reported by O'Donohue et al. (2000). Ethical approval for the UK study was obtained from the Leeds East Research Ethics committee. The European centres obtained ethical approval from the relevant local ethics committees.

### *3.2.2 Inclusion criteria and patients eligibility*

For inclusion in the study, patients needed to meet at least one out of three diagnostic criteria: 1) clinical jaundice or bilirubin > 40 μmol/l, 2) alanine aminotransferase (ALT) >5x the upper normal limit (ULN) or 3) alkaline phosphatase (ALP) >2x ULN and bilirubin > ULN. The patients who met the above criteria were also required to be over 18 years of age, able to give informed consent and their hepatotoxicity was suspected to be due to either flucloxacillin or co-amoxiclav. Confirmation that the cases appeared likely to represent DILI induced by the relevant drugs was made by expert adjudicating hepatologists (Professor Guruprasad Aithal, Nottingham University and Dr Einar Bjornsson, Reykjavik) using international consensus criteria (RUCAM scoring) (Aithal et al., 2011; Benichou, 1990).

### *3.2.3 Causality assessment*

The RUCAM method was used to assess DILI causality. RUCAM scores were calculated as a sum of points for the 7 factors described above; each of those factors is given a score between -3 to 3 and the net score is used to determine the level of causality.

### *3.2.4 Stratification of cases*

DILI cases were stratified based on their gender and duration of drug exposure into shorter (≤ 10 days) or longer courses (> 10 dayss). Also, the patients were subgrouped according to their jaundice status and phenotypic characteristics (cholestatic, hepatocellular or mixed phenotypes (those which show both cholestatic and hepatocellular clinical manifestations)). DNA from all flucloxacillin DILI cases had been genotyped for rs2395029, a tag SNP for HLA-B*57:01, previously by Ms Julia Patch. These data were used to classify cases as positive or negative for HLA-B*57:01. The patients were further classified as a result of DILI severity into those without jaundice (score 1, mild DILI) or jaundiced patients. The cases with jaundice were additionally divided into 3 groups; the patients with moderate DILI (score 2), severe (score 3) or those with the very severe DILI form (score 4 which involves cases underwent liver transplantation or died due to DILI).

### *3.2.5 Statistical analysis*

Fisher's exact test P-values were calculated for categorical covariates such as gender and pattern of liver damage using Graphpad PRISM version 5.0. Calculations of means and standard deviations of clinical parameters such as age were performed using SPSS 21.0 program and the differences were measured using the 2-independent samples t-test or binary logistic regression test.

## 3.3 Results

### *3.3.1 Effect of clinical characteristics on DILI susceptibility due to flucloxacillin*

Among the 155 flucloxacillin DILI cases, the causality of the drug in the majority of selected patients were considered as highly probable (48.4%) or probable (40.6%) based on RUCAM scoring (Table 3.2). In contrast, a lower number of reported cases (n=17) showed a possible causality (RUCAM score of 3-5). All other cases with RUCAM scores below 3 which were classified as unlikely had been excluded from this study. The patients were on drug therapy for an average period of 10 days before they developed DILI reactions on an average of 23.5 days thereafter at the mean age of 62.6 years. About 58% of the cases were above 60 years and more than two thirds of the affected cases were females. In line with the previous report (Daly et al., 2009), the majority of cases showed cholestatic and mixed reactions (91.6%) and also 84.5% of the cases were positive for HLA-B*57:01 when genotyped for the tag SNP rs2395029. Five cases showed a high level of severity (score of 3 or 4), with one undergoing liver transplantation and one patient dying 25 days post-diagnosis but the majority of cases had a score of 1 or 2.

It was decided to perform further analysis dividing on the basis of males versus females (Table 3.3), those who used flucloxacillin for 10 days or under and those used it over 10 days (Table 3.4) and those positive and negative for HLA-B*57:01 (Table 3.5). The parameters compared were age, gender, the percentage of cases that were cholestatic or mixed, the presence of jaundice, length of time on the drug and HLA-B*57:01 genotype. Comparison of certain parameters including age, development of jaundice, cholestatic and mixed reactions and B*57:01 genotype between male and female patients showed no differences (Table 3.3). However, significant age difference was noted (P=0.022) between patients who used flucloxacillin for more than 10 days and others who were drug-exposed for 10 days or less (mean age ±SD= 66.28±12.17 vs 60.90±13.62, respectively) (Table 3.4). Dividing the cases into HLA-B*57:01 positive or negative failed to demonstrate an association with any of the selected parameters shown in Table 3.5.

**Table 3.2: Clinical and biochemical variables in DILI patients exposed to flucloxacillin (n=155).**

| | |
|---|---|
| Sex (F/M) | 108 (69.7) / 47 (30.3) |
| Age at onset (yrs) | 62.6 ± 13.4 |
| Time to onset (days) | 23.5 ± 17.8 |
| Total days on drug | 10.3 ± 6.2 |
| Pattern of liver injury | |
| Cholestatic | 95 (61.3) |
| Hepatocellular | 13 (8.4) |
| Mixed | 47 (30.3) |
| RUCAM scoring | |
| 3-5 (possible) | 17 (11.0) |
| 6-8 (probable) | 63 (40.6) |
| >8 (highly probable) | 75 (48.4) |
| Peak Bilirubin (µmol/l) | 264.3 ± 227.1 |
| Peak ALT (U/l) | 400.4 ± 253.9 |
| Peak ALP (U/l) | 569.6 ± 668.5 |
| Severity of DILI | |
| Mild | 11 (7.1) |
| Moderate | 139 (89.7) |
| Severe | 3 (1.9) |
| Very severe | 2 (1.3) |
| Cases with jaundice | 144 (92.9) |
| Time taken for ALT/ALP to decrease to ≥ 50% after discontinuation (days) | 68.4 ± 74.3 |
| Cases positive for B*57:01 (%) | 131(84.5) |

Percentages are shown in parentheses.

**Table 3.3: Comparison of selected parameters between female and male flucloxacillin DILI cases.**

| | Female (n=108) | Male (n=47) | P |
|---|---|---|---|
| Age | 64.72±11.90 | 61.62±13.93 | 0.19 |
| Cholestatic & mixed | 99 (91.7%) | 43 (91.5%) | 1.0 |
| Jaundice | 99 (91.7%) | 43 (91.5%) | 1.0 |
| Exposure > 10 days | 32 (29.6%) | 15 (31.9%) | 0.78 |
| HLA-B*57:01-positive | 93 (86.1%) | 37 (80.4) | 0.37 |

**Table 3.4: Comparison of selected parameters between patients on flucloxacillin for 10 days or less versus those on longer courses.**

| | ≤ 10 days (n=105) | >10 days (n=47) | P |
|---|---|---|---|
| Age | 60.90±13.62 | 66.28±12.17 | 0.022 |
| Female | 76 (72.3%) | 32 (68.1%) | 0.78 |
| Cholestatic & mixed | 98 (93.3%) | 44 (93.6%) | 0.76 |
| Jaundice | 98 (93.3%) | 44 (93.6%) | 0.76 |
| HLA-B*57:01-positive | 91 (86.7%) | 39 (83.0%) | 0.81 |

**Table 3.5: Comparison of selected parameters between HLA-B*57:01-positive and negative flucloxacillin DILI cases.**

| | HLA-B*57:01-positive (n=130) | HLA-B*57:01-negative (n=24) | P |
|---|---|---|---|
| Age | 62.52±13.94 | 62.30±10.10 | 0.94 |
| Female | 93 (86.1%) | 15 (62.5%) | 0.37 |
| Cholestatic & mixed | 119 (91.5%) | 22 (91.7%) | 1.0 |
| Jaundice | 119 (91.5%) | 22 (91.7%) | 1.0 |
| Exposure > 10 days | 39 (30.0%) | 8 (33.3%) | 0.75 |

### *3.3.2 Effect of clinical characteristics on DILI susceptibility due to co-amoxiclav*

Similar to flucloxacillin cases, RUCAM classification of co-amoxiclav DILI patients indicated strong drug causality with 87.3% of the cases which were considered either highly probable or probable (Table 3.6). Severe DILI toxicity (score of 3 or 4) was noticed in five co-amoxiclav cases; two of them required liver transplantation while one patient died shortly as a consequence of DILI development. The period of drug exposure was about 9 days and the onset of DILI was noticed on an average of 20 days post-exposure at the mean age of 63 years. More than 64% of the cases were older than 60 years and the number of male patients was slightly larger (52.1%) than females. The cases showed severe elevation of liver enzymes with an average about 11.7 times ULN of bilirubin, around 9.3 times ULN of ALT and an approximate 4.3 times ULN of ALP. As for flucloxacillin, cholestatic and mixed (83.6%) were the predominant histological phenotypes among co-amoxiclav cases. Only 2 cases failed to show noticeable reduction of their abnormal ALT and ALP after several months of co-amoxiclav discontinuation. In contrast, the majority of patients had their liver enzymes reduced by more than 50% of excess over upper limits of normal in about one and a half months.

When male and female cases were compared for similar parameters (Table 3.7), age at the time of DILI was significantly lower in females compared with males with the mean age of male cases about 5.5 years more than the mean age of females (P=0.006). Males were also found to be at higher risk of developing jaundice due to co-amoxiclav than females (P=0.009). There was also a trend towards significance (p=0.086) in the proportion with cholestatic and mixed disease in the male group. When a range of parameters was compared in those who used co-amoxiclav for 10 days or under and patients on longer durations (Table 3.8), no significant differences noted between tested groups in terms of age or gender and also no differences seen in the percentages showing cholestatic/mixed DILI or the percentage with jaundice. Further analysis was performed to compare the gender and phenotype of co-amoxiclav cases with the flucloxacillin DILI group. The results indicated a significantly higher percentage of female cases in the flucloxacillin group (P=0.00011) than in the co-amoxiclav DILI cohort (about 70% versus 48%, respectively) (Table 3.9). More frequent cholestatic and mixed reactions were also

noted in the flucloxacillin cases rather than co-amoxiclav group with a borderline significant difference (P=0.042). Additionally, the flucloxacillin DILI cases showed a higher mean age (64.7 versus 61.6 years, respectively) and slightly higher jaundice incidence rate (91.6% versus 90.9%, respectively) but these differences were not statistically significant.

**Table 3.6**: **Clinical and biochemical variables in DILI patients exposed to co-amoxiclav (n=165).**

| | |
|---|---|
| Sex (F/M) | 79 (47.9) / 86 (52.1) |
| Age at onset (yrs) | 63.1 ± 12.8 |
| Time to onset (days) | 19.9 ± 19.6 |
| Total days on drug | 9.2 ± 10.4 |
| Pattern of liver injury | |
| Cholestatic | 108 (65.4) |
| Hepatocellular | 27 (16.4) |
| Mixed | 30 (18.2) |
| RUCAM scoring | |
| 3-5 (possible) | 21 (12.7) |
| 6-8 (probable) | 71 (43.0) |
| >8 (highly probable) | 73 (44.3) |
| Peak Bilirubin (µmol/l) | 221.4 ± 161.8 |
| Peak ALT (U/l) | 420.4 ± 422.7 |
| Peak ALP (U/l) | 516.8 ± 393.4 |
| Severity of DILI | |
| Mild | 15 (9.1) |
| Moderate | 145 (87.9) |
| Severe | 2 (1.2) |
| Very severe | 3 (1.8) |
| Cases with jaundice | 150 (90.9) |
| Time taken for ALT/ALP to decrease to ≥ 50% (days) after discontinuation | 44.4 ± 42.3 |

Percentages are shown in parentheses.

**Table 3.7: Comparison of certain parameters between female and male co-amoxiclav DILI patients.**

| | Female (n=79) | Male (n=86) | P |
|---|---|---|---|
| Age | 60.18±14.16 | 65.71±10.78 | 0.006 |
| Cholestatic & mixed | 62 (78.5%) | 76 (88.4%) | 0.086 |
| Jaundice | 67 (84.8%) | 83 (96.5%) | 0.009 |
| Exposure >10 days | 14 (17.7%) | 23 (26.7%) | 0.17 |

**Table 3.8: Comparison of selected parameters between patients on co-amoxiclav for 10 days or less versus those on longer courses.**

| | ≤ 10 days (n=125) | >10 days (n=36) | P |
|---|---|---|---|
| Age | 62.61±13.17 | 64.81±11.18 | 0.36 |
| Male | 63 (50.4%) | 23 (63.9%) | 0.17 |
| Cholestatic & mixed | 105 (84.0%) | 33 (91.7%) | 0.30 |
| Jaundice | 116 (92.8%) | 34 (94.4%) | 0.81 |

**Table 3.9: Comparison of some parameters between both DILI cohorts.**

| | Flucloxacillin (n=155) | Co-amoxiclav (n=165) | P |
|---|---|---|---|
| Age | 64.72±11.90 | 61.62±13.93 | 0.19 |
| Female | 108 (69.7%) | 79 (47.9%) | 0.00011 |
| Cholestatic & mixed | 142 (91.6%) | 138 (83.6%) | 0.042 |
| Jaundice | 142 (91.6%) | 150 (90.9%) | 0.85 |
| Exposure >10 days | 47 (30.3%) | 37 (22.4%) | 0.13 |

## 3.4 Discussion

This analysis of clinical data involved the largest number of DILI cases relating to both flucloxacillin and co-amoxiclav compared with all previous published studies (Andrade et al., 2005; Bell and Chalasani, 2009; Bjornsson et al., 2013; Chalasani et al., 2008; Daly et al., 2009; de Abajo et al., 2004; De Valle et al., 2006; Derby et al., 1993; Fairley et al., 1993; Ghabril et al., 2010; Hautekeete et al., 1999; Koek et al., 1994; Larrey et al., 1992; Lucena et al., 2006; Lucena et al., 2009; Lucena et al., 2011; O'Donohue et al., 2000; Russmann et al., 2005; Sgro et al., 2002; Shin et al., 2013). Though overall numbers are high, there are limitations in using the clinical data collected here as an epidemiological survey. Most cases are retrospective so this may result in the most severe cases not being included due to patient deaths. In addition, it is not a survey of DILI in any particular region or country. The samples were primarily collected for genetic studies by investigators interested in the area of genetics of DILI.

Data analysis of the flucloxacillin group (104 newly recruited cases in addition to those analysed by Daly et al. (2009) (n=51)) has investigated the effect of biochemical variables and other non-genetic factors on flucloxacillin DILI susceptibility. Our data have confirmed the previously reported findings that cholestatic reactions are the most common DILI form related to flucloxacillin and the incidence rate is highly noticeable in female patients and those older than 60 years (Daly et al., 2009; de Abajo et al., 2004; De Valle et al., 2006; Derby et al., 1993; Fairley et al., 1993; Koek et al., 1994; Russmann et al., 2005). Both Koek et al. (1994) and de Abajo et al. (2004) have reported that their cases showed more frequent cholestatic and mixed liver injury due to flucloxacillin (72.7% and 75%, respectively) and comparably higher percentage (91.6%) was seen in our data. In addition, all the flucloxacillin cases (n=8) reported by De Valle et al. (2006) were cholestatic and mixed DILI which induced due to drug exposure for a period mean of 10 days. The vast majority of flucloxacillin DILI patients in our study were jaundiced (92.9%) which is consistent with the results seen in de Abajo et al. (2004) and (De Valle et al., 2006) studies (75% and 87.5%, respectively).

The Australian flucloxacillin study (Fairley et al., 1993) as well as the Netherlands report (Koek et al., 1994) showed more incidents of DILI in female than male patients (29 females versus 22 males in the Australian study and 7 females versus 4 males in the Netherlands study) and the average age at the time of diagnosis of the Australians was 60.1 years with 60.8% of the patients aged above 55 years whereas the mean age of Dutch cases was 57 years; these data are consistent with our findings. In the study of Russmann et al. (2005) which involve 283,097 patients as first-time users of flucloxacillin, 24 cholestatic DILI cases were identified within a range of time to onset between 14 to 44 days (average is 25.5 days) after an exposure to flucloxacillin for 7 days whereas 5 other cases were developed after 46 to 90 days. Our findings supported Russmann et al. (2005) results who reported that the majority of flucloxacillin cases (72.4%) were females (female to male ratio=2.6) though female gender was not identified as a risk factor for DILI development. Despite only 25% of all the participants of Russmann et al. (2005) study being above 60 years, it was demonstrated that 69% of the patients who developed DILI toxicity were older than 60 and the average age of onset was 62.6 years in line with our results. More recently, De Valle et al. (2006) found that 71.4% of the flucloxacillin cases involved in the Swedish study were older than 60 years too and most of the cases were women (57.1%).

Use of flucloxacillin for longer period over two weeks was reported to significantly increase the risk of DILI by 7 times as seen in the Australian study that recruited 51 cases versus 199 drug-exposed controls (Fairley et al., 1993). This report was later supported by de Abajo et al. (2004) study which indicated that even drug usage for more than a week has still the potential to increase DILI risk. Our data analysis has confirmed those findings in older patients only. Interestingly, delayed onset (46-90 days) of liver injury was mostly noticed (in 80% of the cases) in patients who used the drug for less than 6 days in the Russmann et al. (2005) study while the delayed onset cases (n=10) recruited in our study were treated by flucloxacillin for an average period of 16 days.

In the case of co-amoxiclav, the analysis combined the UK DILIGEN cases (n=77) described in Lucena et al. (2011) with an additional 88 new cases (n=165 altogether). The Lucena et al. study also included cases from Spain and the US for which the clinical data was not available to us so it is useful to compare the current

findings with these earlier findings. Similar to Lucena et al. (2011) and O'Donohue et al. (2000), a slight excess of male cases over females was noted here (percentage of males is 50.6%, 54.5% in Lucena and O'Donohue studies versus 52.1% in our study). In contrast, the only 4 co-amoxiclav DILI cases involved in Sgro et al. (2002) report were all females. The time on drug treatment before developing DILI was slightly longer in our patient group (9 ± 10 days in our study versus 8 ± 6 days for Lucena and an average of 7 days for O'Donohue), days to DILI onset were longer in our analysis (20 ± 20 days) compared with the Lucena and O'Donohue studies (15 ± 14 days for Lucena and mean of 17 days for O'Donohue) and the mean age of onset was above 60 years in our samples and in Lucena study (63 versus 61 years, respectively) but was 59.1 years in O'Donohue report. More than 64% of our cases were above 60 years but significant mean age difference between male and female patients in our co-amoxiclav DILI cohort was noted (P=0.006). A separate French study (Larrey et al., 1992) that involved 15 co-amoxiclav DILI cases showed quite similar findings to ours with a comparable mean age of 64 years but a higher percentage of males than females was seen (80% versus 20%) and the cases were exposed to co-amoxiclav for longer durations (average drug exposure of 18 days) and more times were required to detect DILI development (average time of DILI onset was 29 days after start of drug treatment). Cholestatic and mixed DILI were found to be the prominent pattern of liver injury due to co-amoxiclav in our data (83.6%) and similar findings were obtained previously by Lucena and O'Donohue as well (70% and 94.1%, respectively).

Twenty one DILI cases due to co-amoxiclav were reported in a very recent study conducted in Iceland (Bjornsson et al., 2013); six of them initiated co-amoxiclav in the hospital while the remaining 15 cases started their drug therapy as outpatients; 40% of the outpatient cases developed jaundice. Ten of the Iceland cases were recruited to our study with 80% of these were jaundiced and the overall incidence of this in all co-amoxiclav cases involved in our data was extremely high (90.9%). The lower frequency of jaundiced patients in the Iceland study compared to ours may refer to the criteria of jaundice used by Bjornsson et al. (2013) where bilirubin level has to be greater than 50 µmol/L in order for the cases to be classified as jaundiced, while in our study the level of bilirubin of ≥40 µmol/L is enough to consider a patient as jaundiced. In addition, some of the cases included by Bjornsson et al.

(2013) may not have fulfilled the required ALT increase for inclusion in our study. The risk to develop jaundice due to co-amoxiclav in our cases is higher in the male group compared with female patients. This result is in line with the Belgian study which stated that 68.6% of 35 jaundiced patients involved were males (Hautekeete et al., 1999). However, more females (n=12) than males (n=10) were observed among the jaundiced cases reported by O'Donohue et al. (2000). Furthermore, the mean age of patients who showed cholestatic and mixed phenotypes was significantly higher (P=0.011) than hepatocellular patients (64.2 versus 57.3 years). This finding is consistent with several previous reports that observed more frequent cholestatic and mixed toxicity type in older patients (Chalasani et al., 2008; Ghabril et al., 2010). A previous study by Lucena et al. (2006) reported that 65% of 69 co-amoxiclav DILI cases were older than 55 years and more recently Lucena et al. (2009) stated that cholestatic patients who represented 61% of 149 DILI cases recruited over 13 years period (between April 1994 to August 2007) were above 60 years. These reports were also supported by the findings of DILIN study in the United States which involved 300 DILI cases and showed that the average age of cholestatic and mixed DILI patients was 54 years versus 44 years for those who developed hepatocellular pattern (Chalasani et al., 2008). Besides, our results indicated that males developing cholestatic and/or mixed DILI due to co-amoxiclav are overrepresented compared with females (55.1% vs 44.9%, respectively). Similar findings were seen by Hautekeete et al. (1999) who reported that the 77.4% of the cases with cholestatic and mixed hepatotoxicity due to co-amoxiclav were above 60 years and 67.7% of the cases were males.

The increased frequency of liver injury among older patients seen in both DILI cohorts involved in our study may partly be explained by the higher consumption of both drugs by older than younger people. The prescribing trends seen in the Scottish population for example showed clearly that the amount of both drugs dispensed to general community was increasing proportionally with the increase of patients' age in different age groups (Scottish Antimicrobial Prescribing Group, 2010). Patients at the age range of 30-59 were prescribed 190 items/ day of β-lactamase resistant penicillins (e.g. flucloxacillin) and an average of 85 items/ day of penicillin combinations (e.g. co-amoxiclav); the amount dispensed increased to 240 and 130 items/ day, respectively for the age group of 60-79 and more noticeably the amount

was almost doubled to 390 and 180 items/ day, respectively for patients aged over 80.

Significant differences were also noted in the cases of flucloxacillin DILI than in co-amoxiclav group with regard to sex distribution (P=0.00011). The flucloxacillin DILI cohort had more than twice (2.3 times) as many females as males which is in line with the previous reports that suggested a link between female gender and liver injury due to certain drugs (Banks et al., 1995; Bell and Chalasani, 2009; de Abajo et al., 2004). A number of studies have shown more frequent distribution of females among hepatocellular DILI cases only as seen in the Swedish (De Valle et al., 2006) and the US studies (Chalasani et al., 2008) (females represented 63% and 65% of hepatocellular cases, respectively). In the Spanish study that involved 461 DILI cases (Andrade et al., 2005) due to multiple agents, co-amoxiclav was reported as the leading drug causing DILI and the female sex was identified as an independent risk factor; however, both our results as well as the findings reported in the American study by Chalasani et al. (2008) failed to observe a significant association between female gender and DILI development. Among both sexes, the percentage of cholestatic and mixed reactions was seen higher in the flucloxacillin group than in co-amoxiclav cases. These findings are consistent with de Abajo et al. (2004) results who also demonstrated higher cholestatic and mixed phenotypes in a flucloxacillin DILI cohort than in co-amoxiclav cases (75% versus 61.5%).

In summary, analysis of both DILI groups confirmed the predominantly cholestatic and mixed nature of DILI relating to both drugs, the high percentage of patients with DILI who developed jaundice and the mean age of recruited individuals was above 60 years. The older patients are at higher risk of hepatotoxicity when exposed to flucloxacillin for more than 10 days and males are more susceptible to jaundice than females when treated with co-amoxiclav. Also, females seem to develop co-amoxiclav DILI at a younger age than males.

# Chapter 4. Investigation of Biologically-Relevant Candidate Genes as Risk Factors for Drug-Induced Liver Injury Relating to Flucloxacillin and Co-amoxiclav

## 4.1 Introduction

This chapter considers polymorphisms in genes of possible biological relevance to DILI due to flucloxacillin and co-amoxiclav as risk factors for disease development. Genes were selected on the basis of either having a possible role in disposition of the drugs or because of previous reports of having a more general role in DILI susceptibility either due to their role in the biology of the liver or in protecting the liver against oxidative stress.

Flucloxacillin undergoes limited metabolism by the cytochrome P450 CYP3A4 but as far as is known neither amoxicillin nor clavulanic acid are metabolized. Previous studies on flucloxacillin DILI found no evidence for an effect by CYP3A4 or CYP3A5 genotype on susceptibility to DILI (Andrews et al., 2010; Chamberlain, 2014). Further candidate gene studies in relation to drug metabolism are therefore unlikely to be of value. Drug transporters play a more general role than metabolic enzymes in regulating hepatic drug levels and the genes coding for these proteins are therefore worthwhile candidates for a role in susceptibility. The possibility that four different ABC transporter genes, ABCB1, ABCC2, ABCB4 and ABCB11 might affect susceptibility to DILI due to flucloxacillin or co-amoxiclav in our study population has already been investigated and no significant associations found (Bhatnagar et al., 2008) (see Section 1.6.3). However, the OATP transporters encoded by the SLCO1B genes are relevant to the transport of penicillins (Yamaguchi et al., 2011) across the sinusoidal membrane of hepatocytes and have not been studied previously as risk factors for DILI. As discussed in Section 5.1, SLCO1B1 has shown to be a risk factor for several other types of adverse drug reactions. Though a related transporter SLCO1B3 may also contribute to hepatic penicillin uptake (Yamaguchi et al., 2011), data on functional significance of polymorphisms in this second transporter is limited. It was decided therefore to study only SLCO1B1 as a candidate gene for flucloxacillin and co-amoxiclav-related DILI focusing on SLCO1B1*15 since this allele has been shown to be associated with slower hepatic uptake of a number of different drugs.

Two recent reports suggest that genotype for UGT1A1 and the haemachromatosis gene (HFE) affect susceptibility to DILI due to lapatinib and pazopanib respectively (Spraggs et al., 2012; Xu et al., 2011). Both genes have important roles in the normal

physiology of the liver and it is possible that they could also be relevant to DILI due to additional drugs. In the case of UGT1A1, expression of this gene is affected by the number of TA repeats in the TATA box. Gilbert's syndrome, the common familial mild hyperbilirubinemia, is associated with homozygosity for a (TA)7 repeat allele (UGT1A1*28) instead of the more common (TA)6 allele which is associated with higher UGT1A1 expression. Patients with Gilbert's syndrome may be more susceptible to drug induced-liver injury due to lapatinib (Spraggs et al., 2012) though the relationship seen was complex and HLA class II genotype also needed to be considered in assessing risk of DILI. There is also an additional report suggesting that carriers of UGT1A1*28 are more likely to develop DILI when treated with pegvisomant for acromegaly (Bernabeu et al., 2010). In view of these reports, UGT1A1 was chosen for further investigation as a risk factor in flucloxacillin and co-amoxiclav-induced liver injury.

The high iron Fe (HFE) gene is located on chromosome 6 in the MHC region but despite its location it does not appear to be an immune function gene (Bahram et al., 1999). It encodes a protein which competes with transferrin molecules in binding with their receptor. Mutations in HFE gene were repeatedly shown to be risk factors in patients with hereditary haemochromatosis in several human and animal studies (Barton et al., 1999; Beutler, 1998; Feder et al., 1996; Zhou et al., 1998). The majority of haemochromatosis cases (85%) were found positive for a C282Y variant though other variants can contribute and the C282Y shows limited penetrance with only a small percentage of individuals homozygous for this variant showing clinical symptoms of iron overload. Two SNPs (rs2858996 and rs707889), in strong linkage disequilibrium ($r^2$=0.99), in HFE were recently reported to be associated with reversible ALT elevation in 115 patients treated with the anti-cancer drug pazopanib (Xu et al., 2011) and the results were replicated in 128 additional cases. This study encouraged us to investigate the relevance of HFE to susceptibility to DILI caused by other drugs.

Oxidative stress within the liver may be important in the development of DILI and a number of previous genetic studies have focused on genes coding for enzymes that protect against oxidative stress. These include superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPX1) which have a direct role in detoxicating reactive oxygen species (ROS) together with the GST enzymes GSTM1 and T1 which may

detoxicate compounds such as malondialdehyde and 4-hydroxynonenal which are produced when ROS causes lipid peroxidation. The SOD2 gene, which encodes the mitochondrial manganese-dependent enzyme MnSOD, transforms superoxide radicals ($O_2^-$) into hydrogen peroxide ($H_2O_2$) which is then detoxified by GPX1 into water molecule ($H_2O$) (Bastaki et al., 2006; Michelson, 1982). Occurrence of cellular damage in response to impaired enzyme activity due to polymorphisms in oxidative stress genes is well recognized (Hensley et al., 2000). In addition to the DILI associations with SOD2 previously mentioned in Chapter 1 (Section 1.6.6), the non-synonymous polymorphism C47T (rs4880) in SOD2 was described as a risk factor for a number of complex diseases including progeria (aging rare genetic disease) (Rosenblum et al., 1996), nonfamilial idiopathic cardiomyopathy (Hiroi et al., 1999) and diabetic nephropathy in Japanese patients with type 2 diabetes (Nomiyama et al., 2003) and in European populations with type 1 diabetes (Mollsten et al., 2009; Mollsten et al., 2007). The T variant of rs4880 was also seen more commonly in Japanese patients with non-alcoholic fatty liver disease (NAFLD) (Namikawa et al., 2004). This finding was later confirmed by Al-Serri et al. (2012) in a UK-based study. The GPX1 gene which encodes the antioxidant glutathione peroxidase 1 enzyme is the most commonly expressed gene in liver compared to other family members which include GPX2 (expressed mainly in gastrointestinal cells), GPX3 (plasma) and GPX4 (mainly in phospholipids) (Brigelius-Flohe et al., 2000; Margis et al., 2008). GPX1 (rs1050450) was recently reported to be associated with drug-induced cholestatic hepatotoxicity in a Spanish study (Lucena et al., 2010).

The detoxicating genes GSTM1 and GSTT1 (encoding the glutathione S-transferases (GSTs) isoforms mu 1 and theta 1) which are highly expressed in liver were also selected to study in this project. The aim was to replicate the association obtained by Lucena et al. (2008) between both GST genes and co-amoxiclav-related DILI and to examine the possibility that these genes play a more general role in DILI susceptibility through testing an additional DILI cohort (flucloxacillin DILI cases). The frequency of null mutations of these genes varies among different ethnic groups ranging between 23 to 62% for GSTM1 and 20 to 50% for GSTT1 (Board et al., 1990; Cotton et al., 2000). GSTM1 is deleted in more than 50% in individuals of European ancestry whereas frequency of GSTT1 null allele exceeds 20%. Deletion of one or both GSTM1/GSTT1 genes was reported previously as a contributing

factor in DILI development due to several drugs (e.g. antituberculous agents and troglitazone) (see Section 1.6.2).

Seven genes (UGT1A1, HFE, SLCO1B1, SOD2, GPX1, GSTM1 and GSTT1) of known function were therefore selected for study in the candidate gene studies described in this chapter.

## 4.2 Methods

### *4.2.1 Control samples*

The cases studied were described in detail in Chapter 3. As controls for the DILI cases, several different population controls groups were used for the analyses described in this chapter. The population controls used were not necessarily drug-treated since it is widely accepted to use drug non-exposed controls in genetic studies involving comparisons with very rare diseases such as DILI due to the very low likelihood that controls would ever develop the disease. Also, recruitment of controls for the study who had been prescribed flucloxacillin and/or co-amoxiclav and following them up would be costly and time consuming and was not feasible given limited resources. The exception to this was a set of controls (n=64) who had been exposed to flucloxacillin without developing DILI (described by Daly et al., 2009). This group was used as a control group in a limited number of the analyses described in this thesis but in view of small numbers was not suitable for general use as a control group.

The community control groups used in the current chapter varied depending on the gene being studied. For genes already studied in the flucloxacillin and co-amoxiclav GWAS, control data on the POPRES control group (n=282) was used. UK individuals from this cohort described by Nelson et al. (2008). The Population Reference Sample, POPRES: a resource for population, disease, and pharmacological genetics research have already been shown to be a good genetic match for the flucloxacillin and co-amoxiclav cases included in the GWAS for DILI due to these drugs (Daly et al., 2009; Lucena et al., 2011). For other genes, up to 334 apparently healthy individuals recruited from North-East England were used as a community control group and genotyped directly. This group has been described in detail previously (Velaga et al., 2004). For HFe genotyping only, the control data

used was from 379 UK controls in the 1000 genomes project (http://www.1000genomes.org/).

### *4.2.2 UGT1A1*28 assay*

The primers used to genotype for UGT1A1*28 were described by (Lin et al., 2009). The PCR methodology described in Section 2.3.2 was followed; however, 5% DMSO was added to the master mix in this experiment to enhance PCR amplification. Cycling conditions were 30 cycles of 1 min at 95°C denaturation, 1 min at 55 °C annealing and 72 °C elongation for 1 min, followed by a 7 min extension at 72ºC resulting in 77 bp or 79 bp products. For DNA analysis, a 12.5% polyacrylamide gel (19:1, Acrylamide/Bisacrylamide) was used to visualize the 2 bp difference between both PCR products. Co-amoxiclav cases (n=120) and flucloxacillin DILI cases (n=89) were compared to 162 community controls samples.

### *4.2.3 TaqMan assays*

TaqMan SNP genotyping assays were used to genotype both DILI cohorts for SLCO1B1, SOD2 and GPX1 polymorphisms. A readymade assay (reference number: C___8709053_10, Cat. # 4351379) designed by Applied Biosystems was used to genotype SOD2 (rs4880) while custom TaqMan assays were ordered for the SNPs rs4149056 in SLCO1B1 and rs1050450 in GPX1. Allelic discrimination analysis was performed using a Step-One Real-Time PCR machine. Cases genotyped in these experiments were 164 co-amoxiclav and 155 flucloxacillin DILI cases which were compared to our community controls (n=334) in the case of SOD2 and GPX1 whereas previously obtained data from POPRES controls (n=282) (Daly et al., 2009) were used in the SLCO1B1 genotyping comparison with cases. A small number of cases failed genotyping in each assay used.

### *4.2.4 PCR- RFLP assay for HFE (rs2858996)*

PCR-RFLP was used to genotype samples for the polymorphism rs2858996 in the HFE gene. To amplify the PCR product, the nucleotide sequences 5′-GGGTGTCAAAGGAAAGAATG-3′ and 5′-AACCCAATATCCCAAAAGAC-3′ were used as forward and reverse primers, respectively. Cycling conditions were 35 cycles of 1 min at 94°C denaturation, 1 min at 53 °C annealing and 1 min at 72 °C elongation, followed by a 7 min extension at 72ºC resulting in 316 bp product. A 20 µl PCR product was digested overnight at 37 °C using 2 U of Dde I enzyme. The

enzyme cuts at the (T) allele, the variant type, resulting in fragments of 118 bp and 198 bp, whilst (G) allele remains uncut. As these studies were carried out earlier in the project, lower number of cases of each DILI cohort (101 co-amoxiclav and 84 flucloxacillin cases) was genotyped. Control data was obtained from UK population data (n=379) in the 1000 genomes study.

### *4.2.5 GSTM1 and GSTT1 assays*

164 co-amoxiclav cases, 155 flucloxacillin cases and 326 community controls were genotyped by PCR. The primers used to amplify GSTM1 (219 bp) and GSTT1 genes (459 bp) were obtained from Xiong et al. (2001). The primers used for the positive internal control (TNF-α, 340 bp) were obtained from Parnes et al. (2010). PCR cycling conditions used for both assays were similar to the conditions used by Xiong et al. (2001). The primers of internal positive control were added to each assay to confirm successful PCR amplification. The PCR products were visualized on 2% agarose gel stained with ethidium bromide. The absence of GSTM1 or GSTT1 fragment indicates the null genotype. However, absence of the TNF-α DNA band showed that the PCR amplification was unsuccessful and the experiment must be repeated.

## 4.3 Results

### *4.3.1 Association of UGT1A1*28 (-53(TA) 6-7) with DILI*

A typical genotyping assay which shows amplified fragments of different TA repeat length in the promoter region of UGT1A1 is shown in Figure 4.1. Genotype frequency results for DILI cases and community controls are described in Table 4.1. Comparison of co-amoxiclav case and control subjects showed slightly higher variant (TA7) allele frequency in cases (36.7%) than in controls (33.3%), however, the difference was not statistically significant (OR=1.46, 95% CI=0.90–2.40; P=0.14). Investigating cholestatic or mixed cases of co-amoxiclav DILI only (n=71) did not show an association with UGT1A1*28 (OR=1.6, 95% CI=0.88–2.85; P=0.15).

Similarly, no influence was seen for this gene in the other DILI cohort (flucloxacillin DILI) which showed a slightly higher but not significant genotype frequency difference in cases ( 34.8%) than in controls (33.3%) (OR=0.85, 95% CI=0.51–1.43; P=0.60).

Tag SNP genotype for HLA-B*57:01 reported by Chamberlain (2014) and Daly et al. (2009), was used to separate cases into positive and negative genotype groups. The possibility that UGT1A1*28 might be a risk factor in cases negative for the "at risk" HLA B*57:01 genotype in line with a previous report (Spraggs et al., 2012) could not be confirmed and the B*57:01 positive cases also failed to show any association (n=71) (OR=0.82, 95% CI=0.48–1.38; P=0.50).

Distribution of minimum allele frequency (MAF) (TA7) in the tested controls and in the flucloxacillin cases that developed cholestatic or mixed phenotypes was exactly the same (33.3%). Combining all DILI cases (n=206) in the comparison to the community controls has also shown negative association (OR=1.08, 0.71–1.64; P=0.75).

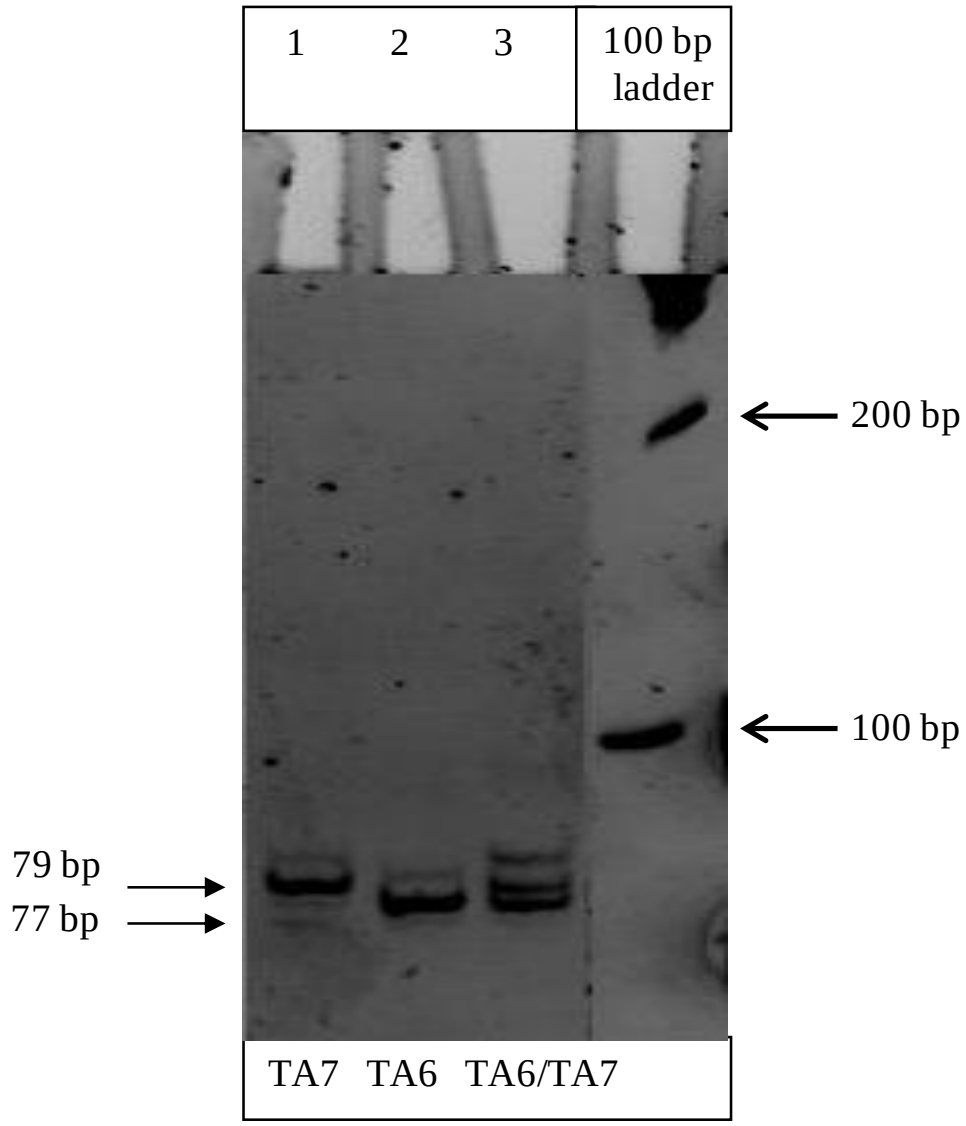


**Figure 4.1: 12.5% polyacrylamide gel (19:1, Acryl/Bis) showing UGT1A1 gene (-53(TA) 6-7)**. Lane 1 is homozygous mutant, Lane 2 is homozygous wild-type and Lane 3 is heterozygous.

**Table 4.1: UGT1A1*28 (-53(TA) 6-7) genotyping results in both cohorts of DILI cases and community controls.**

| Samples | No. of TA repeats | | | TA7% | P-value | OR | 95% CI |
|---|---|---|---|---|---|---|---|
| | 6/6 (%) | 6/7 (%) | 7/7 (%) | | | | |
| All co-amox cases (n=120) | 41 (34.2) | 70 (58.3) | 9 (7.5) | 36.7 | 0.14 | 1.46 | 0.90–2.40 |
| Cholestatic & Mixed co-amox cases (n=71) | 23 (32.4) | 44 (62.0) | 4 (5.6) | 36.6 | 0.15 | 1.60 | 0.88–2.85 |
| All fluclox cases (n=89) | 42 (47.2) | 32 (36.0) | 15 (16.8) | 34.8 | 0.60 | 0.85 | 0.51–1.43 |
| Fluclox cases +ve for rs2395029 (B*57:01) (n=85) | 41 (48.2) | 29 (34.1) | 15 (17.7) | 34.7 | 0.50 | 0.82 | 0.48–1.38 |
| Cholestatic & Mixed fluclox cases (n=75) | 36 (48.0) | 28 (37.3) | 11 (14.7) | 33.3 | 0.57 | 0.82 | 0.48–1.43 |
| All DILI cases (n=206) | 85 (41.3) | 99 (48.0) | 22 (10.7) | 34.7 | 0.75 | 1.08 | 0.71–1.64 |
| Controls (n=162) | 70 (43.2) | 76 (46.9) | 16 (9.9) | 33.3 | | | |

### *4.3.2 Genotyping results for SLCO1B1 (rs4149056, T/C)*

The variant rs4149056 in SLCO1B1 is a nonsynonymous mutation that results in an amino acid change from valine (T allele) to alanine (C allele) at codon 174. Analysis of TaqMan genotyping results of both co-amoxiclav and flucloxacillin DILI cohorts did not show an evidence of association with the tested groups (OR=0.71, 95% CI= 0.46–1.12; P=0.17 for flucloxacillin cases and OR=0.87, 95% CI= 0.56–1.33; P=0.58 for co-amoxiclav) (Table 4.2). Despite the observed slightly lower frequency (22.8%) of positive flucloxacillin cases compared to POPRES controls (29.4%), this difference was not significant. Subgroup analysis of flucloxacillin cases to include only those cases positive for rs2395029 (n=125) also failed to detect a role for SLCO1B1 in flucloxacillin DILI susceptibility (OR=0.76, 95% CI=0.47–1.23; P=0.28). Additionally, we investigated the contribution of SLCO1B1 to DILI in the cases that only show cholestatic or mixed DILI within the flucloxacillin group but no effect was observed (OR=0.90, 95% CI=0.57–1.41; P=0.64).

### *4.3.3 Genotyping results of HFE (rs2858996, G/T)*

The HFE genotyping assay showing digested and undigested DNA fragments is illustrated in Figure 4.2. No associations were seen between the intronic variant rs2858996, located in HFE on chromosome 6 in either DILI group. Genotypes of the tested samples described in Table 4.3 indicating that the frequency of minor allele (MAF) in flucloxacillin cases (18.4%) was almost similar to that seen in the community controls genotyped in the 1000 Genome project (19.4%) (OR=0.9, 95% CI=0.50–1.5; P=0.8) whereas co-amoxiclav group has shown a slightly higher MAF frequency distribution (21.8%) but the difference was not statistically significant (OR=1.3, 95% CI= 0.80–2.0; P=0.34). Subgroup analysis by histological type also showed no statistically significant associations.

**Table 4.2: SLCO1B1 (rs4149056) genotyping results in both DILI cohorts compared to POPRES community controls.**

| Genotype | TT (%) | CT (%) | CC (%) | P-value | Odds ratio | 95% CI |
|---|---|---|---|---|---|---|
| Fluclox cases (n=149) | 115 (77.2) | 31 (20.8) | 3 (2.0) | 0.17 | 0.71 | 0.46 – 1.12 |
| Fluclox cases positive for rs2395029 (tag SNP for B*57:01) (n=125) | 95 (76) | 27 (21.6) | 3 (2.4) | 0.28 | 0.76 | 0.47 – 1.23 |
| Fluclox cases with cholestatic or mixed phenotypes (n=136) | 104 (76.5) | 29 (21.3) | 3 (2.2) | 0.64 | 0.90 | 0.57 – 1.41 |
| Co-amox cases (n=162) | 119 (73.4) | 40 (24.7) | 3 (1.9) | 0.58 | 0.87 | 0.56 – 1.33 |
| POPRES controls (n=282) | 199 (70.6) | 74 (26.2) | 9 (3.2) | | | |

*Cases with mixed phenotypes are those which show both cholestatic and hepatocellular clinical manifestations.

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2 tailed test was used to calculate significance.

**Table 4.3: HFE (rs2858996) genotyping results in both DILI cohorts compared to 1000 Genome controls.**

| Genotype | GG (%) | GT (%) | TT (%) | P-value | Odds ratio | 95% CI |
|---|---|---|---|---|---|---|
| Fluclox cases (n=84) | 57 (67.8) | 23 (27.4) | 4 (4.8) | 0.80 | 0.90 | 0.50 – 1.5 |
| Cholestatic & mixed fluclox cases (n=75) | 50 (66.7) | 21 (28.0) | 4 (5.3) | 0.89 | 0.96 | 0.57 – 1.6 |
| Co-amox cases (n=101) | 61 (60.4) | 36 (35.6) | 4 (4.0) | 0.34 | 1.3 | 0.80 – 2.0 |
| Cholestatic & mixed co-amox cases (n=74) | 47 (63.5) | 25 (33.8) | 2 (2.7) | 0.79 | 1.1 | 0.66 – 1.8 |
| Hapatocellular co-amox cases (n=27) | 14 (51.9) | 11 (40.7) | 2 (7.4) | 0.21 | 1.8 | 0.81 – 3.9 |
| 1000 Genome controls (n=379) | 249 (65.8) | 113 (29.8) | 17 (4.4) | | | |

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2 tailed test was used to calculate significance.

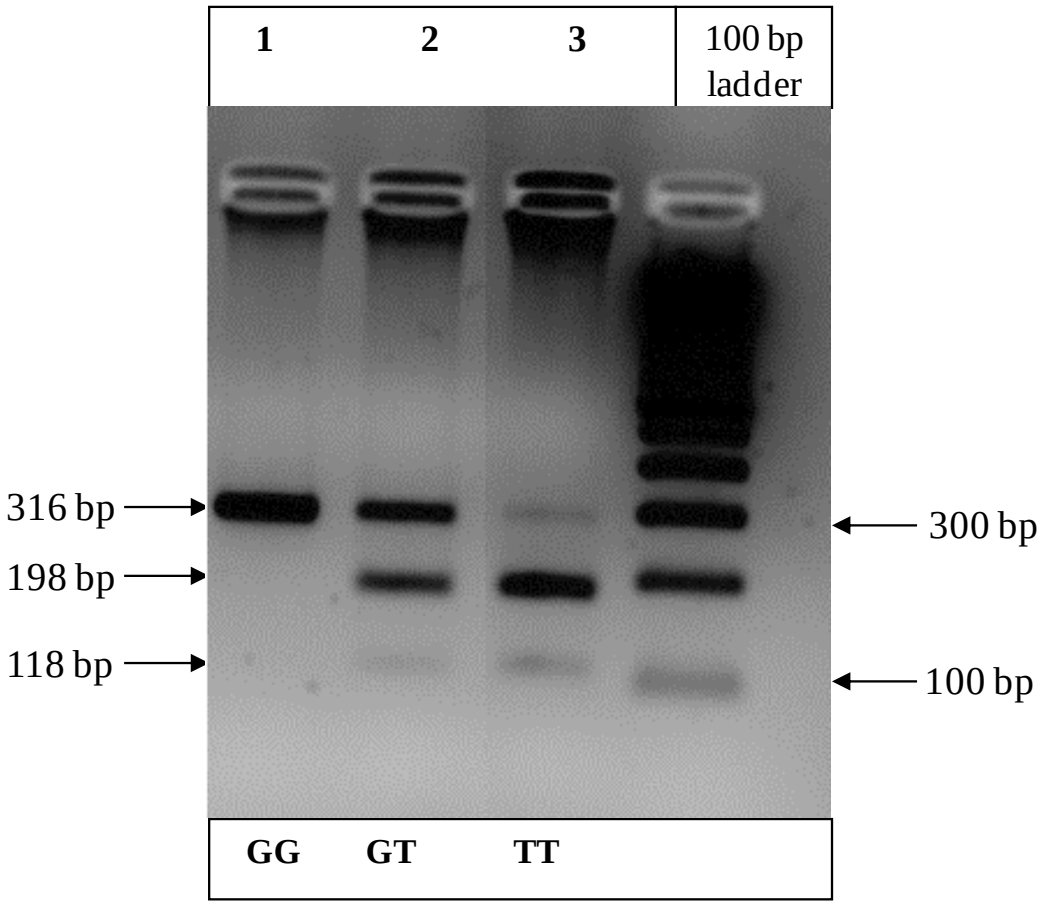


**Figure 4.2**: **2% agarose gel showing HFE gene digested with Dde I enzyme.** Lane 1 is homozygous wild-type, lane 2 is heterozygous, lane 3 is homozygous mutant.

### *4.3.4 Genotyping results for SOD2 (rs4880, C/T)*

The variant rs4880 of the SOD2 gene is a nonsynonymous SNP where a substitution of wild-type (C) allele into the mutant (T) allele results in the change of an alanine amino acid into valine at codon 16 (A16V). As summarized in Table 4.4, there was no significant difference in genotype distributions between cases and community controls for either flucloxacillin DILI (OR=0.82, 95% CI=0.54–1.25; P=0.38) or co-amoxiclav DILI (OR=1.1, 95% CI=0.7–1.7; P=0.66). Further, the difference of genotypes frequency distribution between controls and flucloxacillin cases that are only positive for rs2395029 (B*57:01) was not statistically significant (OR=0.72, 95% CI= 0.46–1.11; P=0.17). Moreover, similar genotype frequencies were observed upon comparing all DILI cases (combining both DILI groups, n=319) with the controls (OR=0.85, 95% CI=0.67–1.34; P=0.79).

### *4.3.5 Genotyping results of GPX1 (rs1050450, C/T)*

The rs1050450 polymorphism is also a nonsynonymous polymorphism which leads to a substitution of proline (C, wild-type allele) to leucine (T, variant allele) at codon 198. Genotypes frequency of DILI cases and community controls summarized in Table 4.4 showed no significant differences which indicated no role for GPX1 in DILI development. In the case of flucloxacillin cases, the genotype frequency was almost identical with controls giving an odds ratio of 1 and a p-value of 1. Limiting the case-control comparison to involve those cases positive for rs2395029 (B*57:01) did not identify any risk for DILI (OR=0.89, 95% CI=0.6–1.3; P=0.6). GPX1 genotype also showed no effect on risk of DILI related to co-amoxiclav (OR=1.17, 95% CI=0.8–1.7; P=0.44). Enlarging the sample size by combining both DILI cohorts to give 326 cases in the comparison with our community controls (n=334) also failed to show any significant association between GPX1 and risk of DILI (OR=1.1, 95% CI=0.8–1.5; P=0.59). Further analysis that involve cholestatic and mixed DILI in the cases positive for both SOD2 (alanine) and GPX1 (leucine) was performed (Table 4.5), however, this homozygosity for both genes was found to be irrelevant to flucloxacillin DILI (OR=0.87, 95% CI=0.57–1.33; P=0.59) neither to co-amoxiclav hepatotoxicity (OR=0.94, 95% CI=0.63–1.40; P=0.76).

**Table 4.4: SOD2 (rs4880) and GPX1 (rs1050450) genotyping results of both DILI cohorts and community controls.**

| | Genotype | TT (%) | CT (%) | CC (%) | P-value | Odds ratio | 95% CI |
|---|---|---|---|---|---|---|---|
| SOD2 (rs4880) | Fluclox cases (n=155) | 36 (23.2) | 72 (46.5) | 47 (30.3) | 0.38 | 0.82 | 0.54–1.25 |
| | Fluclox cases positive for rs2395029 (B*57:01) (n=130) | 26 (20) | 61 (46.9) | 43 (33.1) | 0.17 | 0.72 | 0.46–1.11 |
| | Co-amox cases (n=164) | 42 (25.8) | 82 (50.3) | 40 (23.9) | 0.66 | 1.1 | 0.7–1.7 |
| | All DILI cases (n=319) | 78 (24.5) | 154 (48.3) | 87 (27.2) | 0.79 | 0.95 | 0.67–1.34 |
| | Controls (n=331) | 85 (25.7) | 159 (48) | 87 (26.3) | | | |
| GPX1 (rs1050450) | Fluclox cases (n=154) | 9 (5.8) | 63 (40.9) | 82 (53.3) | 1.0 | 1.0 | 0.7–1.5 |
| | Fluclox cases positive for rs2395029 (B*57:01) (n=129) | 8 (6.2) | 48 (37.2) | 73 (56.6) | 0.60 | 0.89 | 0.6–1.3 |
| | Co-amox cases (n=163) | 9 (5.5) | 73 (45.5) | 81 (49) | 0.44 | 1.17 | 0.8 – 1.7 |
| | All DILI cases (n=326) | 19 (5.8) | 140 (42.9) | 167 (51.2) | 0.59 | 1.1 | 0.8 – 1.5 |
| | Controls (n=334) | 18 (5.4) | 137 (41) | 179 (53.6) | | | |

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2 tailed test was used to calculate significance.

**Table 4.5: Combined genotypes of SOD2 (rs4880) and GPX1 (rs1050450) in both DILI cohorts versus community controls.**

| | positive for rs4880 and rs1050450 | positive for one of the SNPs | Cases negative for both SNPs | P-value | OR (95% CI) |
|---|---|---|---|---|---|
| Flucloxacillin cases (n=154) | 47 (30.5) | 83 (53.9) | 24 (15.6) | 0.21 | 0.76<br>0.50 – 1.15 |
| Cholestatic and mixed flucloxacillin cases (n=140) | 47 (33.6) | 74 (52.8) | 19 (13.6) | 0.59 | 0.87<br>0.57 – 1.33 |
| Co-amoxiclav cases (n=162) | 57 (35.2) | 90 (55.5) | 15 (9.3) | 0.76 | 0.94<br>0.63 – 1.40 |
| Cholestatic and mixed co-amoxiclav cases (n=93) | 32 (34.4) | 54 (58.1) | 7 (7.5) | 0.71 | 0.91<br>0.56 – 1.47 |
| All DILI cases (n=316) | 104 (32.9) | 173 (54.8) | 39 (12.3) | 0.35 | 0.85<br>0.61 – 1.18 |
| Community controls (n=300) | 110 (36.7) | 145 (48.3) | 45 (15) | | |

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2 tailed test was used to calculate significance.

### *4.3.6 Genotyping results of GSTM1 and GSTT1*

Association of gene deletions related to GSTM1 and GSTT1 with hepatic injury induced by flucloxacillin, co-amoxiclav or both was investigated by genotyping with a PCR assay (Figure 4.3). Generally, the frequency of null genotypes of one or both tested genes did not show significant differences in DILI cases compared with community controls (Table 4.6). No altered risk for DILI was seen in flucloxacillin cases with single (deletion of either GSTM1 or GSTT1) or double gene mutations (deletion of both genes) (OR=1.1, 95% CI=0.7–1.6; P=0.84 for GSTM1, OR=0.85, 95% CI=0.5–1.4; P=0.53 for GSTT1 and OR=0.82, 95%=0.4–1.5; P=0.55 for double null genotypes) and similar results were obtained in cases positive for rs2395029 (a tag SNP for B*57:01) (OR=1.0, 95% CI=0.7–1.5; P=1.0 for GSTM1, OR=0.96, 95% CI=0.6–1.6; P=0.9 for GSTT1 and OR=0.86, 95%=0.5–1.6; P=0.75 for double null genotypes). The effect of GSTT1 on co-amoxiclav DILI development was neutral and no increased risk was seen in cases where both genes are deleted (OR=1.1, 95% CI=0.7–1.7; P=0.81 for GSTT1 and OR=0.89, 95%=0.5–1.6; P=0.77 for double null genotypes) whereas a borderline significant association was noted between GSTM1 genotype for co-amoxiclav cases (OR=0.7, 95% CI=0.5–1.0; P=0.083) with a protective effect for the null genotype. No further associations were seen when DILI cases were combined (n=318) and compared with the community controls (OR=0.86, 95% CI=0.6–1.2; P=0.38 for GSTM1, OR=0.95, 95% CI=0.6–1.4; P=0.84, for GSTT1 and OR=0.83, 95% CI=0.5–1.4; P=0.46 for double null genotypes). Subgroup analysis of the co-amoxiclav cohort has shown negative association with those that show cholestatic or mixed phenotypes.

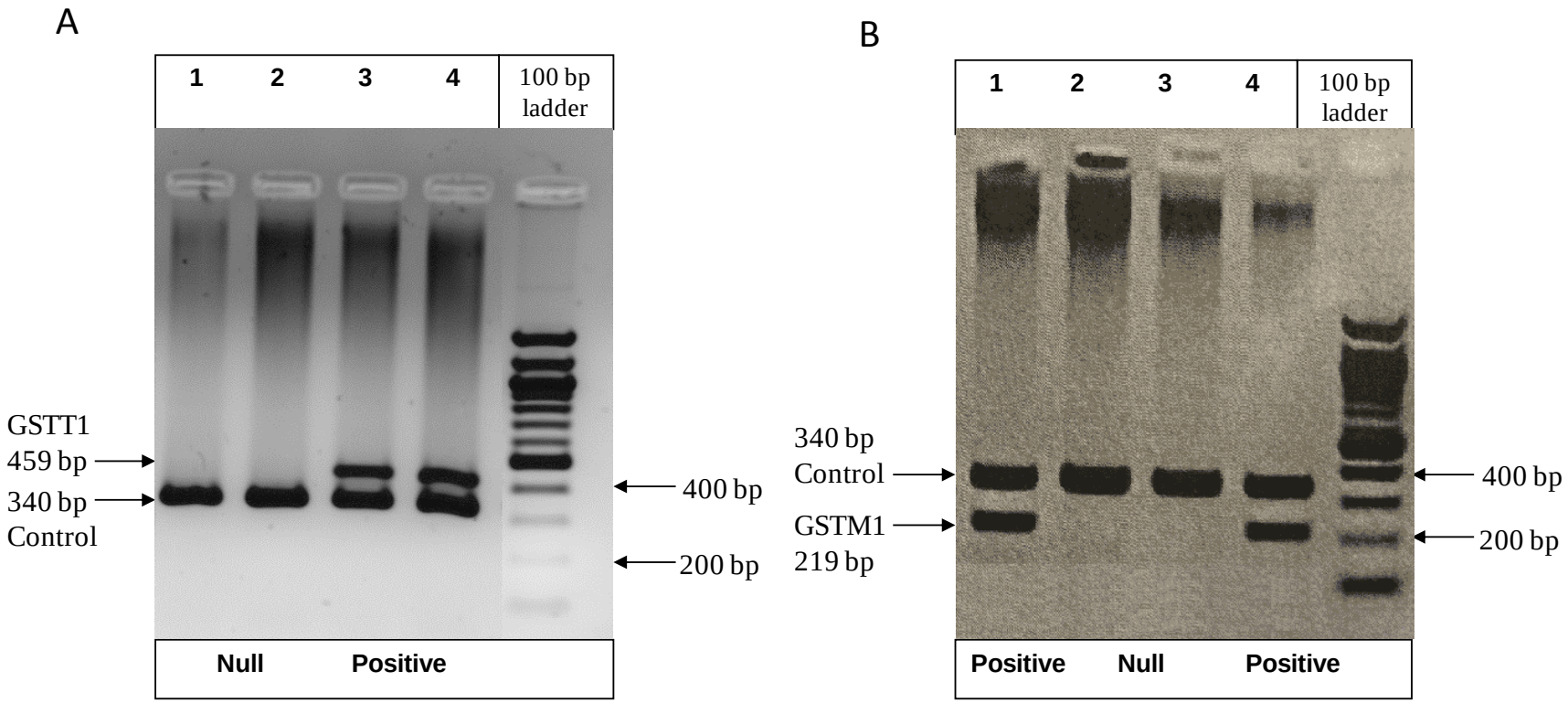


**Figure 4.3**: **2% agarose gel showing GSTM1 and GSTT1 genotypes**. (A) Lanes 1 and 2 are null genotypes of GSTT1, while lanes 3 and 4 are indicating the presence of the gene (positive). (B) Lanes 1 and 4 are showing the positive genotype of GSTM1, whereas lanes 2 and 3 are representing the deleted gene form.

**Table 4.6: GSTM1 and GSTT1 genotype distribution and number of GST positive genotypes among DILI patients and community controls.**

| | *GSTM1* Genotype, | | *GSTT1* Genotype, n (%) | | No. of Positive Genotypes, n (%) | | |
|---|---|---|---|---|---|---|---|
| | **Null** | **Positive** | **Null** | **Positive** | **Two** | **One** | **None** |
| Controls (n=326) | 183 (56.1) | 143 (43.9) | 65 (19.9) | 261 (80.1) | 117 (35.9) | 169 (51.8) | 40 (12.3) |
| Fluclox cases (n=155) | 89 (57.4) | 66 (42.6) | 27 (17.4) | 128 (82.6) | 55 (33.3) | 84 (56.8) | 16 (9.9) |
| OR (95% CI)<br>P value | 1.1 (0.7 – 1.6)<br>0.84 | | 0.85 (0.5 – 1.4)<br>0.53 | | 1.0 (0.7 –1.5)<br>1.0 | 1.1 (0.7-1.6)<br>0.70 | 0.82 (0.4-1.5)<br>0.55 |
| Fluclox cases +ve for rs2395029 (B*57:01) (n=130) | 73 (56.1) | 57 (43.9) | 25 (19.2) | 105 (80.8) | 46 (35.4) | 70 (53.8) | 14 (10.8) |
| OR (95% CI)<br>P value | 1.0 (0.7 – 1.5)<br>1.0 | | 0.96 (0.6 – 1.6)<br>0.9 | | 0.98 (0.6 –1.5)<br>1.0 | 1.1 (0.7-1.6)<br>0.76 | 0.86 (0.5-1.6)<br>0.75 |
| Co-amox cases (n=162) | 77 (47.5) | 85 (52.5) | 34 (21.0) | 128 (79.0) | 68 (42.0) | 76 (46.9) | 18 (11.1) |
| OR (95% CI)<br>P value | 0.7 (0.5 – 1.0)<br>**0.083** | | 1.1 (0.7 – 1.7)<br>0.81 | | 1.3 (0.9 – 1.9)<br>0.23 | 0.8 (0.6–1. 2)<br>0.34 | 0.89 (0.5 –1.6)<br>0.77 |
| Co-amox cases with cholestatic or mixed phenotypes (n=133) | 66 (47.5) | 67 (52.5) | 66 (47.5) | 67 (52.5) | 53 (39.8) | 65 (48.9) | 15 (11.3) |
| OR (95% CI)<br>P value | 0.77 (0.5 – 1.2)<br>0.21 | | 0.77 (0.5 – 1.2)<br>0.21 | | 1.18 (0.8 –1.8)<br>0.46 | 0.89 (0.6 – 1.3)<br>0.61 | 0.91 (0.5 – 1.7)<br>0.87 |
| All DILI Cases (n=318) | 167 (47) | 151 (53) | 61 (20.7) | 257 (79.3) | 124 (43.3) | 161 (46.3) | 33 (10.4) |
| OR (95% CI)<br>P value | 0.86 (0.6 – 1.2)<br>0.38 | | 0.95 (0.6 – 1.4)<br>0.84 | | 1.1 (0.8–1.6)<br>0.46 | 0.95 (0.7–1. 3)<br>0.81 | 0.83 (0.5 –1.4)<br>0.46 |

## 4.4 Discussion

Based on the assumption that some genetic factors may play a general role for DILI susceptibility rather than being drug specific (Daly and Day, 2009), a number of potential candidate genes with known associations to DILI development were selected to study in this project according to their involvement either in drug transport, hepatic physiology or protection against oxidative stress.

A polymorphism in UGT1A1 gene which leads to a reduction of enzyme activity and considered the main cause of elevated serum bilirubin in Gilbert's syndrome (Ferraris et al., 2006) was investigated. The TA repeat variation of the promoter region polymorphism described by UGT1A1*28 did not show any evidence of association with DILI due to either co-amoxiclav or flucloxacillin. Different methodologies were used to analyze the obtained results including combining both DILI cohorts and examining various subgroups but these approaches failed to detect a significant association for UGT1A1 gene with the hepatic injury related to the examined antibiotics in our study. These findings suggest a lack of important influence of UGT1A1 on DILI development. The previous study on lapatinib DILI found that UGT1A1 genotype was a risk factor for DILI in patients who were negative for the HLA class II risk alleles for this form of DILI (Spraggs et al., 2012). This was partly due to patients with Gilbert's syndrome showing higher plasma bilirubin levels which made it more likely that even with a relatively small elevation of liver enzymes such as ALT they might be classed as suffering DILI. The inconsistency between our findings and lapatinib study results in term of association between UGT1A1 and DILI may refer to the fact that the majority of HLA-negative cases involved in Spraggs et al. (2012) study were mostly homozygous mutant for UGT1A1 (*28/*28) whereas a lower frequency of variant homozygosity was seen in our cases. This may indicate that the reported association by Spraggs et al. (2012) with lapatinib DILI is related more to the carriage of homozygous mutation of UGT1A1 rather that HLA negativity.

The SLCO1B1 rs4149056 SNP (SLCO1B1*15) was not a significant DILI risk in relation to either flucloxacillin or co-amoxiclav and subgroup analyses also failed to show any significant associations. However, in view of the apparently more important role for SLCO1B3 compared with SLCO1B1 in the transport of penicillins

and related compounds (Yamaguchi et al., 2011) genotyping to include SNPs in this second transporter gene would be worthwhile in the future. SLCO1B1 and 1B3 are both located on chromosome 12p12 and there is linkage disequilibrium between polymorphisms in both genes. In particular, a recent report has shown that the Val174Ala SNP genotypes are in linkage disequilibrium with several SNPs in SLCO1B3 (Nies et al., 2013). Some of these linked SNPs in SLCO1B3 are nonsynonymous and associated with impaired transport of the immunosuppressant tacrolimus (Boivin et al., 2013). The lack of effect we observed for SLCO1B1*15 would tend to suggest that SLCO1B3 is unlikely to be very important in penicillin-related DILI but it would still be appropriate to study this gene directly.

Also, our findings indicated that the association between the downstream polymorphism rs2858996 in HFE gene and elevation of liver enzymes (transaminases) observed in patients exposed to pazopanib (Xu et al., 2011) cannot be generalized to DILI induced by co-amoxiclav or flucloxacillin. The biological basis for a role for HFE in DILI seems relatively weak though iron overload does cause oxidative stress. The fact that this study did not find a significant association with C282Y may indicate that the observed association by Xu et al. (2011) does not relate directly to HFE. Importantly, HFE is situated in the MHC region of chromosome 6 and the possibility that the observed association with pazopanib DILI is due to an HLA association cannot be excluded. Though Xu and colleagues indicate that they could find no evidence for an actual HLA association in their study, they also point out that they cannot rule out an association with a rare HLA allele since their study was an exploratory candidate gene analysis not a GWAS and detailed HLA typing was not performed. If the findings for pazopanib are due to a novel HLA association with a rare allele, the failure to see any association with rs2858996 for DILI due to flucloxacillin and co-amoxiclav is not surprising.

Despite the available evidence of a possible contribution of the oxidative stress genes particularly SOD2 and GPX1 in DILI susceptibility (Huang et al., 2007; Lucena et al., 2010), our genotyping experiments which involve these genes failed to show similar evidences in our DILI cohorts which were larger than those used in the earlier studies. The DILI association reported by Huang et al. (2007) was limited to anti TB drugs in Taiwanese population. It is important to realize that significant ethnic variability in genotypes of SOD2 between Taiwanese and European controls used

was noted (Lucena et al., 2010). Such difference may possibly affect the overall analysis of gene-disease association among populations of different ancestries. The positive findings of Lucena et al. (2010), on the other hand, were related to DILI due to multiple drugs but did involve 37 co-amoxiclav cases. These cases which were the largest DILI group among Lucena samples did not show significant SOD2 genotype distribution differences in cases with cholestatic/mixed phenotypes (25 in total for co-amoxiclav) compared with the drug-matched control group. The most significant findings reported by Lucena et al. (2010) relate to combined genotypes for SOD2/GPX1 and cholestatic/mixed DILI. The current study has larger numbers of cholestatic/mixed DILI cases due to two different drugs but was unable to confirm any of the findings from the earlier study. One important difference is that Lucena et al. (2010) suggest that the association they observe is more important for drugs that cause toxicity by targeting mitochondria. It is unlikely this is an important target in DILI for either flucloxacillin or co-amoxiclav. However, the fact that Urban et al. (2012) failed to find any evidence that SOD2 and GPX1 were important in a larger number (n=783) of cholestatic/mixed DILI due to a wide range of drugs also should be considered. It remains possible that the Lucena et al. (2010) findings represent an artefact due to small numbers of cases especially since to see any significance it was necessary for them to do subgroup analysis.

Our investigation of the possible role of null genotypes of the glutathione S-transferases GSTM1 and GSTT1 on DILI development due to co-amoxiclav or flucloxacillin showed lack of contribution to drug hepatotoxicity in both tested groups. The frequency of combined, single or no GSTM1/T1 deletions did not show significant differences between cases and control group. GSTM1 null genotype, but not GSTT1, was previously reported as a risk factor for developing DILI in a number of Asian studies involving Indian, Taiwanese and Chinese population (see Section 1.6.2). One Asian study on the now withdrawn drug troglitazone did report that a combined null genotype for GSTM1 and T1 was a risk factor for serious DILI (Watanabe et al., 2003). In one Spanish study, those with a deletion of GSTM1 showed a lower risk of drug hepatotoxicity (Lucena et al., 2008) whereas the GSTT1 deletion (not the GSTM1) was more common in anti TB DILI cases of another Spanish cohort than in matched controls (Leiro et al., 2008). Lucena et al. (2008) found that, in agreement with the Watanabe et al. (2003) findings, carriage of double

mutation (GSTM1-/GSTT1-) increased the risk for DILI development due to use of multiple drugs by 2.7 fold whereas a weaker association was seen in co-amoxiclav cases only. The inconsistency noticed between our results and the findings seen in the previous studies may refer to the fact that most of those studies used multiple drug DILI cases rather than stratifying the cases according to the possible causative drugs. Secondly, the majority of positive associations reported between GST genes and DILI were more specific to anti TB and NSAIDs. Thirdly, the studies conducted mainly involved Asian populations where GST genotypes frequency differs significantly to European populations (Leiro et al., 2008). The increased risk of developing hepatic injury shown in co-amoxiclav cases only with combined gene deletions emphasized in Lucena study (2008) may possibly detected by chance as a result of the limited number of cases used (n=32). This conclusion is supported by our findings which involved much larger numbers of co-amoxiclav cases (n=162) and showed non-significant associations with null genotypes of both GST genes and hence contradicts Lucena's suggestion that GST enzymes play a general role in DILI susceptibility. The large study by Urban et al. (2012) which includes cases of DILI due to a large number of different drugs failed to detect any signals involving GST genes, either when GWAS was performed or a smaller number of metabolism-related genes including the GSTs were studied.

In conclusion, although the candidate genes studied in this chapter are biologically plausible causes of DILI, show high expression in the liver and previous associations with liver toxicity, none of them were found to be an important risk factor for DILI in patients treated by co-amoxiclav or flucloxacillin.

# Chapter 5. Replication of Reported Associations from Previous Genome-Wide Association Studies on Flucloxacillin and Co-amoxiclav-Induced Liver Injury

## 5.1 Introduction

For the development of strategies for optimizing drug treatment, a GWAS approach has been increasingly used by pharmacogenetic researchers to identify predisposing genetic factors associated with particular drug toxicities and to detect susceptibility markers related to therapeutic failure in certain patients versus others who achieved satisfactory drug response. Up to the present, the majority of published studies have been concerned with efficacy of treatment with fewer GWAS examining factors concerned with adverse drug reaction development (Daly, 2010). Examples of GWAS concerned with drug dosing and efficacy include those performed in relation to dose requirement for warfarin and its analogue acenocoumarol. These studies confirmed earlier candidate gene studies and showed significant associations for VKORC1, CYP2C9 and CYP4F2 (Cooper et al., 2008; Takeuchi et al., 2009). Acenocoumarol dosing was also found affected by a CYP2C18 polymorphism in addition to the factors reported for warfarin (Teichert et al., 2009). Other GWAS findings showed significant associations between response to interferon-α, a hepatitis C treatment, with IL28B genotype (Ge et al., 2009) and clopidogrel response with CYP2C19 (Shuldiner et al., 2009). Similarly, genome-wide significant associations were obtained for serious drug induced toxicities (e.g. flucloxacillin with HLA-B*57:01 (Daly et al., 2009) and both methotrexate and simvastatin with SLCO1B1 gene (Link et al., 2008; Trevino et al., 2009)). Though strong associations with particular HLA alleles have been detected by GWAS for both flucloxacillin and co-amoxiclav-induced liver injury, additional non-HLA SNPs show associations that are close to genome-wide significance. Since using HLA genotype alone to predict susceptibility to these reactions shows relatively low predictive value, it is likely that additional genetic factors contribute to susceptibility though the observed effect sizes may be lower than those for the HLA risk factors. This chapter will further investigate the role of non-HLA markers detected as risk factors for drug-induced hepatotoxicity at levels close to genome-wide significance using the data from the previously published GWAS (Daly et al. (2009) and Lucena et al. (2011)). Additional cases of DILI relating to both these drugs are now available and will be used to further investigate these markers.

### *5.1.1 Overview of genes to be studied*

#### *5.1.1.1 ST6GAL1*

A SNP in ST6GAL1 was previously shown to be a genome-wide significant genetic risk factor for DILI due to flucloxacillin in patients positive for B*57:01 only (Daly et al., 2009). ST6GAL1 (ST6 beta-galactosamide alpha-2,6-sialyltransferase 1) is a member of the sialyltransferase enzyme family. These enzymes are located in the Golgi apparatus of the cell. Sialyltransferases add sialic acid to the terminal portions of the sialylated glycolipids (gangliosides) or to the N- or O-linked sugar chains of glycoproteins (Harduin-Lepers et al., 2001; Hennet et al., 1998; Takashima, 2008). The modified molecules are then either exported to the plasma membrane or secreted (Dall'Olio, 2000). ST6GAL1 adds sialic acid with an alpha-2,6 linkage to galactose or N-acetylgalactosamine acid units whereas different sialyltransferases produce an alpha-2,8 linkage as in the formation of proteins with polysialic acid residues.

Typical proteins that are modified by ST6GAL1 in this way include both the B cell receptor and the TNF receptor 1 or "death" receptor which binds TNF and participates in apoptosis. Sialylation has a role in cell-cell interactions. Siglecs (Sialic acid-binding immunoglobulin-type lectins) are cell surface proteins that bind sialic acid bound to other proteins. Each Siglec contains an N-terminal V-type immunoglobulin domain which acts as the binding receptor for sialic acid. These proteins are classified as "I-type lectins" because the carbohydrate binding domain is an immunoglobulin fold. All Siglecs are extended from the cell surface by C2-type Ig domains which have no carbohydrate binding activity and individual Siglecs differ in the number of domains. In the protein encoded by ST6GAL1, a linkage is formed between the 2 position of the 6 membered ring of sialic acid (which has 9 carbons in total) and the 6 position of the galactose ring. Other members of the sialyltransferase family may generate sialic acid joined to glycoproteins and glycolipids with other linkages involving either sialic acid with an alpha-2,3 linkage to galactose or sialic acid to other sialic members of the immunoglobulin superfamily. Siglecs vary in their binding specificity for sialic acid with some recognising sialic acid bound through the 2 to 6 linkage produced by ST6GAL1 but others can specifically bind the alpha-2,3 and alpah-2,8 linkages described above.

Mice lacking ST6Gal1 show impaired development of the thymus and granulocytes together with defective B cell maturation and antibody production (Hennet et al., 1998; Marino et al., 2008; Nasirikenari et al., 2006). Macrophages from transgenic mice overexpressing ST6Gal1 are protected from TNF-alpha-induced apoptosis (Liu et al., 2011).

High levels of ST6GAL1 mRNA are seen mainly in haematopoietic cells, in B-lymphocytes and in lactating mammary glands with the highest levels expressed in liver where the concentration of alpha-2,6 enzyme exceeds alpha-2,3 by 10 fold , whereas it could be expressed minimally in brain and heart (Paulson et al., 1989). This may explain a finding of increased hepatic expression of ST6GAL1 during acute inflammation (Hennet et al., 1998; Kaplan et al., 1983), though enhanced expression was also found to be correlated with human cancer progression (e.g. colon adenocarcinoma) (Hedlund et al., 2008; Shaikh et al., 2008). More recently, proteins modified by ST6GAL1 have demonstrated the ability to bind to the protein CD33 expressed on mature cells of the innate immune system (e.g. neutrophils, mast cells). CD33 has a recognized role in the regulation of cellular proliferation and differentiation (Crocker et al., 2007). Modification of multiple cell-surface differentiation antigens, HB-6, CDw75, and CD76, required for lymphocyte function, was found to be performed by ST6GAL1 (Bast et al., 1992). Also, recent published studies have confirmed the role of ST6GAL1 enzyme in sialylation of sugars linked to Asn 297 on the Fc region of immunoglobulin G (IgG) (Figure 5.1), which is a key step for IgG in mediating its anti-inflammatory effect (Jones et al., 2012) (Lauc et al., 2013).


**Figure 5.1: Linkage of the sialylated sugar (O-glycan) to the immunoglobulin G (IgG) at Asn 297 (Wang, 2005).**

Three different transcripts, which share the same coding region but different five prime untranslated region (5'UTR) sequences, are expressed from the ST6GAL1 gene (Wang et al., 1993; Xu et al., 2003). These transcripts are generated from 3 alternative promoters and, hence, mutations at these areas can have implications for disease development (Eric, 2008 ) (Figure 5.2). Promoter 1 (P1), a sequence upstream of exon 1 and lacking exons Y, Z and X, is responsible for the transcription of hepatic form (H form). The H form can be expressed in many tissues but most commonly in liver (Aas-Eng et al., 1995). The second isoform (X form), which is transcribed from promoter 2 (P2), contains exon X and has been isolated specifically from B cells (Lo and Lau, 1996; Lo and Lau, 1999) while the YZ form, which is transcribed from promoter 3 (P3), contains Y and Z exons and appears to be expressed in cell lines derived from various tissues like human placenta, breast cancer , bronchial or kidney tissues (Eric, 2008 ).

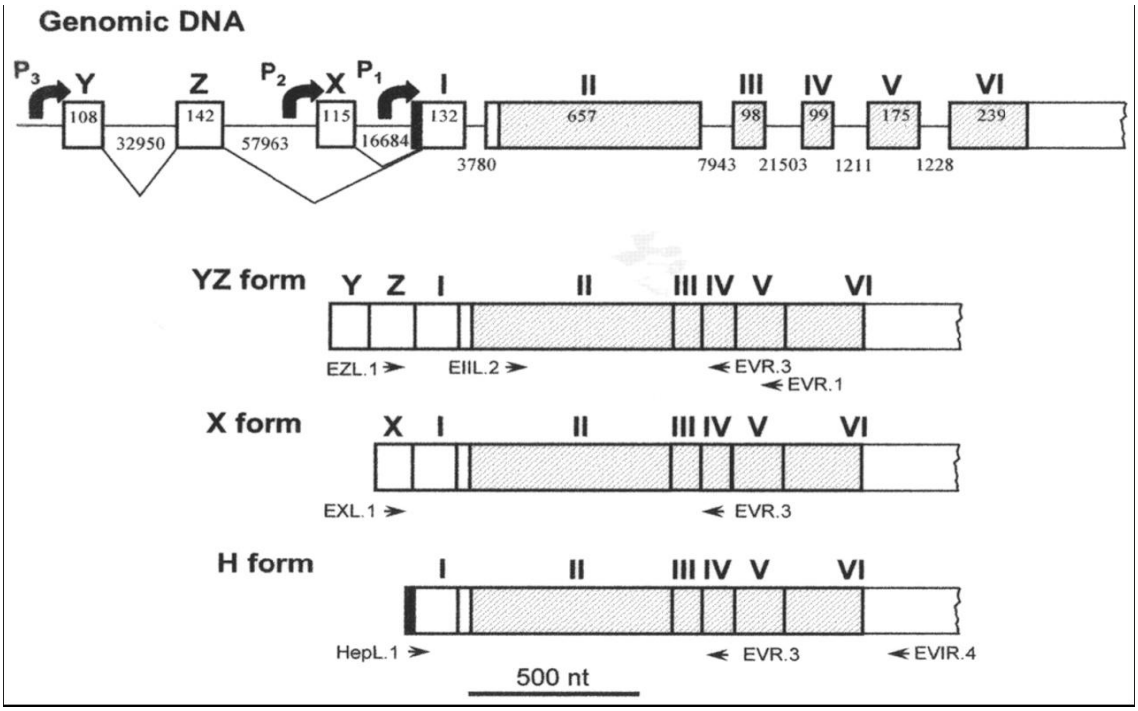


**Figure 5.2: Location of the ST6GAL1 promoters and their mRNA transcripts (Dall'Olio *et al.*, 2004).** The three different promoters are designated in P1, P2 and P3. Shaded area represents the coding exon. Three different isoforms are named as YZ, X and H respectively.

In the GWAS on flucloxacillin-induced liver injury (Daly et al., 2009), a SNP upstream of ST6GAL1 (rs10937275) also showed genome-wide significance (OR 4.1; P =1.4 x $10^{-8}$) but in HLA-B*57:01 carrier cases only. More recently, a GWAS conducted on 5,561 South Asian type 2 diabetic (T2D) patients in comparison to 14,458 healthy controls suggested another SNP in ST6GAL1 (rs16861329) as a susceptibility marker for diabetes (OR=1.12; P=2.3 x $10^{-5}$). This result was reproduced on larger numbers of cases (n=13,170) and controls (n=25,398) who were from London but with Asian ancestry and in Pakistani and Singaporeans (OR=1.07; P=1.6 x $10^{-4}$) but no effect was seen in individuals of European ethnicity (P=0.62) (Kooner et al., 2011). In Chinese population with T2D, rs16861329 was also found to be significantly associated with higher levels of triglycerides (median=1.05 mmol/l (0.8-1.34)) in cases than in controls (median=0.91 mmol/l (0.73-1.23)) (P=0.008) (Lu et al., 2012). Additionally, positive cases for rs16861329 have shown lower high-density lipoprotein (HDL) (median=1.17 mmol/l (1.02-1.33) than in the CC carriers (median=1.21 mmol/l (1.05-1.41) (P=0.034). In this project, we decided to replicate the GWAS findings related to ST6GAL1 (rs10937275) and to further investigate other SNPs located in the promoter regions of ST6GAL1based on the following criteria: (i) SNP is located within 1600 bp upstream of transcription start sites of P1, P2 or P3, (ii) non-synonymous, (iii) the variant is common among European population (MAF>0.05) or (iv) located within a splice site.

#### *5.1.1.2 PTPN22*

In a recent study on DILI due to co-amoxiclav, a SNP in PTPN22 was reported to be a significant risk factor for development of this condition (Lucena et al., 2011). The gene tyrosine phosphatase nonreceptor type 22 (PTPN22), located on chromosome 1 encodes the enzyme tyrosine phosphatase 22 which is expressed primarily in lymphoid tissues and known as lymphoid-specific phosphatase (LYP). It is an intracellular regulatory enzyme, consisting of 807-amino acid residues and negatively affects T-cell-receptor (TCR) signalling and T-cell development. The PTPN22 molecule is characterized by its four proline-rich sequence motifs (P1-P4) located in the last 200 amino acid residues where the Src homology 3 (SH3) domain of the first motif (P1) tends to bind the Csk tyrosine kinase. Csk exerts an inhibitory

effect on TCR signalling through phosphorylation of the negative regulatory tyrosine in the C-terminus (Figure 5.3) (Gjorloff-Wingren et al., 1999).

rs2476601 is a nonsynonymous SNP (C1858T giving rise to R620W) located in exon 14 of PTPN22 which has been reported to be associated with a range of autoimmune diseases e.g. rheumatoid arthritis (RA) (Begovich et al., 2004), Graves' disease (Smyth et al., 2004), type 1 diabetes mellitus (T1D) (Bottini et al., 2004) (Smyth et al., 2004), systemic lupus erythematosus (SLE) (Orru et al., 2009), juvenile idiopathic arthritis (JIA) (Viken et al., 2005), autoimmune thyroid disease (Hinks et al., 2005), generalized vitiligo (Canton et al., 2005) and other autoimmune diseases (Bottini et al., 2006). At least one additional variant (rs1217411) in the PTPN22 gene region may also affect RA susceptibility (Carlton et al., 2005). The R620W amino acid substitution associated with rs2476601 occurs in the first of the four SH3 (proline-rich) domain binding sites in PTPN22 which disrupts binding to Csk (Lee et al., 2005). The GWAS study conducted on 201 cases of DILI due to co-amoxiclav collected from the UK, Spain and in the US (Lucena et al., 2011) versus 466 community controls genotyped for rs2476601 did not show genome-wide significance but when a substudy involving SNPs previously shown to contribute to autoimmune disease was performed, this SNP showed an association with co-amoxiclav hepatotoxicity which was significant after correction for multiple testing (OR=2.1; $P=1.3 \times 10^{-4}$).

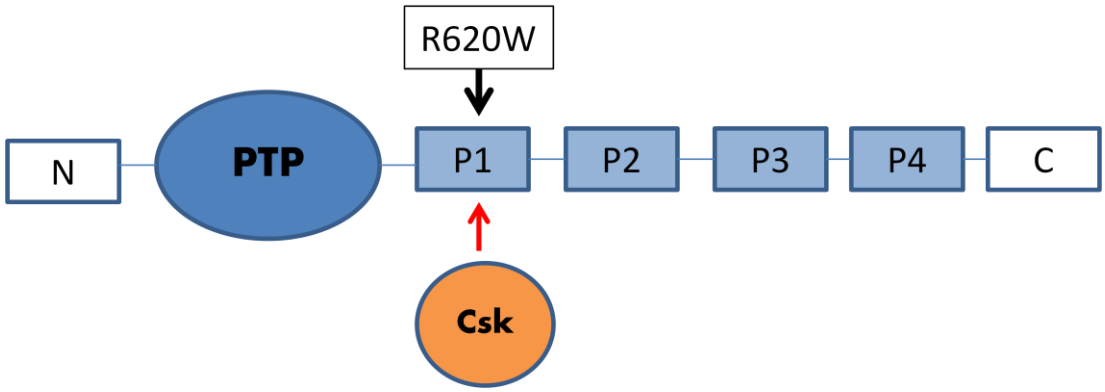


**Figure 5.3**: **Schematic representation of PTPN22 structure showing the 4 proline-rich domains.** Mutation in P1 domain interrupts binding with Csk tyrosine kinase which counteracts its inhibitory effect on T-cell receptor signalling.

The frequency of rs2476601 ranges from 2-6% in southern European (e.g. in Italy and Spain) to 13-15% in individuals from northern European countries (e.g. in the UK and Finland) (Bottini et al., 2006) while it is absent in Asian (e.g. Japanese, Chinese and Koreans) (Zheng and She, 2005) and African populations (e.g. Nigerians) (Lins et al., 2010). Instead, Kawasaki et al. (2006) has detected another variant located in the PTPN22 promoter region (C-1123G, rs2488457) that showed significant association with acute-onset type of insulin dependent diabetes in Japanese and Korean patients (OR=1.41, 95% CI=1.09–1.82; P=0.0105). This result was further confirmed in a Caucasian populations (n=472) in which stronger association observed toward the promoter SNP C-1123G than the association seen with the more common SNP C1858T (P=0.019 versus P=0.046 respectively), though both SNPs were in partial linkage disequilibrium ($r^2$= 0.57). Previous studies demonstrated a stronger association between C1858T polymorphism and T1D in 689 non-Hispanic white North American individuals (Bottini et al., 2004) and in a larger group of UK population (n=8210, the U.K. Genetic Resource Investigating Diabetes (GRID) study) (Smyth et al., 2004) (OR=1.83; P=0.0005 and OR=1.78; P=6.02 x $10^{-27}$, respectively). The C1858T variant was also investigated in several other autoimmune diseases including primary sclerosing cholangitis (Viken et al., 2005), celiac disease (Rueda et al., 2005), psoriasis (Huffmeier et al., 2006), psoriatic arthritis (Hinks et al., 2005), irritable bowel disorder (IBD) (Prescott et al., 2005) and systemic sclerosis but no associations were detected (Wipff et al., 2006).

#### *5.1.1.3 Additional SNPs from co-amoxiclav GWAS study showing borderline significance*

As summarised in Table 5.1, a number of SNPs outside the MHC region of chromosome 6 gave relatively low p values in the co-amoxiclav DILI GWAS described by Lucena et al., (2011). As there was insufficient funding available to genotype all new available co-amoxiclav cases samples for these SNPs, it was decided to select a small subset based on the following criteria: (i) SNP is located within a gene (ii) the p value for the previous UK cases was <0.001 and in Spanish <0.05, (iii) more than one SNP in the region showed this level of significance. Four SNPs in the KCNJ1 gene fulfilled these criteria and therefore the gene was chosen for further study.

KCNJ1 (potassium inwardly-rectifying channel, subfamily J, member 1) is located on chromosome 11 and encodes the renal outer medullary potassium channel (ROMK). The main function of this protein is to regulate cellular potassium homeostasis (Yano et al., 1994).

Tobin et al. (2008) found that 5 SNPs located in KCNJ1 showed significant associations with mean 24-hour systolic or diastolic blood pressure. This study also reported an association between several SNPs in KCNJ1 and left ventricular mass as assessed by measuring voltage signal on electrocardiograms (ECG). The effect was in the same direction as seen with BP and the strongest association noted was with rs675759. Brochard et al. (2009) have also detected ten rare mutations in KCNJ1 gene associated with antenatal Bartter syndrome type 2 (hyperprostaglandin E syndrome), which is characterized by salt wasting, hypokalemic alkalosis, hypercalciuria, and low blood pressure. The relevance of KCNJ1 to a disease affecting the liver is slightly unclear but in view of the relatively strong signal seen in the GWAS, it was decided to perform genotyping for the SNP showing the lowest p value.

**Table 5.1: Top markers detected from GWAS after removal of chromosome 6 SNPs (Lucena et al., 2011).**

| | | | UK (fisher, 66 cases, 291 controls) | | | Spain (Fisher, 53 cases, 167 controls) | | |
|---|---|---|---|---|---|---|---|---|
| Gene | SNP | Chr | P | OR | MAF | P | OR | MAF |
| AKAP6 | rs17522991 | 14 | $2.3x10^{-6}$ | 2.47 | 0.266 | 0.2478 | 1.32 | 0.350 |
| | rs17523067 | 14 | $2.3x10^{-6}$ | 2.47 | 0.266 | 0.2478 | 1.32 | 0.350 |
| CACNA1E | rs4282766 | 1 | 0.0063 | 3.12 | 0.028 | 0.0269 | 2.45 | 0.054 |
| CADM2 | rs7619493 | 3 | 0.0069 | 1.87 | 0.151 | 0.1847 | 1.47 | 0.159 |
| | rs9816329 | 3 | 0.0072 | 1.85 | 0.153 | 0.3292 | 1.33 | 0.189 |
| | rs9310001 | 3 | 0.0103 | 1.82 | 0.155 | 0.4069 | 1.28 | 0.195 |
| DOK6 | rs11662320 | 18 | 0.0176 | 0.59 | 0.294 | 0.0053 | 0.45 | 0.296 |
| | rs17081109 | 18 | 0.0327 | 0.58 | 0.230 | 0.0010 | 0.32 | 0.225 |
| | rs12969411 | 18 | 0.0330 | 0.59 | 0.229 | 0.0010 | 0.32 | 0.225 |
| DPP9 | rs2109069 | 19 | 0.0025 | 1.78 | 0.311 | 0.0003 | 2.34 | 0.240 |
| FAM107A | rs13088795 | 3 | 0.0012 | 2.21 | 0.115 | 0.0963 | 1.70 | 0.114 |
| FREM1 | rs12236053 | 9 | 0.0044 | 0.36 | 0.136 | 0.0578 | 0.40 | 0.111 |
| GADD45G | rs16905942 | 9 | $3.6x10^{-5}$ | 3.02 | 0.081 | 0.0723 | 1.966 | 0.072 |
| | rs2890109 | 9 | 0.0002 | 2.91 | 0.065 | 0.0023 | 4.181 | 0.027 |
| | rs2890110 | 9 | $2.5x10^{-5}$ | 3.00 | 0.084 | 0.0509 | 2.037 | 0.075 |
| | rs620311 | 9 | 0.0027 | 2.27 | 0.089 | 0.0413 | 2.572 | 0.039 |
| INPP4B | rs1497393 | 4 | 0.0005 | 1.92 | 0.330 | 0.0365 | 1.62 | 0.329 |
| | rs7666932 | 4 | 0.0015 | 1.84 | 0.328 | 0.0269 | 1.67 | 0.323 |
| KCNJ1 | rs2855798 | 11 | 0.0006 | 0.39 | 0.213 | 0.0060 | 0.39 | 0.213 |
| | rs2847381 | 11 | 0.0006 | 0.39 | 0.213 | 0.0018 | 0.31 | 0.207 |
| | rs2855790 | 11 | 0.0006 | 0.39 | 0.213 | 0.0012 | 0.31 | 0.210 |
| | rs3016774 | 11 | 0.0008 | 0.39 | 0.212 | 0.0018 | 0.31 | 0.207 |
| KCNJ3 | rs1823003 | 2 | 0.0100 | 0.58 | 0.341 | 0.0123 | 0.50 | 0.305 |
| ODZ2 | rs7715979 | 5 | 0.0052 | 1.99 | 0.122 | 0.0499 | 1.87 | 0.117 |
| PARP4 | rs9511249 | 13 | 0.0048 | 1.73 | 0.318 | 0.1255 | 1.46 | 0.319 |
| | rs1050112 | 13 | 0.0048 | 1.73 | 0.318 | 0.1002 | 1.47 | 0.317 |
| RBPMS | rs6988150 | 8 | 0.0245 | 1.56 | 0.263 | 0.0001 | 2.53 | 0.240 |
| SIPA1L3 | rs8107385 | 19 | 0.0012 | 0.54 | 0.473 | 0.0186 | 0.58 | 0.482 |
| | rs2304132 | 19 | 0.0016 | 0.54 | 0.469 | 0.0186 | 0.58 | 0.482 |
| SLC30A2 | rs3121763 | 1 | 0.0078 | 0.34 | 0.112 | 0.0003 | 0.19 | 0.168 |
| SMOX | rs1741327 | 20 | 0.0003 | 2.03 | 0.273 | 0.0604 | 1.56 | 0.320 |
| | rs1051904 | 20 | 0.0007 | 1.92 | 0.278 | 0.0162 | 1.74 | 0.356 |
| SYNPO2 | rs6828669 | 4 | 0.0101 | 1.63 | 0.349 | 0.0139 | 1.78 | 0.317 |
| TRIM63 | rs7553840 | 1 | 0.0079 | 0.33 | 0.113 | 0.0003 | 0.22 | 0.183 |

## 5.2 Methods

### *5.2.1 Cases and controls*

The cases studied were described in detail in Chapter 3. As controls for the DILI cases, several different population controls groups were used for the analyses described in this chapter. Most of these have been discussed in more detail previously (Section 4.2.1). However, in the case of ST6GAL1, an additional control group consisting of individuals from North-East England who had been prescribed flucloxacillin (n= 62) without developing DILI (see Daly et al., 2009 for more detail) was used.

### *5.2.2 Studies on ST6GAL1 gene expression*

#### *5.2.2.1 Cell culture*

HepG2 cells (hepatocellular carcinoma cell lines), obtained from the European Tissue Collection (Porton Down, UK), were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich) supplemented with 10% fetal calf serum (Gibco-Invitrogen), 100 U/ml penicillin (BioWhittaker), 100 µg/ml streptomycin (BioWhittaker), 200 mM L-Glutamine (BioWhittaker), 0.1 mM none essential amino acid (NEAA; BioWhittaker), and 5 ml gassed 4.4% sodium bicarbonate with 0.4% phenol red. Cells were grown and maintained at an appropriate temperature and gas mixture (37°C, 5% CO2) in a cell incubator. Cells were detached with 0.25% trypsin EDTA (Gibco-Invitrogen) when 70-80% confluent reached. B cell leukemia (Ramos) and breast cancer cell lines (T47D) cell lines were obtained from Ms Fiona Fenwick (Institute of Cellular Medicine, Newcastle University). We did not culture these cells but extracted RNA directly from a thawed frozen stock. mRNA from primary human hepatocytes purchased from Life Techologies, Warrington, UK was prepared and supplied by Dr Ching-Soon Ng.

#### *5.2.2.2 RNA extraction using Trizol*

Trizol reagent (Life Techologies, Warrington, UK) (1 ml) was added to the cultured cells on 6-well plates ($1.5 \times 10^6$ cells per well) after removal of medium and washing briefly with phosphate-buffered solution (PBS, Merck biosciences). The cell lysate in Trizol was removed from the well and left in a sterile screw-cap tube for 5 min at room temperature. Chloroform (0.2 ml) was added and the mixture was shaken

vigorously for 15 – 30 seconds to get cloudy appearance solution which was centrifuged at 12 000 rpm for 15 min at 2 to 8°C. A clear upper (aqueous) layer was separated, carefully removed and transferred to a new tube and mixed with 0.5ml isopropanol. After incubation at room temperature for 10 min, the solution was further centrifuged at 10 000 rpm for 10 minutes. Thereafter, the solution was discarded and the pellet at the bottom of the tube was treated with 1 ml 75% ethanol (diluted with readymade diethylpyrocarbonate (DEPC)-treated water; Fisher Scientific,UK) and vortexed for a few seconds to be mixed before a final centrifugation took a place at 7500 rpm for 5 minutes. Afterwards, the supernatant was carefully removed, and the pellet allowed to dry before 20 µl DEPC water was added to dissolve the RNA pellet.

#### *5.2.2.3 cDNA synthesis (Reverse transcription)*

First strand cDNAs were synthesized by mixing 1 µg of extracted RNA with a mixture containing 1 µl random hexamer, 1 µl of deoxyribonucleotide triphosphate (dNTPs, 10 mM) and 6 µl DEPC water followed by incubation at a temperature of 65°C for 10 min. The RNA sample was then cooled on ice for 2 min and further mixed with another mixture containing 2 µl 10Xbuffer, 0.1 µl RNase inhibitor, 0.25 µl reverse transcriptase enzyme and 7.65 µl DEPC water to be incubated at 37°C for 50 min. Lastly, additional incubation at 70°C for 15 min were performed to inactivate any remaining enzyme before storing the cDNA at -20°C.

#### *5.2.2.4 Expression of ST6GAL1 transcripts*

In order to confirm the previously reported findings that expression of different ST6GAL1 transcripts is cell type specific, primers specific to Exon I, Exon X and Exon Y (Table 5.2), were used to amplify cDNA prepared from mRNA derived from different cell types (breast cancer cell lines, B cell leukemia cell lines, HepG2 cells and primary human hepatocytes). The nucleotide sequence 5′-TTCTTTTCCTTCCACACACAGATG-3′ from Exon II was used as a primer for reverse transcription. This sequence in addition to those used for Exon I, X and Y were designed by Xu et al. (2003).

**Table 5.2: Probes sequences of main exons characterizing ST6GAL1 promoters, product sizes and annealing temperatures.**

| Exon | Probes | PCR size | Annealing temp. |
|---|---|---|---|
| Exon I | 5′-CTTTCTGTCTCTTATTTTTTGCCTTTGCAG-3′ | 271 bp | 54 °C |
| Exon X | 5′-ACAACCAGGGAGGGCGTGGAAGCT-3′ | 377 bp | 57 °C |
| Exon Y | 5′-GCCCGGCGTTAACAAAGGGAGCCG-3′ | 636 bp | 59 °C |

### *5.2.3 TaqMan assay for rs10937275 in ST6GAL1*

A predesigned TaqMan SNP genotyping assay prepared by Applied Biosystems, catalogue number of 4351379 (C_2679367_20), was used to genotype for rs10937275 in ST6GAL1. The fluorescent allelic discrimination analysis was performed using a One-Step Real-Time PCR machine (Applied Biosystems). To replicate GWAS findings, genotypes for 100 newly recruited flucloxacillin DILI cases (see Section 3.2.3) were compared to genotypes for the POPRES control group (n=282), genotyped in the Daly et al. (2009) study, which were matched for ethnicity to the original 51 flucloxacillin DILI cases in that study.

### *5.2.4 Genotyping for ST6GAL1 promoter region SNPs*

To investigate other SNPs located in the ST6GAL1 promoter regions, 36 newly reported flucloxacillin DILI cases ( in addition to the 51 cases used in Daly et al. (2009) study) and 147 control samples (62 drug-exposed and 85 non-exposed) were sent to KBiosciences, Herts for genotyping using a real time PCR assay. This experiment was performed at the early stage of this project, therefore, a limited number of cases were available to study at that time. A 96-well plate were prepared (as described previously in Section 2.5) with 75 µl (50 ng/µl) DNA per well based on manufacturer's recommendations which needed a minimum amount of 5 ng per sample per reaction. Certain samples in cases and in controls failed to be genotyped for particular SNPs.

### *5.2.5 RFLP-PCR Assays for PTPN22 and KCNJ1*

PCR-restriction fragment length polymorphism analysis was used for PTPN22 (rs2476601) and KCNJ1 (rs2855790) genotyping. All assays were developed in our laboratory using unique primers shown below in Table 5.3.

#### *5.2.5.1 PTPN22 (rs2476601) assay*

PCR cycling conditions used for PTPN22 amplification were 35 cycles of 1 min at 94°C denaturation, 1 min at 59 °C annealing and at 72 °C elongation for 1 min, followed by a 7 min extension at 72 ºC resulting in 541 bp product. A 20 µl PCR product was digested overnight at 37 °C using 2 U of Xcm I digestion enzyme. The enzyme was predicted to cut at the (T) allele, the variant type, resulting in fragments

of 296 bp and 245 bp, whilst the common (C) allele is predicted to remain uncut. A 2% agarose gel was used for DNA optimization and analysis. Two cohorts of DILI patients were genotyped to study PTPN22 including an additional 99 co-amoxiclav DILI cases to those described in the Lucena et al. (2011) study and 154 flucloxacillin DILI cases (both the cases in the Daly et al. (2009) study and new cases) and the results were compared to the previously genotyped POPRES controls (Daly et al., 2009).

#### *5.2.5.2 KCNJ1 assay*

In the case of KCNJ1 (rs2855790 C/T), similar PCR cycling conditions of PTPN22 (rs2476601) amplification were used except the annealing temperature which was 54 °C for 1 min and the PCR amplicon size was 290 bp. The KCNJ1 PCR product was digested with BseYI which was predicted to digest the common allele (C), whereas DNA fragments carrying the minor (T) allele remained uncut. When digested homozygous wild-type DNA was separated by electrophoresis on a 10% polyacrylamide gel, two fragments of 141 bp and 149 bp were predicted to be seen.

The role of KCNJ1 gene in DILI susceptibility was investigated through genotyping 73 newly reported co-amoxiclav DILI cases and 75 community controls supplied by Dr Peter Donaldson (Velaga et al., 2004). The data obtained were analysed for its odds ratio (OR) value and Fisher's exact test was used to generate a P value.

**Table 5.3: Primer sequences, product sizes and annealing temperatures.**

| Gene | SNP | Forward and reverse primers | PCR size | Annealing temp. |
|---|---|---|---|---|
| PTPN22 | rs2476601 | 5′-TCAAGTGATCCTCTCACCTC-3′<br>5′-GCAAAAACCTCCTGGGTTTG-3′ | 541 bp | 59 °C |
| KCNJ1 | rs2855790 | 5′-TCACTCACTTAACTGCCACG-3′<br>5′-GAGGTGTTTCTCCTCTTACC-3′ | 290 bp | 54 °C |

## 5.3 Results

### *5.3.1 ST6GAL1 transcript analysis*

The aim of this experiment was to confirm the expression specificity of different ST6GAL1 transcripts in particular cell lines as reported by Lo and Lau (1999) and Xu et al. (2003). As shown in Figure 5.4, these studies confirmed that Exon I sequence, *bottom panel*, is expressed in all cell lines examined while Exon X sequence, *2$^{nd}$ panel,* is identified exclusively in B cell lines. On the other hand, Exon Y which represents transcript 3 (YZ form) of ST6GAL1, *upper panel*, was found expressed in the breast cancer cell lines (T47D). Other types of cell lines (e.g. lung or kidney tissues) normally express the YZ form too but were not examined in this experiment.

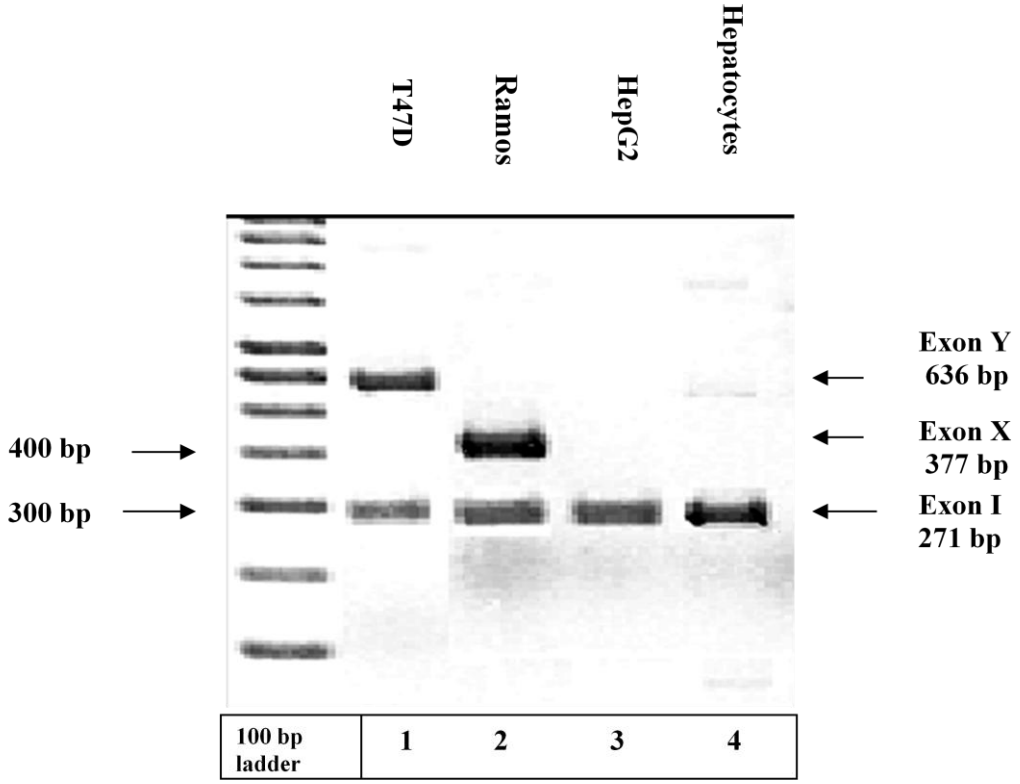


**Figure 5.4**: **PCR expression analysis of ST6GAL1 mRNA derived from multiple tissues**. PCR products were run on 2% agarose gel and stained with ethidium bromide. cDNA from T47D, breast cancer cell lines (lane 1), Ramos (B cells, lane 2), HepG2 (lane 3) and primary human hepatocytes (lane 4) were used as target DNA in the PCR analysis. The PCR product shown represent the following: Upper panel is Exon Y (transcript 3); 2$^{nd}$ panel is Exon X (transcript 2); and the bottom panel is Exon I (transcript 1).

### *5.3.2 Genotyping of SNPs in ST6GAL1*

Several promoter SNPs located on P1, P2 and P3, which are more likely to affect transcriptional activity of ST6GAL1 gene (Dall'Olio *et al.*, 2004), were selected to investigate relevance of ST6GAL1 to flucloxacillin DILI susceptibility. The sites of the selected SNPs are described in **Figure 5.5**. Four SNPs (rs1468906, rs1468905, rs28366038 and rs28366036) were located in P3 (-399, -471, -736 and -753, respectively) upstream of Exon Y, 1 SNP (rs4686837) located in P2 (Intron/Exon X boundary), 3 SNPs (rs73071448, rs6444196 and rs3936289) located in P1 (-149, -648 and -1598, respectively) upstream of Exon I, and the 9$^{th}$ SNP (rs113928218) is located within a splice site at the exon II / intron II boundary (Ensembl genome database). These studies were performed early in the project when only smaller numbers of flucloxacillin DILI cases (n= 51 from Daly et al. (2009) plus 36 newer cases) were available for study.

Genotyping frequency results for cases and controls are described in Table 5.4. Comparison of studied subjects, shown in Table 5.5, failed to detect any association significances and the frequency of variant allele of the two SNPs rs73071448 and rs113928218 found to be nil in the UK population. A slight increase in frequency for the variant alleles of the SNPs rs1468906, rs28366038, rs28366036 and rs6444196 were detected in the flucloxacillin DILI cases compared with the drug-exposed controls though this was not statistically significant (P= 0.12, 0.46, 0.12, and 0.08, respectively). Borderline significant associations were detected between 3 candidate SNPs (rs1468906, rs28366036 and rs6444196) and flucloxacillin DILI in B*57:01 (rs2395029)-positive cases (n=70) when compared with drug-exposed controls (n=62) (P= 0.05, 0.07, and 0.07, respectively) (Table 5.6). However, these associations were lost when the cases compared to a larger number of control samples (combined exposed and non-exposed controls, n=147) (P=0.31, P=0.16 and P=0.15, respectively).

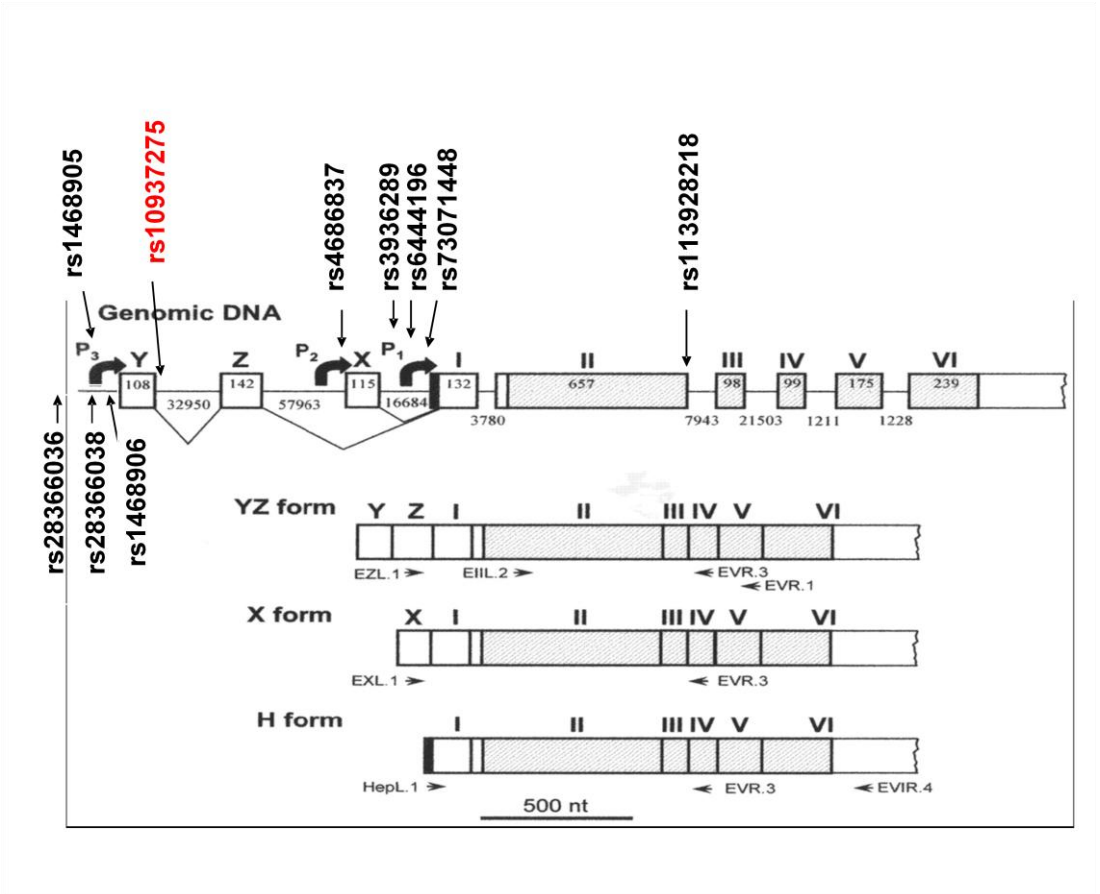


**Figure 5.5: The polymorphisms chosen to study the relevance of ST6GAL1 to flucloxacillin DILI in this project are indicated by arrows.** The SNP rs10937275 indicated by red colour (located at position +2167 bp downstream of exon Y) was reported to be a significant risk factor for the flucloxacillin DILI in a GWAS study (Daly et al., 2009).

**Table 5.4: Genotype distribution (%) of candidate ST6GAL1 SNPs in flucloxacillin DILI case and controls.**

| ST6GAL1(SNP) | Genotypes | All cases (n=87) | rs2395029 (B*57:01) positive cases (n=70) | Drug-exposed controls (n=62) | Non-exposed controls (n=85) |
|---|---|---|---|---|---|
| rs1468906 | A:A | 31 (0.36) | 23 (0.33) | 30 (0.50) | 28 (0.33) |
| | G:A | 38 (0.44) | 31 (0.44) | 24 (0.40) | 38 (0.45) |
| | G:G | 17 (0.20) | 16 (0.23) | 6 (0.10) | 18 (0.21) |
| rs1468905 | A:A | 1 (0.01) | 0 | 3 (0.05) | 3 (0.03) |
| | G:A | 25 (0.30) | 20 (0.30) | 20 (0.33) | 26 (0.31) |
| | G:G | 58 (0.69) | 47 (0.70) | 37 (0.62) | 56 (0.66) |
| rs28366038 | G:G | 73 (0.84) | 57 (0.81) | 51 (0.89) | 73 (0.87) |
| | A:G | 14 (0.16) | 13 (0.19) | 5 (0.09) | 11 (0.13) |
| | A:A | 0 | 0 | 1 (0.02) | 0 |
| rs28366036 | C:C | 17 (0.20) | 15 (0.22) | 5 (0.08) | 17 (0.20) |
| | A:C | 37 (0.45) | 30 (0.45) | 25 (0.42) | 38 (0.45) |
| | A:A | 29 (0.39) | 22 (0.33) | 29 (0.49) | 30 (0.35) |
| rs4686837 | A:A | 10 (0.12) | 9 (0.13) | 5 (0.08) | 9 (0.11) |
| | G:A | 37 (0.43) | 28 (0.40) | 35 (0.56) | 34 (0.40) |
| | G:G | 39 (0.45) | 33 (0.47) | 22 (0.35) | 42 (0.49) |
| rs6444196 | A:A | 14 (0.16) | 13 (0.19) | 2 (0.03) | 16 (0.19) |
| | G:A | 45 (0.52) | 36 (0.51) | 30 (0.50) | 41 (0.49) |
| | G:G | 27 (0.31) | 21 (0.30) | 28 (0.47) | 27 (0.32) |
| rs3936289 | T:T | 46 (0.53) | 35 (0.50) | 35 (0.59) | 45 (0.54) |
| | C:T | 36 (0.41) | 31 (0.44) | 23 (0.39) | 33 (0.39) |
| | C:C | 5 (0.06) | 4 (0.06) | 1 (0.02) | 6 (0.07) |
| rs73071448 | C:C | 87 (1.00) | 70 (1.00) | 62 (1.00) | 85 (1.00) |
| rs113928218 | G:G | 87 (1.00) | 70 (1.00) | 62 (1.00) | 85 (1.00) |

**Table 5.5: Statistical analysis summary of genotyping results for 7 candidate polymorphisms selected to further study the relevance of ST6GAL1 to flucloxacillin DILI.**

| SNP | Genotypes | Cases vs drug-exposed controls | | Cases vs non-exposed controls | |
|---|---|---|---|---|---|
| | | OR (95% CI) | P value | OR (95% CI) | P value |
| ***rs1468906*** | AA vs AG+ GG | 1.77 (0.91-3.47) | 0.12 | 0.89 (0.47-1.67) | 0.75 |
| ***rs1468905*** | GG vs GA + AA | 0.72 (0.36-1.45) | 0.38 | 0.87 (0.45-1.65) | 0.74 |
| ***rs28366038*** | GG vs AG + AA | 1.63 (0.59-4.53) | 0.46 | 1.27 (0.54-2.99) | 0.67 |
| ***rs28366036*** | AA vs AC + CC | 1.80 (0.91-3.56) | 0.12 | 1.02 (0.54-1.91) | 1 |
| ***rs4686837*** | GG vs GA + AA | 0.66 (0.34-1.30) | 0.24 | 1.18 (0.65-2.15) | 0.65 |
| ***rs6444196*** | GG vs GA + AA | 1.91 (0.97-3.78) | 0.08 | 1.04 (0.54-1.97) | 1 |
| ***rs3936289*** | TT vs CT + CC | 1.30 (0.67-2.54) | 0.5 | 1.03 (0.56-1.88) | 1 |

**Table 5.6: Associations between 7 candidate SNPs in ST6GAL1 and flucloxacillin DILI in rs2395029 (B*57:01)-positive cases.**

| SNP | Genotypes | rs2395029 positive cases (n=70) vs drug-exposed controls (n=62) | | rs2395029 positive cases (n=70) vs non-exposed controls (n=85) | |
|---|---|---|---|---|---|
| | | OR | P value | OR | P value |
| ***rs1468906*** | AA vs AG+ GG | 2.04 (1.00-4.16) | **0.05*** | 1.02 (0.52-2.01) | 1.00 |
| ***rs1468905*** | GG vs GA + AA | 0.68 (0.33-1.43) | 0.35 | 0.82 (0.41-1.64) | 0.60 |
| ***rs28366038*** | GG vs AG + AA | 1.94 (0.69-5.48) | 0.22 | 1.51 (0.63-3.63) | 0.38 |
| ***rs28366036*** | AA vs AC + CC | 1.98 (0.96-4.68) | **0.07*** | 1.12 (0.57-2.19) | 0.86 |
| ***rs4686837*** | GG vs GA + AA | 0.62 (0.31-1.24) | 0.22 | 1.095 (0.58-2.06) | 0.87 |
| ***rs6444196*** | GG vs GA + AA | 2.04 (0.99-4.19) | **0.07*** | 1.11 (0.56-2.20) | 0.86 |
| ***rs3936289*** | TT vs CT + CC | 1.46 (0.72-2.94) | 0.38 | 1.15 (0.61-2.18) | 0.75 |

*No significant difference obtained when cases were compared to the combined controls (n=147).

The SNP rs10937275, which is located within the 5' UTR (2167 bp downstream of exon Y, **Figure 5.5**), in ST6GAL1 gene had been previously reported to be a significant risk factor for the flucloxacillin DILI in a GWAS study that involved smaller group of samples (n=51) (Daly et al., 2009). Genotyping of larger numbers of additional flucloxacillin DILI cases was performed using TaqMan SNP genotyping assay. Table 5.7 summarizes the results obtained. Carriage of the mutant allele (A) showed no difference between the newly recruited flucloxacillin DILI cases (n=100) and POPRES controls (OR=1.5, 95% CI=0.9–2.7; P=0.14). Additional analysis on cases positive for HLA class I B*57:01 only (genotyped by Daly et al. (2009) and Chamberlain (2014)) showed that 26.5% were also positive for ST6GAL1 (rs10937275) gene . However, this higher variant distribution frequency in cases compared to controls (18%) did not reach a statistically significant level (OR=1.67, 95% CI=0.94–2.97; P=0.085). This replication genotyping therefore failed to confirm the previously reported genome-wide significance (OR=4.1; $P=1.4x10^{-8}$).

Genotypes in cases with cholestatic and mixed phenotypes only were also compared to POPRES controls but these also showed no significant difference (OR=1.64, 95% CI= 0.94–2.86; P=0.097).

**Table 5.7: ST6GAL1 (rs10937275) genotyping results in flucloxacillin DILI cases compared to POPRES community controls.**

| Genotype | GG (%) | GA (%) | AA (%) | P-value | Odds ratio | 95% CI |
|---|---|---|---|---|---|---|
| Cases (n=100) | 75 (74) | 23 (24.1) | 2 (1.9) | 0.14 | 1.5 | 0.90 – 2.67 |
| Cases positive for rs2395029 (a tag SNP for B*57:01) (n=83) | 61 (73.5) | 20 (24.1) | 2 (2.4) | 0.085 | 1.67 | 0.94 – 2.97 |
| Cases with cholestatic or mixed phenotypes (n=92) | 68 (73.9) | 22 (23.9) | 2 (2.2) | 0.097 | 1.64 | 0.94 – 2.86 |
| POPRES controls (n=282) | 232 (82.2) | 49 (17.4) | 1 (0.4) | | | |

*Cases with mixed phenotypes are those which show both cholestatic and hepatocellular clinical manifestations.

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2 tailed test was used to calculate significance.

### *5.3.3 PTPN22 genotyping results*

Figure 5.6 shows the PCR-RFLP genotyping assay, designed for the SNP rs2476601 in PTPN22. The analysis of PTPN22 genotypes confirmed the significant association with co-amoxiclav DILI ($P=4.1 \times 10^{-4}$) (Table 5.8) and also showed an association for the flucloxacillin DILI group (P=0.02) (Table 5.9). The results showed that 29.3% of the co-amoxiclav DILI cases carried the variant T allele while only 13.2% of the community controls were found to be positive for this allele. Patients who carry the risk allele would be at 2.74 times higher risk compared with normal controls. A lower effect of the PTPN22 SNP on flucloxacillin DILI was detected. rs2476601 positive cases among flucloxacillin DILI cohort were found at 1.9 higher risk to develop hepatotoxicity compared to 282 community controls.

Subgroup analysis was done to assess a possible interaction of PTPN22 with other HLA gene of functional significance for DILI development. Data for tag SNP (rs2395029) genotypes for HLA-B*57:01 alleles previously associated with flucloxacillin hepatotoxicity were available (Chamberlain, 2014; Daly et al., 2009). Combined HLA-B*57:01-PTPN22 genotypes were investigated. Positivity for both PTPN22 and rs2395029 in flucloxacillin DILI cases did not show a signal of synergy between genes. An identical risk to that seen in all flucloxacillin cases was observed when only those positive for rs2395029 were studied (OR=1.9; P=0.02 for both groups).

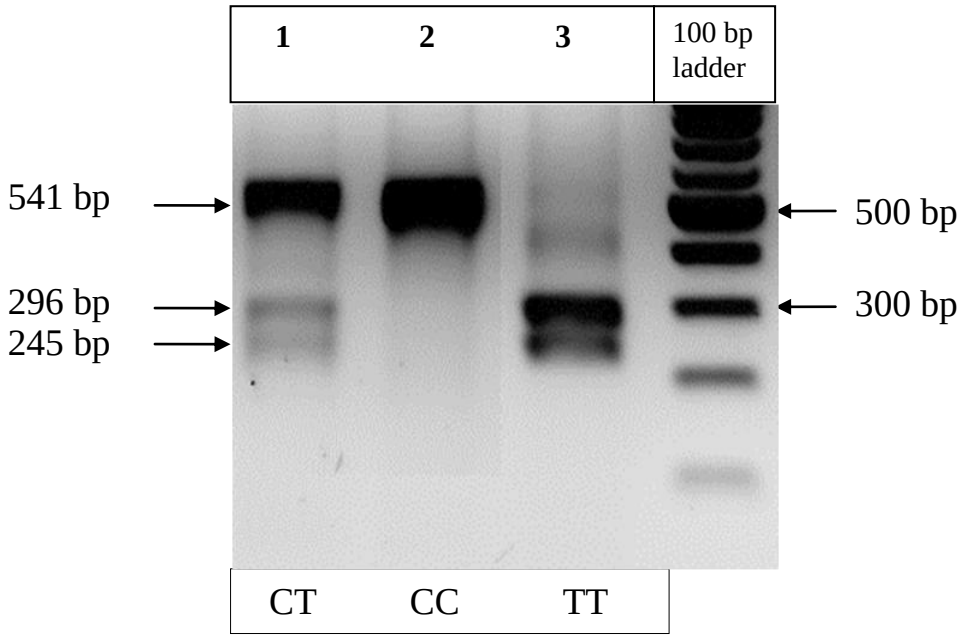


**Figure 5.6**: **2 % agarose gel showing PTPN22 PCR product digested with XcmI enzyme.** Lane 1 is heterozygous, lane 2 is homozygous wild-type and lane 3 is homozygous mutant.

**Table 5.8: Comparison of both DILI cohorts with POPRES controls genotyped for PTPN22 (rs2476601).**

| Genotypes | CC (%) | CT (%) | TT (%) | P-value | Odds ratio | 95% CI |
|---|---|---|---|---|---|---|
| New co-amoxiclav cases (n=99) | 70 (70.7) | 29 (29.3) | 0 | 0.00041 | 2.74 | 1.58 – 4.77 |
| All co-amoxiclav cases (new cases and DILIGEN cases included in Lucena et al. (2011) (n=165) | 122 (73.9) | 43 (26.1) | 0 | 0.00082 | 2.33 | 1.43 – 3.81 |
| All flucloxacillin cases (n=154) | 120 (77.9) | 32 (20.8) | 2 (1.3) | 0.02 | 1.9 | 1.1– 3.1 |
| Flucloxacillin cases positive for rs2395029, a tag SNP for B*57:01 (n=131) | 102 (77.9) | 27 (20.6) | 2 (1.5) | 0.02 | 1.9** | 1.1 – 3.3 |
| POPRES controls (n=282) | 245 (86.9) | 36 (12.8) | 1 (0.4) | | | |

** Combined odds ratio for disease development in flucloxacillin cases positive for both rs2476601 (PTPN22) and rs2395029 (B*57:01, OR=46.3, 95% CI=29.8–79.8; P=1.9x $10^{-62}$) =48.2.

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2 tailed test was used to calculate significance.

### *5.3.4 KCNJ1 Genotyping results*

Four polymorphisms (rs3016776 rs2855798, rs2847381 and rs2855790) in KCNJ1 gene, located in chromosome 11, were suggested as risk factors for co-amoxiclav DILI in the GWAS study performed by Lucena et al. (2011) although the association was not genome-wide significant ($P=4.26x10^{-5}$, $P=6.57x10^{-5}$, $P=3.93x10^{-5}$ and $P=3.7x10^{-5}$, respectively). We decided to study the SNP rs2855790 in this project based on its location at position 112 on exon 1 close to the promoter region of KCNJ1 gene. In addition, the SNP rs2855790 was found in strong linkage disequilibrium (LD) with the other 3 variants ($r^2=0.97$ with both rs3016776 and rs2847381 while $r^2=0.94$ with rs2855798). rs2855790 was also found in complete LD ($r^2=1$) with the polymorphism rs2186832 which was previously reported as a factor in systolic and diastolic blood pressure (BP) reduction (Tobin et al., 2008). This SNP (rs2186832) as well as the entire 4 SNPs identified by GWAS study are located in one block ($2^{nd}$ block) on the KCNJ1 gene Haploview (Figure 5.7) which indicates the strength of LD between variants. To replicate the possible association between KCNJ1 and co-amoxiclav DILI proposed by the GWAS study, a PCR-RFLP assay was designed for the SNP rs2855790 (Figure 5.8) and the results indicated a lower variant allele frequency in cases (15.8%) than in controls (24.7%); however, the difference was not statistically significant (OR=0.58, 95% CI=0.29–1.1; P=0.13) (Table 5.9). Further comparison to a larger control group (POPRES, n=282) also failed to replicate GWAS findings (P=0.22 for this study versus $P=3.7x10^{-5}$ for GWAS study).

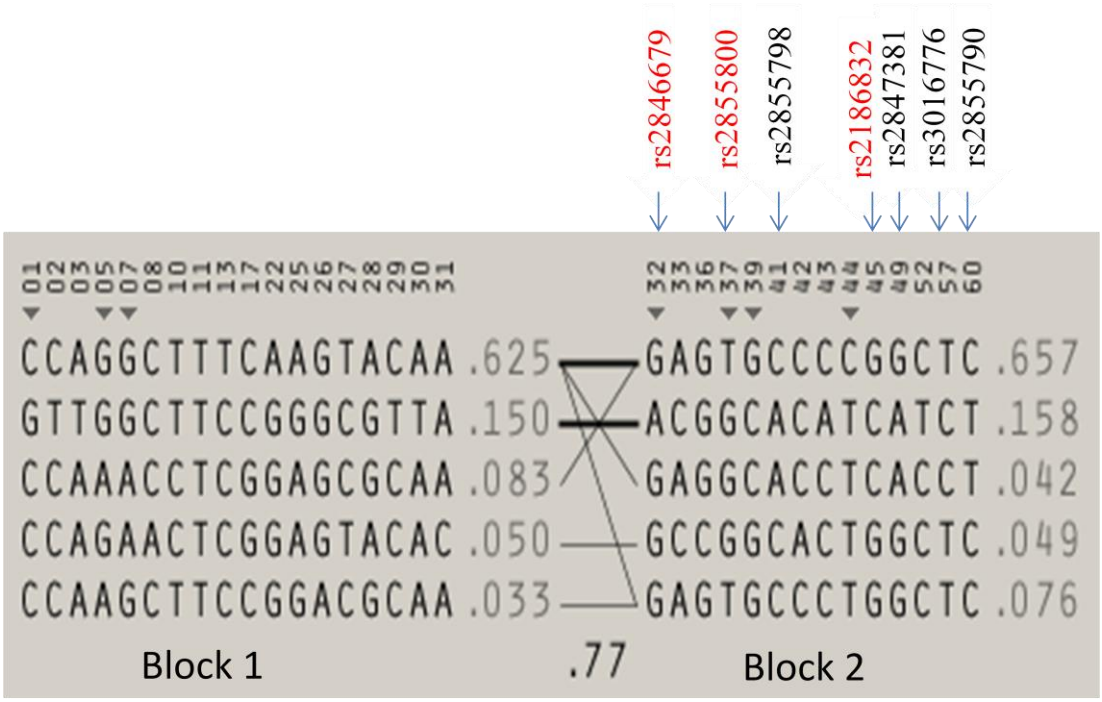


**Figure 5.7**: **Block 1 and 2 from Haploview for the KCNJ1 gene showing 4 GWAS suggested SNPs in BLACK and 3 SNPs in RED associated with lower blood pressure (Tobin et al., 2008).** The numbers below the arrows indicate the SNP number on Haploview. SNP number 60 (rs2855790) was chosen to study in this project.

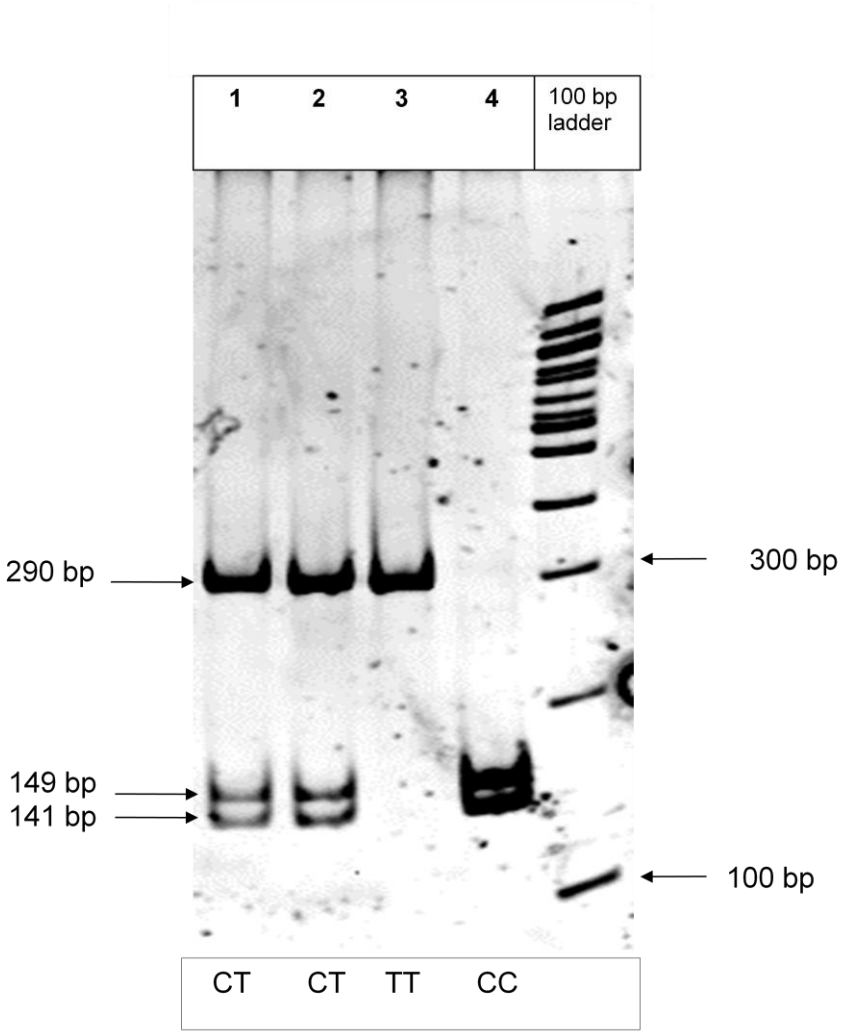


**Figure 5.8: 10 % polyacrylamide gel showing KCNJ1 gene digested with BseYI enzyme.** Lane 1 and 2 are heterozygous, lane 3 is homozygous mutant and lane 4 is homozygous wild-type.

**Table 5.9: KCNJ1 (rs2855790) genotyping results in co-amoxiclav DILI cases and two healthy community controls.**

| | | Samples | | |
|---|---|---|---|---|
| | | Co-amoxiclav cases (n=73) | Community controls (n=75) | POPRES controls (n=282) |
| Genotypes | CC | 50 (68.5) | 42 (56) | 169 (60) |
| | CT | 23 (31.5) | 29 (38.7) | 104 (36.8) |
| | TT | 0 | 4 (5.3) | 9 (3.2) |
| MAF (%) | | 15.8 | 24.7 | 20 |
| P value | | 0.13 | | 0.22 |
| OR | | 0.58 | | 0.67 |
| 95% CI | | 0.29 - 1.1 | | 0.4 – 1.2 |

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2 tailed test was used to calculate significance.

## 5.4 Discussion

The key findings from this chapter are that the previously reported association with a SNP in ST6GAL1 with flucloxacillin DILI could not be confirmed in a new cohort together with a confirmation of the previously reported association of PTPN22 genotype with co-amoxiclav DILI and the demonstration that it also appears to be a novel genetic risk factor for flucloxacillin DILI.

Using GWAS as a powerful tool to identify novel genetic associations with complex diseases and drug induced toxicities is increasingly feasible. This valuable approach was able to detect a significant effect of HLA loci on emergence of drug related hepatic injury, particularly associations of HLA-B*57:01 with flucloxacillin DILI and HLA-A*02:01 with co-amoxiclav hepatotoxicity. In addition to these findings, the GWAS study performed by Daly et al. (2009) found a possible effect of a non-HLA marker, an intronic polymorphism (rs10937275) located in ST6GAL1, on DILI development in cases exposed to flucloxacillin ($P=1.4x10^{-8}$, OR=4.1). ST6GAL1 is an immune-related gene which codes for a protein with a possible role in B cell differentiation (Stamenkovic et al., 1990). ST6GAL1 deficient individuals are known to have an interrupted IgG anti-inflammatory activity (Jones et al., 2012) and more prone to exacerbate certain types of infections and inflammations (Shinya et al., 2006) (Nicholls et al., 2007). There is also a suggested role in for ST6GAL1 in apoptosis (Liu et al., 2011). All these observations indicate that it is a biologically plausible gene for a role in drug-induced liver injury. Genotyping of an additional 100 flucloxacillin DILI cases for rs10937275 did not show a significant difference despite the higher number of cases (26%) carrying the variant allele (A) than those positive samples in POPRES controls (17.8%). Subgroup analysis was performed to include only the cases that are positive for rs2395029 (a tag SNP for B*57:01) (n=83) but the results still failed (P=0.085) to prove the previous GWAS findings. Further comparison of controls to a different cases category, mixed and cholestatic flucloxacillin DILI (n=92) also failed to show an association. Using of small sample sizes as in the case of Daly's GWAS study (51 cases vs 64 controls) may not be sufficiently robust to identify variants with small effect. The inconsistency between the current project and the previous GWAS results could reflect false-positive findings in the GWAS study as a result of using smaller number of cases in contrast

to the increased number of samples involved in this project which is predicted to provide more accurate representative figure. This lack of influence of ST6GAL1 on DILI was also augmented by the findings obtained in my previous project (Al-Shabeeb M. A, 2009) when 100 co-amoxiclav DILI cases were genotyped for rs13061952, a marker in linkage disequilibrium with rs10937275 ($r^2$=0.876). There was no evidence that ST6GAL1 was a risk factor for DILI due to co-amoxiclav (OR=1.11, 95% CI=0.60-2.07; P=0.75); this result confirmed the absence of an association relating to rs10937275 reported by Lucena et al. (2011) in the GWAS study involving 68 UK co-amoxiclav DILI cases (OR=1.2, 95% CI=0.6–2.3; P=0.60).

Prior to the completion of all the replication studies on ST6GAL1, we decided to study several other variants located in or close to the 3 known promoter regions. Two SNPs (rs73071448 and rs113928218) were found to be non-polymorphic in the UK population while only 3 out of the remaining tested SNPs showed borderline significance. Despite the possible role of ST6GAL1 in immunomodulation which makes it a plausible candidate, these results suggested that this gene has no effect on flucloxacillin-related hepatic injury. This project has confirmed that expression of different ST6GAL1 transcripts is cell-type specific regulated by one of the 3 known promoters. Polymorphisms in the promoter 2 region (P2), which confer specific regulated expression in mature B cells, could still play an important role in the immunological pathogenesis mechanism suggested as a cause of drug induced hepatotoxicity.

PTPN22 is another immune related gene studied in this project to replicate and extend findings reported in the previous co-amoxiclav GWAS study (Lucena et al., 2011). To reproduce the previous results that suggested a role for PTPN22 in co-amoxiclav hepatoxicity, additional new cases were investigated. The genotyping results obtained for the new cases confirmed the association of PTPN22 with co-amoxiclav DILI and the level of significance seen was very similar to the reported P value found in the GWAS study (P=4.1 x $10^{-4}$ for this study versus P=1.3 x $10^{-4}$ for GWAS). Findings of our project gave an odds ratio of 2.74 with a 95% confidence interval of 1.6 to 4.8 for possession of the variant allele, though, a slightly lower effect was observed when all cases (including the new (n=99) and those involved in

the GWAS study (n=66)) were compared to the POPRES controls (OR=2.33, CI= 1.4–3.8; P=8.2 x $10^{-4}$). In addition, a smaller but still statistically significant effect was observed for this PTPN22 SNP when another DILI cohort, involving 154 flucloxacillin cases, was also investigated. These results and the known role of PTPN22 variant (rs2476601) in opposing TCR signalling inhibition mediated by a tyrosine kinase enzyme suggests that this SNP might be a general risk factor for drug-induced hepatotoxicity. A recent study of pooled DILI cases from the UK, Spain and the US found no evidence for a role for PTPN22 in DILI induced by drugs other than flucloxacillin and co-amoxiclav was obtained (Urban et al., 2012). However, this study also found no significant relationship for the other DILI cases with HLA genotype and it remains possible that the PTPN22 SNP could be a general risk factor in forms of DILI where there is also a role for HLA genotype. Examples of this type include DILI relating to lumiracoxib, ximelagatran and lapatinib. However, there are no cases of DILI due to these drugs in either the DILIGEN or iDILIC collections (Ann Daly, personal communication) and the data on GWAS studies in relation to these drugs is not publicly available so it was not possible to investigate this issue further. Our study tried to investigate the possibility of genetic interaction between PTPN22 gene and HLA-B*57:01 genotype in flucloxacillin cases on DILI susceptibilty. Despite the fact that both PTPN22 and HLA loci are risk factors for DILI, there was no evidence of any additional risk in individuals positive for both alleles or synergy between the markers with the overall effect observed additive only. These findings suggest an independent effect for the PTPN22 and HLA alleles in their contribution to DILI development. Carriage of variants of both PTPN22 and rs2395029 (B*57:01) among flucloxacillin cases increases the odds ratio for development of hepatotoxicity from 46.3 (P=1.9x $10^{-62}$) for rs2395029 alone to 48.2 for the two SNPs combined.

In the case of the KCNJ1 gene, which encodes the potassium channel known as ROMK, no evidence for an association was seen on genotyping new co-amoxiclav DILI cases for the SNP rs2855790, despite a slightly lower variant allele frequency noticed in the DILI cases. This gene in view of its biological role in the kidney not the liver was not a very biologically plausible candidate as a DILI gene so the final result obtained for this is not too surprising.

Had more resources been available, several of the other genes listed in Table 5.1 represent interesting candidates for further study. In particular, SNPs in AKAP6 which is a protein kinase A anchor protein and GADD45G (growth arrest and DNA-damage-inducible gamma) which shows increased expression under cellular stress and activates the p38/JNK pathway via MTK1/MEKK4 kinase; both showed low p values in the UK cohort. However, further genotyping is currently in progress in a new GWAS study so the possibility that some of the SNPs listed in Table 5.1 are relevant to co-amoxiclav DILI will soon be clearer.

# Chapter 6. Studies on Novel Variants Associated with Co-amoxiclav-Induced Liver Injury Detected in Exome Sequencing Analysis

## 6.1 Introduction

Exome sequencing is a useful tool to diagnose certain rare Mendelian diseases such as Charcot-Marie-Tooth disease (a hereditary motor and sensory neuropathic disorder), retinitis pigmentosa and congenital disorders of glycosylation (a metabolic disorder) where different mutations may occur in particular families. Using other traditional genetic association studies may not be helpful to detect such mutations where large numbers of cases are needed. Exome sequencing is also considered as a potentially valuable approach to identify single nucleotide polymorphisms and rare variants associated with drug toxicities and complex diseases, particularly rare conditions and disorders of unknown etiology (Ku et al., 2012). As described previously in Section 1.7.3, different sequencing approaches are currently used aiming to sequence either part of the genome (e.g. exome capture (targeted genomic regions sequencing) and exome sequencing (coding regions sequencing)) or the whole genome (Imelfort et al., 2009) using high throughput sequencing technology. Newer advanced sequencing techniques become available replacing the old traditional methods like Sanger-based sequencing, used to sequence a DNA fragments of 800 -1000 bp, or pyrosequencing which can sequence shorter portions of 300– 500 bp. At present, the most commonly used platforms are Illumina Genome Analyzer II, the Applied Biosystems SOLiD and the Roche 454 sequencer (the developed form of pyrosequencing). However, variants detected by exome sequencing need further evaluation to be confirmed. Recent exome sequencing performed on a subset of our co-amoxiclav DILIGEN cases (n=66) and 371 population controls at the Broad Institute, Boston, USA (Daly MJ, Goldstein J and Daly AK, unpublished results) suggested a number of apparently significant associations (Table 6.1) which need to be validated and then to be confirmed by genotyping of additional co-amoxiclav DILI cases. After assessment of the various genes in term of possible biological relevance and the individual mutations, it was decided to concentrate on nonsynonymous variants located on 4 particular genes of known function that are expressed in liver and are biologically plausible candidates. These were IL12RB1, SHMT1, FMO5 and TPH1. It was also planned to perform additional functional characterisation of the relevant variants where this was feasible.

**Table 6.1: Top markers detected from exome target study using 66 co-amoxiclav cases and 371 controls.**

| Chr | Gene | SNP | Alleles | Cases (%) | Controls (%) | Chisq | Type | AA change |
|---|---|---|---|---|---|---|---|---|
| 19 | IL12RB1 | rs117511121 | T/C | 7 (10.6) | 3 (0.81) | 23.78 | M | p.283R>Q |
| 7 | CFTR | rs1800098 | C/G | 4 (6.06) | 0 0 | 22.59 | M | p.576G>A |
| 8 | PTK2B | rs61738530 | A/C | 4 (6.06) | 0 0 | 22.59 | M | p.264T>N |
| 19 | FCGBP | rs7249743 | C/T | 22 (33.3) | 39 (10.5) | 22.47 | M | p.1617M>V |
| 1 | FAM131C | rs77667563 | A/G | 33 (50.0) | 76 (20.5) | 22.36 | M | p.245R>W |
| 19 | LRP3 | rs11539586 | T/C | 9 (13.6) | 8 (2.16) | 19.36 | M | p.617A>V |
| 17 | KRTAP9-2 | rs9903833 | C/T | 32 (48.5) | 331 (89.2) | 19.14 | M | p.36P>S |
| 21 | MRPS6 | rs61910679 | T/A | 6 (9.09) | 3 (0.81) | 18.86 | M | p.76E>V |
| 10 | BTBD16 | rs11200524 | A/G | 12 (18.2) | 15 (4.04) | 18.71 | M | p.14R>Q |
| 11 | FAM86C | rs57679800 | T/C | 11 (16.7) | 13 (3.50) | 18.18 | M | p.128P>L |
| 17 | SHMT1 | rs1979277 | A/G | 60 (90.9) | 201 (54.2) | 18.05 | M | p.474L>F |
| 19 | POLRMT | rs2238549 | G/T | 57 (86.4) | 188 (50.7) | 17.69 | M | p.555E>A |
| 19 | C19orf57 | rs111386677 | T/C | 5 (7.58) | 2 (0.54) | 17.46 | M | p.607R>Q |
| 12 | DDX54 | rs2290766 | G/C | 9 (13.6) | 9 (2.43) | 17.46 | M | p.6G>R |
| 19 | ZNF628 | rs34864744 | A/G | 15 (22.7) | 24 (6.47) | 17.37 | M | p.230T>A |
| 19 | CYP2A7 | rs10425169 | G/A | 66 (100) | 233 (62.8) | 17.22 | M | p.64C>R |
| 17 | SMCR8 | rs8080966 | T/C | 60 (90.9) | 204 (55.0) | 17.15 | M | p.524P>L |
| 11 | TPH1 | rs145855109 | T/C | 3 (4.55) | 0 0 | 16.92 | M | p.300A>T |
| 13 | SPERT | rs79707842 | A/C | 13 (19.7) | 19 (5.12) | 16.87 | **NM** | p.286S>C |
| 3 | WWTR1 | rs1055153 | T/G | 20 (30.3) | 40 (10.8) | 16.7 | M | p.74P>Q |
| 9 | KIAA1797 | rs79849792 | T/C | 4 (6.06) | 1 (0.27) | 16.52 | M | p.802A>V |
| 7 | NAH11 | rs2285943 | T/G | 64 (97.0) | 226 (60.9) | 16.43 | **NM** | p.34E>D |
| 1 | FMO5 | rs58351438 | C/T | 6 (9.10) | 4 (1.10) | 15.9 | M | p.166K>E |

M=Missense (non-synonymous), NM= Nonsense (non-synonymous), AA=Amino acid

(Daly MJ, Goldstein J and Daly AK, unpublished results).

Serine hydroxymethyltransferase 1 (SHMT1) codes for a phosphate containing enzyme that plays an important role in folate metabolism (tetrahydrofolate hydrolysis into 5,10-methylene tetrahydrofolate) producing cellular constituents needed for cell growth (Appaji Rao et al., 2003). SHMT1 gene which is highly expressed in liver, biliary system and mainly in brain (Shen-Orr et al., 2010) was found to be completely deleted in 26 patients with Smith-Magenis syndrome (SMS) (Elsea et al. (1995). SMS is "a mental retardation syndrome with distinctive behavioral characteristics including intellectual disability, delayed speech and language skills, distinctive facial features, sleep disturbances, and behavioral problems" (De Leersnyder, 2013).

The flavin-containing monooxygenase (FMO) 5 gene encodes an enzyme which belongs to a family (FMO1-FMO5) of enzymes that have an important role in metabolism of drugs xenobiotics and endogenous substrates via oxidation reactions (Zhang et al., 2009). FMO3 and FMO5 are the most highly expressed enzymes among other FMO forms in the adult human adult liver (Reddy et al., 2010). Also, the two mRNAs of human FMO5 (either the 2.6 or 3.8-kb mRNAs) were accounted for over 50% of the total levels of mRNAs in human fetal liver (Zhang and Cashman, 2006). Investigation of 3 congenital heart disease patients indicated a deletion of a 1q21.1 multigene region which includes FMO5 in all cases but no deletion detected in a control group (Christiansen et al., 2004). This finding was further investigated by Greenway et al. (2009) who noticed significant difference of copy number variants of 1q21.1 region in 114 congenital heart malformation patients and replicated this in a further 398. Mutations in 1q21.1 region which includes FMO5 were also reported in a number of neurological (e.g. mental retardation) (de Vries et al., 2005), psychiatric (e.g. schizophrenia) (Stefansson et al., 2008; Xu et al., 2008) and other developmental and behavioral abnormalities (Brunetti-Pierri et al., 2008; Walsh et al., 2008).

Tryptophan hydroxylase 1 (TPH1) encodes a rate-limiting enzyme that serves as a catalyst in the production of peripheral serotonin (5-hydroxytryptamine, 5-HT); an important hormone and neurotransmitter (Lesurtel et al., 2006). The process of 5-HT synthesis starts with tryptophan monooxygenation into 5-hydroxytryptophan before being decarboxylated into serotonin (Wang et al., 2002). TPH1 is highly expressed in neurons, lung, eye, liver, intestinal and pancreatic cells whereas TPH2 is normally

expressed in brain (Walther et al., 2003). Multiple variations in TPH1 have been reported as risk factors in a variety of medical dysfunctions involved suicidal behavior (Bellivier et al., 2004; Li and He, 2006), major depression (Gizatullin et al., 2006; Viikki et al., 2010), attention deficit hyperactivity disorder in Chinese population (Li et al., 2006) but not in Europeans (Johansson et al., 2010), migraine headache (Erdal et al., 2007), schizophrenia (Allen et al., 2008; Sand, 2007), heroin addictions (Nielsen et al., 2008), bipolar disorder (Chen et al., 2008), endogenous psychoses (Efimov et al., 2009), pancreatic endocrine tumours (Johansson et al., 2009), hepatopulmonary syndrome in patients with advanced liver disease (Roberts et al., 2010), anger-related traits, somatic anxiety and anorexia nervosa (Pinheiro et al., 2010). In an animal studies, TPH1 deficient mice showed increased risk to develop steatohepatitis and lower tendency to regenerate damaged liver cells compared to wild-type species (Lesurtel et al., 2006; Nocito et al., 2007). Therefore, this gene was selected to further investigate its relevance to DILI development.

The interleukin 12 receptor, beta 1 gene (IL12RB1) codes for a subunit of IL12 receptor, also known as CD212. It is expressed on T cells and plays an essential role in interleukin 12 binding (Canda-Sanchez et al., 2009). Co-expression of IL12RB1 and IL12RB2 proteins lead to reconstitution of IL12 dependent signalling (Reddy et al., 2005; Takahashi et al., 2005). IL-12RB1 deficiency is considered the most important genetic risk factor associated with mycobacterial infection (Akahoshi et al., 2003; Fieschi et al., 2003). Mutations in the IL12RB1 have also been reported as predisposing factors for Salmonella infections (de Jong et al., 1998). More frequent candidal infections were recently detected in IL12RB1 deficient patients (Cardenes et al., 2010). Japanese population who are homozygous mutant for two promoter region variants rs436857 at position -2 and rs393548 at position -11, which are in strong linkage disequilibrium, were found to be at risk of developing atopic dermatitis and other allergic manifestations (Takahashi et al., 2005). A higher frequency of the -111 variant allele was also noted among childhood asthmatic patients. In a Chinese study, two common haplotypes of IL12RB1 appeared to be risk factors for development of severe acute respiratory syndrome (SARS) development (Tang et al., 2008). A significant association between rs2305742 in IL12RB1and development of non- Hodgkin's lymphoma was observed in a US study though, this finding could not be confirmed in different American and Australian cohorts (Lan et al., 2011).

## 6.2 Methods

### *6.2.1 Cases and controls*

The cases studied were described in detail in Chapter 3. As controls for the DILI cases, several different population controls groups were used for the analyses described in this chapter. Most of these have been discussed in more detail previously (Section 4.2.1). An additional 371 control samples from the National Institute of Mental Health repository (NIMH) described by (Sklar et al., 2008) were used as controls for selected genotyping assays as data on the relevant SNPs in these controls was already available. For TPH1 genotyping, data from 4293 control individuals from the National Heart, Lung, and Blood Institute Exome Sequencing Project (NHLBI ESP) in Bethesda, Maryland, USA- (https://esp.gs.washington.edu/drupal/)) cohort were used as this was the only source of control data for the variant of interest.

### *6.2.2 TaqMan genotyping*

TaqMan SNP genotyping assays designed by Applied Biosystems were used to genotype for all the variants chosen for study. The unique reference of each assay used and their catalogue numbers are described in Table 6.2. Custom TaqMan assays were ordered for the SNPs rs117511121 in IL12RB1 and rs145855109 in TPH1. Allelic discrimination analysis was performed using a Step-One Real-Time PCR machine.

### *6.2.3 Genomic DNA samples used*

To investigate the associations with co-amoxiclav hepatotoxicity found in the exome sequencing study, we first genotyped the 66 cases involved in the exome sequencing to confirm the obtained results and then 99 cases of newly recruited co-amoxiclav DILI cohort were used to replicate the proposed associations. The calculated statistical power to confirm significance in the replication cohort for the selected genes varied between 75% and 100%. All available co-amoxiclav cases (n=165) were genotyped for two other polymorphisms (rs436857 and rs17852635) in IL12RB1, however, the genotyping assay failed for 4 cases. Also, for variants confirmed to be associated with co-amoxiclav, further genotyping was performed on 155 DILI cases relating to flucloxacillin genotyped for TPH1 (rs145855109) and IL12RB1 (rs117511121).

**Table 6.2: Predesigned TaqMan SNP genotyping assays (Applied Biosystems) used to replicate exome study findings.**

| Gene | SNP | Assay reference number |
|---|---|---|
| IL12RB1 | rs436857 | C_795468_1, (Cat. # 4351379) |
| | rs17852635 | C_34516347_10, (Cat. # 4351379) |
| SHMT1 | rs1979277 | C___3063127_10, (Cat. # 4351379) |
| FMO5 | rs58351438 | C__25597083_20 (Cat. # 4362691) |

### *6.2.4 IL12RB1 sequencing*

A polymerase chain reaction (PCR) product of 1263 base pairs (bp) (1148 bp upstream and 115 downstream of the transcription start site) of IL12RB1 gene was amplified using 5'-CTGGAGCTTGGCATGTGGGGA-3' as a forward primer and 5'-CGTGCCTCCACCCAGCAAGA- 3' as reverse. The PCR product was purified for sequencing using QIAquick PCR Purification Kit Protocol from QIAGEN. Twenty µl of the purified PCR product containing approx. 300 ng DNA and 10 µl of 5 µM primer were sent to Eurofins MWG Operon (London, UK) for sequencing.

### *6.2.5 Haplotype frequency determination*

For studies on the IL12RB1 gene, haplotypes were assigned using Haploview 4.1.

### *6.2.6 Inserts design*

Two different sized-inserts were designed; an insert of 293 bp which covered both the polymorphic -2 and -111 sites of IL12RB1, while the other insert is a 201 bp including the polymorphic -111 site only. The reverse primer used for the 293 bp insert (-265/+28) was 5'-GCATGAGCTC**GGACCACCCAGGTCACCAGC**-3' while the reverse primer used for 201 bp insert (-265/-65), 5'-GCATGAGCTC**CCGTCCCCACTCCGGAACAC**-3', and the common forward primer, 5'-GCATGGTACC**ACCCTGACTTGCTCCAAAGTC**-3', were both described previously by Takahashi et al. (2005). The bold type letters represent the IL12RB1 sequence whereas nonbold ones indicate an overhang sequence containing a restriction site for either *KpnI* or *SacI* to allow ligation to the pGEM T-easy vector and later into the pGL3 basic vector. PCR was amplified using DNA from two samples; one of them was homozygous mutant for the haplotype of interest (-2T/-111T) while the other sample was homozygous wild-type (-2C/-111A).

### *6.2.7 PCR amplification and purification for cloning*

PCR amplification conditions used for both cloning inserts were 35 cycles of 1 min at 94°C denaturation, 1 min at 62 °C annealing and 72 °C elongation for 1 min, followed by a 7 min extension at 72ºC. The amplified PCR products were run on a 2% agarose gel. The gel was placed on a UV transilluminator to visualize the location of DNA bands within the gel and the bands were removed from the gel

using a scalpel blade. DNA bands were purified using QIAGEN clean up kit using the manufacturer's protocol.

### *6.2.8 Ligation and transformation*

The purified PCR product was ligated first to pGEM T-easy vector. The ligation reaction was set up using 5 µl of 10x T4 ligation buffer, 1 µl of 50 ng T-easy vector, 1 µl of T4 DNA ligase and 10 µl of PCR product. Reactions were left overnight at 4 °C. The ligation reaction (10 ul) was then incubated with 100 µl competent JM109 *E.coli* cells (Promega, UK) in a 1.5 ml microfuge tube and left on ice for 20 minutes. This mixture was then exposed to a heat shock in a water bath at 42 °C for 55 seconds and immediately placed on ice for 2 minutes. Following this step, 900 ul Super Optimal Broth (SOB medium) (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 10 mM MgSO4, 10 mM MgCl2, 20 mM glucose) was added to the bacterial mixture. Ultimately, the mixture was incubated at 37 °C in orbital shaker at 150 rpm for 1.5 hours. Different volumes of transformed cells (100 µl and 200 µl) were then spread onto duplicate lysogeny broth (LB) agar plates, containing 100 µg/mL ampicillin, 60µg/mL X-Gal, 0.1 mM IPTG. LB agar plates were then incubated overnight at 37 °C.

### *6.2.9 Extraction of plasmid DNA*

Several white colonies were transported using sterile pipette tips from LB agar plates into 10 ml LB medium containing 100 µg/mL ampicillin and were left to grow overnight at 37 °C in orbital shaker incubator at 150 rpm. Afterwards, the bacterial cells were harvested by centrifugation at 6000 x *g* for 15 min at 4°C. Plasmid DNA was then extracted from bacterial pellet using QIAGEN miniprep plasmid extraction kit.

### *6.2.10 Cloning into pGL3 vector*

Double restriction digestion of the purified plasmid DNA containing pGEM T-easy vector was performed using *SacI* and *KpnI* enzymes. Once inserts (293 bp and 201 bp) were removed from pGEM vector, they were ligated again into pGL3 vector and the transformation and plasmid DNA extraction steps described above in Sections 6.2.8 and 6.2.9 were followed. In order to confirm the cloning of different IL12RB1 promoter fragments into pGL3 vector, the extracted DNA was digested using *SacI* and *KpnI* enzymes and then the digested products were run on a 10%

polyacrylamide gel (Figure 6.1). Orientation of IL12RB1 promoter-pGL3 basic vector and sequence integrity was further confirmed through plasmid DNA sequencing by Eurofins MWG Operon (London, UK).

### *6.2.11 HepG2 cell culturing and seeding*

HepG2 cell lines were cultured as previously described in Chapter 5 (Section 5.2.2.1). Cells were then seeded into 24-well plates at a density of $4x10^{-4}$ cells/ml in complete medium and were left overnight to get 50-80% confluent.

### *6.2.12 Cell transfection*

Growth medium of the seeded cells were replaced with 200 µl fresh complete DMEM before adding 100 µl of transfection mixture to each well. Transfection master mixture was prepared by vortexing 1.5 µl of GeneJuice (Merck biosciences) with 100 µl of serum free medium and incubated at room temperature for 5 minutes, 0.5 µg of each reporter construct or pGL3-basic vector and 50 ng of Renilla vector (Promega) as an internal control were added and incubated at room temperature for 20 minutes. After 24 hours, transfection mixture was removed from the 24-well plates and 1 ml of fresh full medium was added. Seventy two hours post-transfection, Dual-luciferase reporter assay was performed. Each construct was transfected in triplicate.

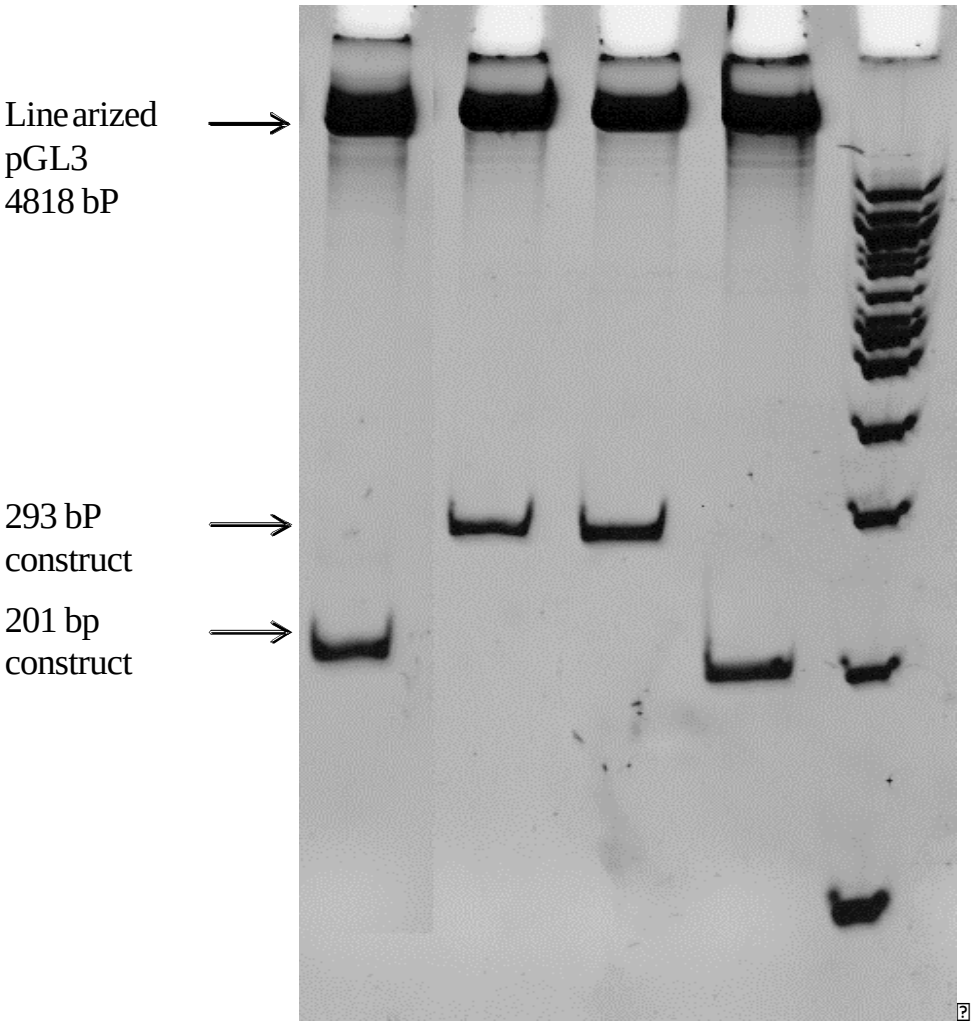


**Figure 6.1**: **Polyacrylamide gel (10%) showing plasmid DNA of two different constructs of IL12RB1 promoter-pGL3 basic digested with *SacI* and *KpnI***. The upper construct includes the -2 and -111 positions while the lower construct includes the -111 position only.

### *6.2.13 Dual –Luciferase reporter assay measurements*

Growth medium was removed; wells were washed by PBS saline and 100 µl of passive lysis buffer (Promega) was added and incubated on a shaker at room temperature for 15 minutes. Cells were harvested and 20 µl of cell lysate was mixed thoroughly with 100 µl of luciferase assay reagent II (LAR II) to measure Firefly luciferase fluorescence (first reading). 1x solution of Stop and Glo reagent (100 µl) was then added to the cell lysate-LAR II mixture and vortexed to measure Renilla Luciferase fluorescence (second reading). Three readings for each luciferase reagent were measured and the mean of readings was plotted as relative reporter activity of Firefly/Renilla luciferase fluorescence.

### *6.2.14 Use of Jurkat cells*

The non-adherent Jurkat cell line, derived from an acute T cell leukemia and obtained from Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, was also used as a transfection host to test IL12RB1 promoter activity. These cells were cultured in RPMI 1640 (Gibco/Invitrogen) medium with similar additives described above used for HepG2 cells. A cell density of $1x10^6$ cells/ml of Jurkat cells were needed for transfection.

### *6.2.15 Statistical analysis*

To test the variances in gene activity of the three means of the studied groups (pGL3 basic, mutant construct and the wild-type construct) at one time, one way analysis of variance test (ANOVA) was used. Tukey's honest significance test (HSD) was used in conjunction with ANOVA to examine differences in reporter genes activities between each two groups. The difference of P value $< 0.05$ was considered statistically significant. IL12RB1 haplotypes frequencies of the tested cases and controls were determined using Haploview version 4.1 and the difference significance among them was calculated by UNPHASED software version 3.1.7. The p value was calculated using a likelihood ratio chi-square test (Dudbridge, 2013). Minitab 16 was used to calculate the statistical power and sample sizes.

## 6.3 Results

### *6.3.1 Genotyping results for SHMT1 (rs1979277, C/T)*

The exome sequencing study suggested a possible role for SHMT1 gene in co-amoxiclav hepatotoxicity (OR=8.46, 95% CI=3.57-20.06; P=$8.76x10^{-9}$). However, genotyping of 99 co-amoxiclav cases which had not been included in the exome sequencing and 266 community controls failed to confirm the association found in the sequencing study (OR=0.88, 95% CI=0.55 - 1.40; P=0.63) (Table 6.3). Comparison of all cases (the new cases and those included in the exome study) with our community controls did not show a significant association either (P=0.32).

### *6.3.2 Association of FMO5 (rs58351438, C/T) and co-amoxiclav DILI*

To confirm the association found in exome sequencing study related to the SNP rs58351438 (OR=9.18, 95% CI=2.52-33.47; P=0.0012), larger numbers of new co-amoxiclav DILIGEN cases (n=99) and 266 local controls were genotyped using a TaqMan genotyping assay. Carriage of the minor allele was slightly higher (3.3%) in co-amoxiclav cases than in controls (2.3%) but the difference was not statistically significant (OR=1.35, 95% CI= 0.33-5.52; P=0.71) (Table 6.3) and therefore failed to replicate the association detected in the exome sequencing study. Similar results were obtained when the previously sequenced cases (n=66) were added to the new cases in the comparison with the community control group (P=0.26).

### *6.3.3 Association of TPH1, rs145855109 (C/T) with co-amoxiclav cases*

This variant was found to be involved in co-amoxiclav hepatotoxicity in the exome sequencing study (OR=6.89, 95% CI=5.48–8.66; P=0.003). Two (1.6%) of the 99 additional co-amoxiclav DILI cases genotyped for rs145855109 were positive for the variant in comparison to only 0.14% positivity in the NHLBI ESP controls (n=4293), which confirm exome study findings (OR=14.73, 95% CI= 2.94–73.92; P=0.013) (Table 6.4). The effect of the TPH1 variant was found to be slightly higher when all available co-amoxiclav cases (n=165) were combined in the comparison with the control group used (OR=17.75, 95% CI=4.96–63.52; P=0.00032). Using a sample size of 165 gave a statistical power of 75% at a two-sided Type-I error rate of 0.05 (Table 6.5). However, no association was detected for flucloxacillin DILI when 155 cases were genotyped for this SNP (OR=1.04, 95% CI=1.03–1.04; P=1.0), suggesting the effect of this SNP as a DILI risk factor is co-amoxiclav specific.

**Table 6.3: SHMT1 (rs1979277) and FMO5 (rs58351438) genotyping results of co-amoxiclav DILI cases compared to community controls.**

| | Genotypes | New cases (n=99) | All cases (n=165) | Controls (n=266) |
|---|---|---|---|---|
| SHMT1 (rs1979277) | TT (%) | 8 (0.08) | 23 (0.14) | 24 (0.09) |
| | CT (%) | 39 (0.41) | 70 (0.43) | 115 (0.43) |
| | CC (%) | 49 (0.51) | 69 (0.43) | 127 (0.48) |
| P value | | 0.63 | 0.32 | |
| OR | | 0.88 | 1.23 | |
| 95% CI | | 0.55-1.40 | 0.83-1.83 | |
| FMO5 (rs58351438) | TT (%) | 1 (0.01) | 1 (0.01) | 0 |
| | CT (%) | 2 (0.02) | 6 (0.04) | 6 (0.02) |
| | CC (%) | 96 (0.97) | 158 (0.95) | 260 (0.98) |
| P value | | 0.71 | 0.26 | |
| OR | | 1.35 | 1.92 | |
| 95% CI | | 0.33-5.52 | 0.63-5.82 | |

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2-tailed test was used to calculate significance.

**Table 6.4: Statistical power value of using 165 cases to replicate associations of genes suggested in the exome study with co-amoxiclav-induced liver injury .**

| | SHMT1 (rs1979277) | FMO5 (rs58351438) | TPH1 (rs145855109) | IL12RB1 (rs117511121) |
|---|---|---|---|---|
| Controls' genotype frequecy (%) | 54.2 | 1.08 | 0.14 | 0.81 |
| Cases' genotype frequecy (%) | 90.9 | 9.09 | 4.55 | 10.6 |
| P value cutoff significance | 0.05 | 0.05 | 0.05 | 0.05 |
| Statistical power | 100% | 92% | 75% | 97% |

**Table 6.5: Comparison of the 1000 Genome controls with both DILI cohorts (co-amoxiclav and flucloxacillin cases) genotyped for TPH1 (rs145855109).**

| | | Flucloxacillin cases (n=155) | New co-amoxiclav cases ( n=99) | All co-amoxiclav cases ( n=165) | NHLBI ESP controls (n=4293) |
|---|---|---|---|---|---|
| Genotype | CT (%) | 0 | 2 (2.02) | 4 (2.42) | 6 (0.14) |
| | CC (%) | 155 (100) | 97 (97.08) | 161 (97.58) | 4287 (99.86) |
| P value | | 1.0 | 0.013 | 0.00032 | |
| OR | | 1.04 | 14.73 | 17.75 | |
| 95% CI | | 1.03 – 1.04 | 2.94 – 73.92 | 4.96 – 63.52 | |

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2-tailed test was used to calculate significance.

### *6.3.4 Genotyping for rs117511121 in IL12RB1*

Based on the exome sequencing findings, the coding polymorphism rs117511121 (C/T) located in exon 8 in IL12RB1 gene (Figure 6.2) on chromosome 19 was among the most significant markers associated with co-amoxiclav hepatotoxicity (OR=14.6, 95% CI=3.7–57.9; P=0.0011). The results now obtained through genotyping larger numbers of newly recruited co-amoxiclav iDILIC cases (n=99) have shown that more than 5% of the cases carried the variant allele T but only 3 positive samples (less than 1%) were found in a community control group (n=371) which was genotyped in the National Institute of Mental Health in Bethesda, Maryland, USA, as a non-treated control cohort (Table 6.6). Patients who carry the risk allele would be at a risk of 6.5 times compared with normal controls (P=0.012). These cases in addition to the cases involved in the exome study were together further compared to the control group. A slightly higher odds ratio was seen with a lower p value (OR=9.62, 95% CI= 2.68–34.57; P= $9.7x10^{-5}$). The sample size involved gave a statistical power of 100% at a two-sided Type-I error rate of 0.05.

This effect of rs117511121 seemed to be co-amoxiclav specific, since 155 flucloxacillin DILI cases were also investigated but no association was identified with cases compared to community controls (OR=1.60, 95% CI=0.27–9.70; P= 0.63).

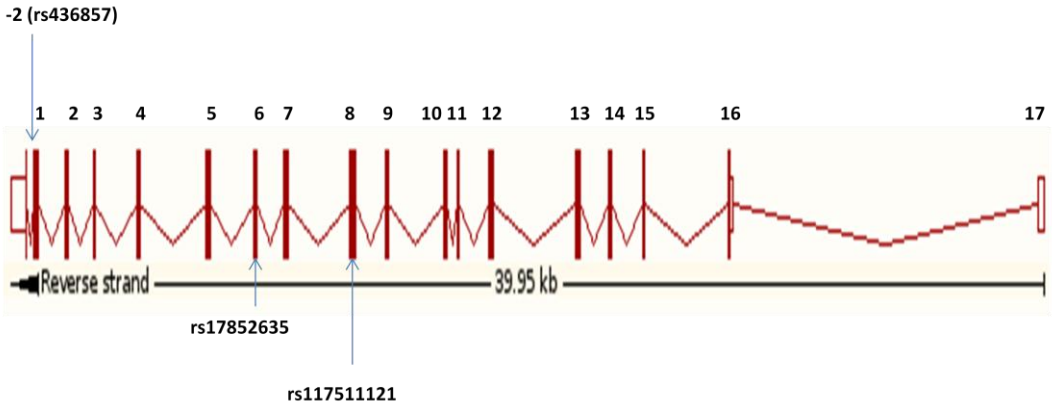


**Figure 6.2**: **The locations of the studied polymorphisms in the IL12RB1 gene are illustrated.**

**Table 6.6: Comparison of the NIMH controls with both DILI cohorts (co-amoxiclav and flucloxacillin cases) genotyped for IL12RB1 (rs117511121).**

| | | Flucloxacillin cases (n=155) | New co-amoxiclav cases ( n=99) | All co-amoxiclav cases ( n=165) | NIMH controls, (n=371) |
|---|---|---|---|---|---|
| Genotype | CT (%) | 2 (1.3) | 5 (5.1) | 12 (7.3) | 3 (0.8) |
| | CC (%) | 153 (98.7) | 94 (94.9) | 153 (92.7) | 368 (99.2) |
| P value | | 0.63 | 0.012 | $9.7x10^{-5}$ | |
| OR | | 1.60 | 6.5 | 9.62 | |
| 95% CI | | 0.27 – 9.70 | 1.5 – 27.8 | 2.68 – 34.57 | |

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2-tailed test was used to calculate significance.

### *6.3.5 IL12RB1 sequencing*

The promoter regions of IL12RB1 from sixteen samples (4 controls and 12 co-amoxiclav DILI) positive for rs117511121 were sequenced to determine whether there were any novel regulatory polymorphisms in linkage disequilibrium with this SNP which though nonsynonymous is predicted not to be damaging for IL12RB1 function (Ensembl genome database). No novel base changes were seen (Figure 6.3, Figure 6.4 and Figure 6.4) but interestingly, the sequenced samples were found all positive, including 3 homozygous mutant and 13 heterozygous samples, for another two promoter region SNPs (rs436857 and rs393549) at positions -2 and -111 which are known to be in complete linkage disequilibrium ($r^2$=1), respectively but were wild-type for other known SNPs in the region (Figure 6.5). These results suggested that the coding SNP rs117511121 and both promoter region SNPs are in linkage disequilibrium (Figure 6.6).

Additional studies on Haploview showed that there were 3 common haplotypes for IL12RB1 (Figure 6.7). In addition to the "wild-type" haplotype and a haplotype which includes the -111 and -2 SNPs detected in the sequencing, there is a third haplotype which includes rs17852635 located at position +11058 in IL12RB1. This common SNP is in linkage disequilibrium with the -111 and -2 SNPs in haplotype 2 but there is also a third haplotype (haplotype 3) including this SNP and an apparently wild-type promoter region sequence. To further analyze the IL12RB1 gene as a candidate gene for co-amoxiclav DILI, it was decided to genotype for both the -2 SNP (rs436857) and for rs17852635.

CTGGAGCTTGGCATGTGGGGAGGTGGGCATTGTGGAGGGCCAGCATGCTGCAAGCTCTTTGT
AACAATTTGTTGGAAGGATGAAATTAGGCCATCCAAGAACACTGCCCTCCTTCAGCTCTTGA
GTCTCAGATTCAGAAGCTGGCGAGATGGCCCGGTGCGGTGGCTCACGCCTGTGATCCCAGAA
TTTTGGTAGGCCAAGGCGGACGGATCATTTGAACCCGGGAGTTTGAGACCAGCCTGGGCAAC
ATGGCAAAACCCCCTCTCTTAAAAAAAAAAAAAATGCAAAAATTAGCTGGGCGTGGTGGTGCA
TGCCTGTAGTCCCAGTTCCTGGGGAGGCTGAGGTAGGAGGATGGCCTGAGCCTGGGGAGGTT
GATGTTGCAGTGAGCCGTGATTGCACCACTGCACTACAGCTGGGCCACAGATCAAGACCCTA
TCTCAAAAGAAGAAAAAAAGAATTTGGCAGCCAGGCACGGTAGCTCACGCCTGTCATCCCAG
CACTTTGGGAGGCTGAGGTGGGTGGATCACTTAGGGTTAGGAGTTCGAGACCAGCCTGGCCA
GCATGGTGAAACCCTGTCTCTACTAAAAATACAAAAATTAGCTCGATGTTGTGGCAGTTGCC
TGTAATTCCAGCTACTCGGGAGGCTGAGGCAGGAGAATTGCTTGAACCCGGGAGGCAGAAGT
TGCAGTGAGTGGAGATCATGCCACCACACTCCAGCCCGGGCGACAGAGCAGGGCTCCATCTC
AAAAAAAAAAAAAAAAAATTGGCTAGAGAGGGCCAGAGACCTGGCCAAGTGCATGGAACCACA
GGTGGCCCATGGGCCCATGGGGCTGGCATCTGGGTGTTCAGTTCAGGTCTGGGGCCAGCAGG
GCAGAACCCCCTAGAACCCTGACTTGCTCCAAAGTCAGACTTGCTCCAGAGTCTGTGGCCGC
CACCATCACCACCACCACGGAGCTGACTTCCTGGGTTTCTCTTTCACTTTGACTTGCCTTAG
GGATGGGCTGTGACACTTTACTTTTTTTCTTTTTTCTTTTTTTTTCTGTCTTTTCTCCTTGCT
CAGCTTCAATGTGTTCCGGAGTGGGGACGGGGTGGCTGAACCTCGCAGGTGGCAGAGAGGCT
CCCCTGGGGCTGTGGGGCTCTACGTGGATCTGATGGAGCCGCTGGTGACCTGGGTGGTCCCC
CTCCTCTTCCTCTTCCTGCTGTCCAGGCAGGGCGGTGAGTCCCCTGACCCTGGCATGGCGGC
TCCTCTTGCTGGGTGGAGGCACG

**Figure 6.3**: **Sequencing results IL12RB1 promoter region (1263 bp) of samples positive for the coding SNP rs117511121 showed mutant alleles at positions -111 and -2 (in red) and 14 other wild-type SNPs (in green).** The gray highlighted sequence indicated the 5'UTR. The orange highlighted sequence represents the coding region of exon 1 in IL12RB1 gene.

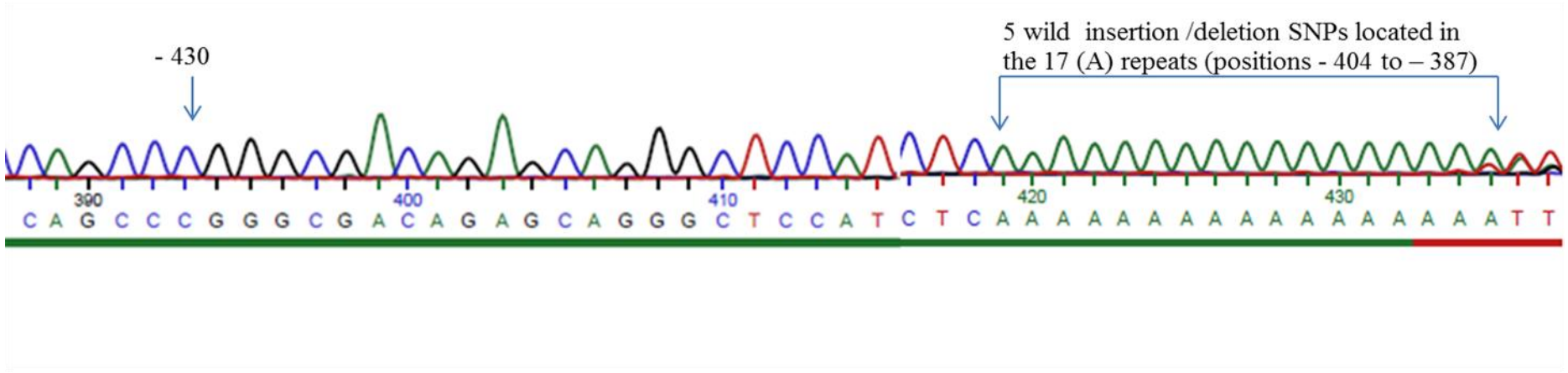


**Figure 6.4**: **Sequencing results of part of the promoter region of IL12RB1 gene. SNPs indicated by arrows were found wild-type.**

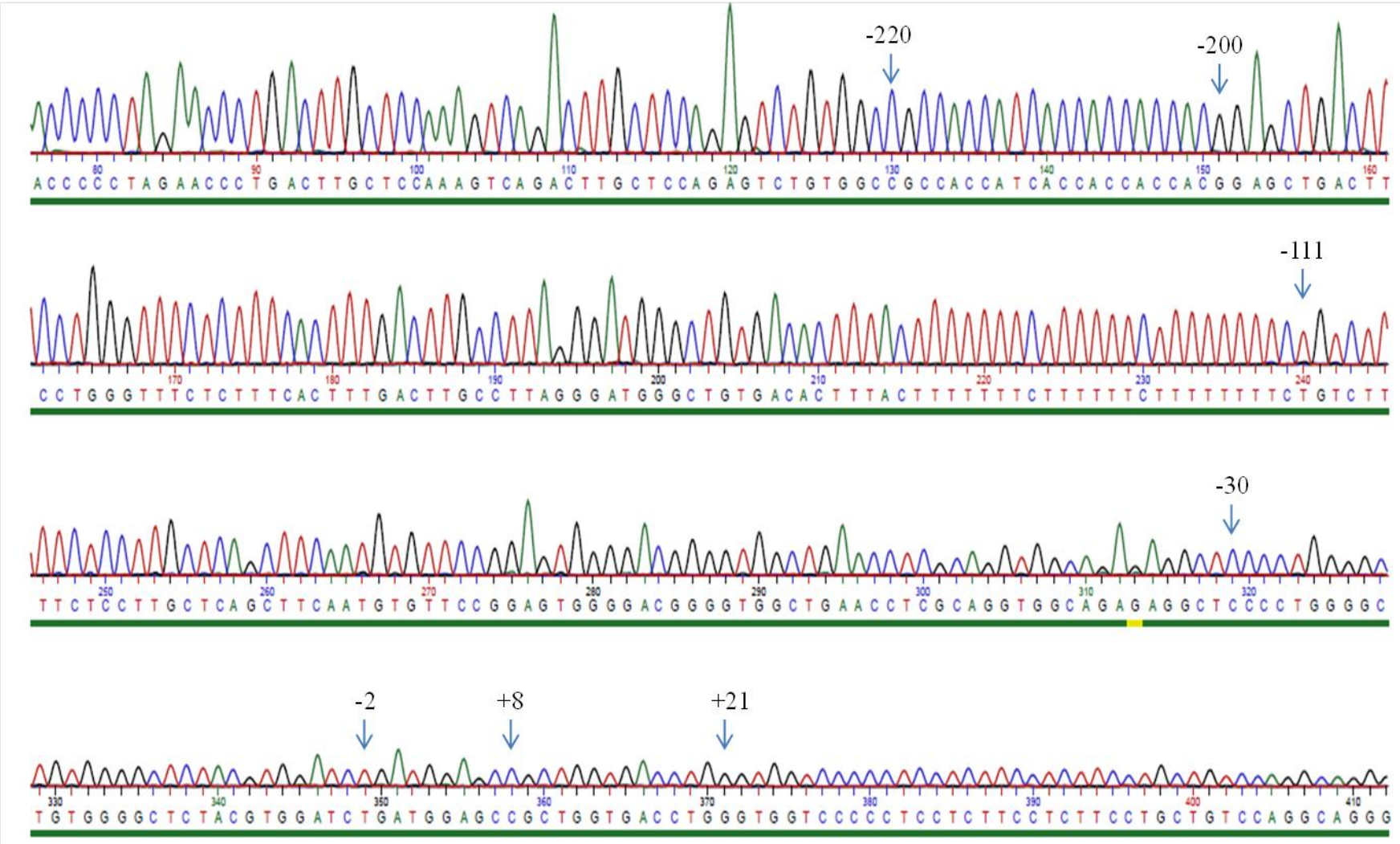


**Figure 6.5**: **Sequecing results UTR of IL12RB1.** Two mutant alleles are indicated at positions -2 and -111 were seen in 3 samples. Heterozygouse genotype was also detected in 13 samples. Other known SNPs at positions -30, +8 and +21 were not detected.

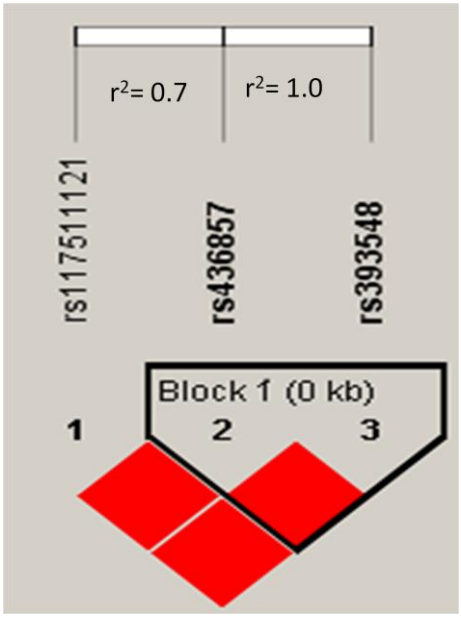


**Figure 6.6**: **LD plot showing linkage disequilibrium between the coding SNP (rs117511121) and 2 promoter SNPs (-2 and -111).**

### *6.3.6 Genotyping results for additional SNPs in IL12RB1*

For rs436857 at -2, the rare TT genotype was significantly more frequent in co-amoxiclav cases compared with the 1000 Genome controls (OR 3.7, 95% CI=1.5– 9.3; P=0.004) (Table 6.7) but this significance was only seen for a recessive model with no significant effect for a co-dominant model. For rs17852635 located at position +11058, we also found significance for a recessive model (OR=2.2, 95% CI=1.3 – 3.9; P=0.007) (Table 6.6) with no significance for a co-dominant model.

To investigate the relationship between the -2 and +11058 SNPs, haplotypes for the three genotypes were assigned using Unphased. Table 6.8 shows the various haplotypes in the co-amoxiclav cases and 1000 Genome controls which indicated a higher frequency distribution (22.5%) of the haplotype that carries the mutant alleles for the tested SNPs (T-T) in cases than in controls (16.7%), which gave an odds ratio of 1.41. The overall association p value which was calculated using the program Unphased suggests a borderline significant association between IL12RB1 haplotypes, particularly the rarer one, and co-amoxiclav DILI (P=0.047).

**Table 6.7: Genotype frequency of co-amoxiclav DILI cases and 1000 Genome controls for the SNPs (rs436857 and 17852635) at position -2 and +11058 in IL12RB1 gene.**

| IL12RB1 SNP | Genotypes | Cases (n=161) | Controls (n=379) | P-value for TT vs CT + CC Recessive model | P-value for TT+ CT vs CC Co-dominant model |
|---|---|---|---|---|---|
| rs436857 | TT (%) | 12 (7.5) | 8 (2.1) | 0.0042 | 0.24 |
| | CT (%) | 51 (31.7) | 120 (31.7) | | |
| | CC (%) | 98 (60.8) | 251 (66.2) | | |
| OR | | | | 3.73 | 1.26 |
| 95% CI | | | | 1.50 – 9.32 | 0.86 – 1.85 |
| rs17852635 | TT (%) | 25 (15.5) | 29 (7.7) | 0.0073 | 0.40 |
| | CT (%) | 69 (42.9) | 177 (46.7) | | |
| | CC (%) | 67 (41.6) | 173 (45.6) | | |
| OR | | | | 2.22 | 1.18 |
| 95% CI | | | | 1.25 – 3.92 | 0.81 – 1.71 |

Number of individuals with each genotype is shown with the percentage frequencies shown in parenthesis. Fisher's exact 2-tailed test was used to calculate significance.

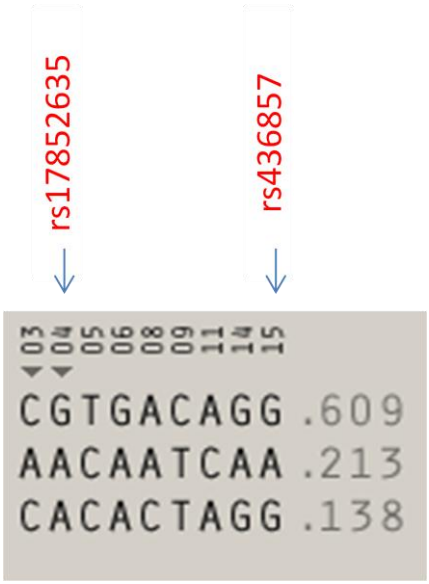


**Figure 6.7**: **Haploview is showing 3 common haplotypes (>10%) of IL12RB1 gene with their frequencies derived from HapMap**. Genotyping of the two SNPs indicated with arrows were sufficient to determine these common haplotypes. The haplotype data from European populations were obtained from HapMap project.

**Table 6.8: IL12RB1 haplotype frequency for common SNPs among co-amoxiclav DILI cases and 1000 Genome controls. The promoter SNP (rs436857) at position -2 and rs17852635 at the 3'end were genotyped to determine these haplotypes.**

| | | Cases (%) n=159 | Controls (%) n=379 | OR (95% CI) |
|---|---|---|---|---|
| Haplotypes | C-T | 46.4 (14.6) | 100.2 (13.2) | 1.23(0.84 -1.81) |
| | T-T | 71.6 (22.5) | 134.8 (16.7) | 1.41 (1.01-1.96) |
| | C-C | 196.6 (61.8) | 521.8 (68.8) | |
| P-value of overall association | | 0.047 | | |

### *6.3.7 Functional studies on the IL12RB1 gene*

#### *6.3.7.1 Sequencing*

Sequencing results of the tested constructs performed by Eurofins MWG Operon showed that the required base changes at the -2 and -111 sites were present in one construct with the other showing the wild-type sequence at these positions. The sequences of both constructs were otherwise identical to the known IL12RB1 sequence for this region (Figure 6.8 and Figure 6.9).

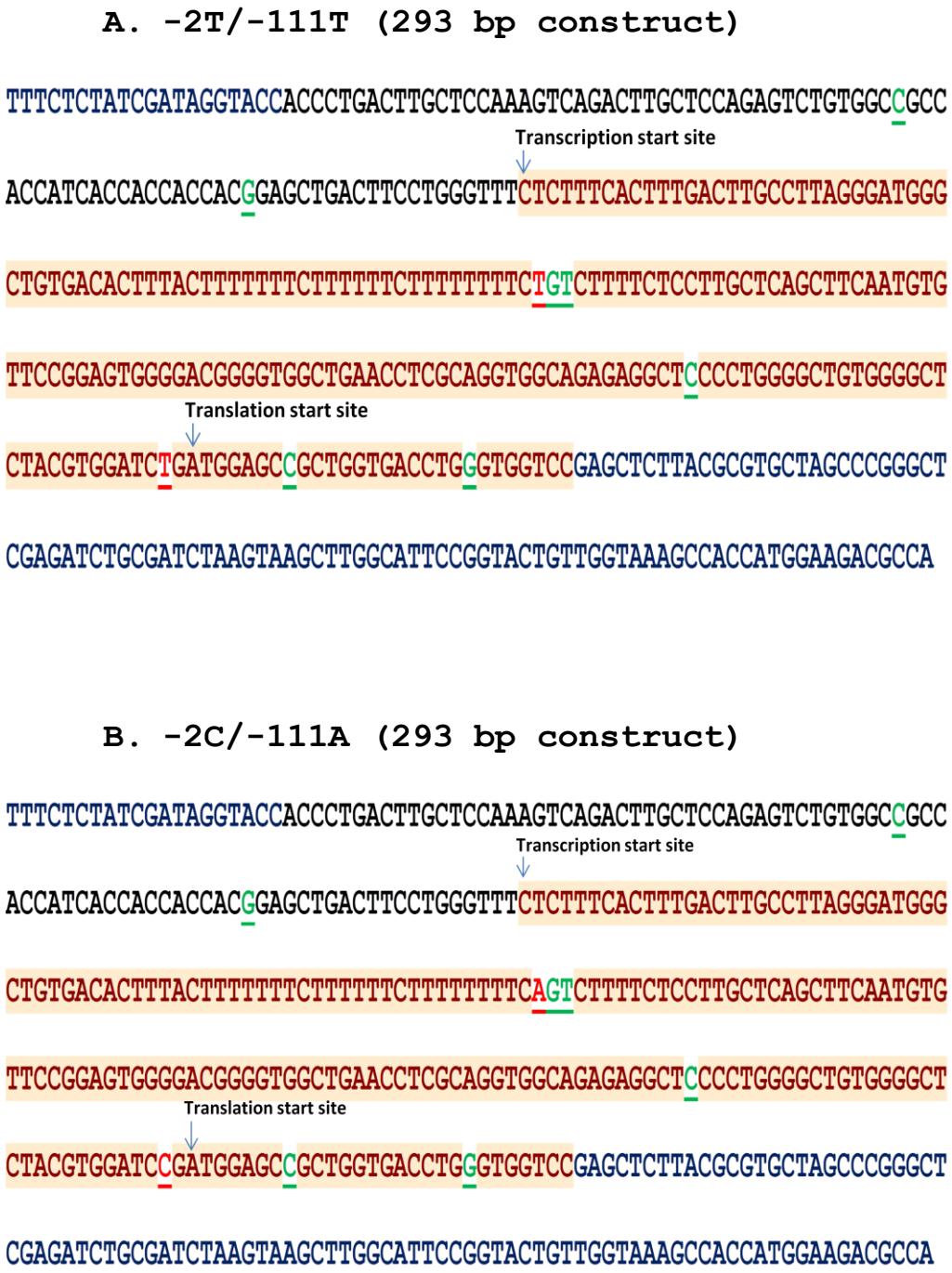


**Figure 6.8**: **Sequences of IL12RB1 promoter inserts (293 bp, black/orange)-pGL3 basic (blue) with variations, red coloured, only at -2 (C/T) and -111 (A/T).** Other SNPs, green coloured, were found wild-type. The sequence (A) represents *mutant haplotype* (-2T/-111T), (B) showing *wild haplotype* (-2C/-111A). The translation start site (+1) indicated by an arrow is the first nucleotide of the start codon (ATG). The indicated transcription start site is located at position -180 (van de Vosse et al., 2013).

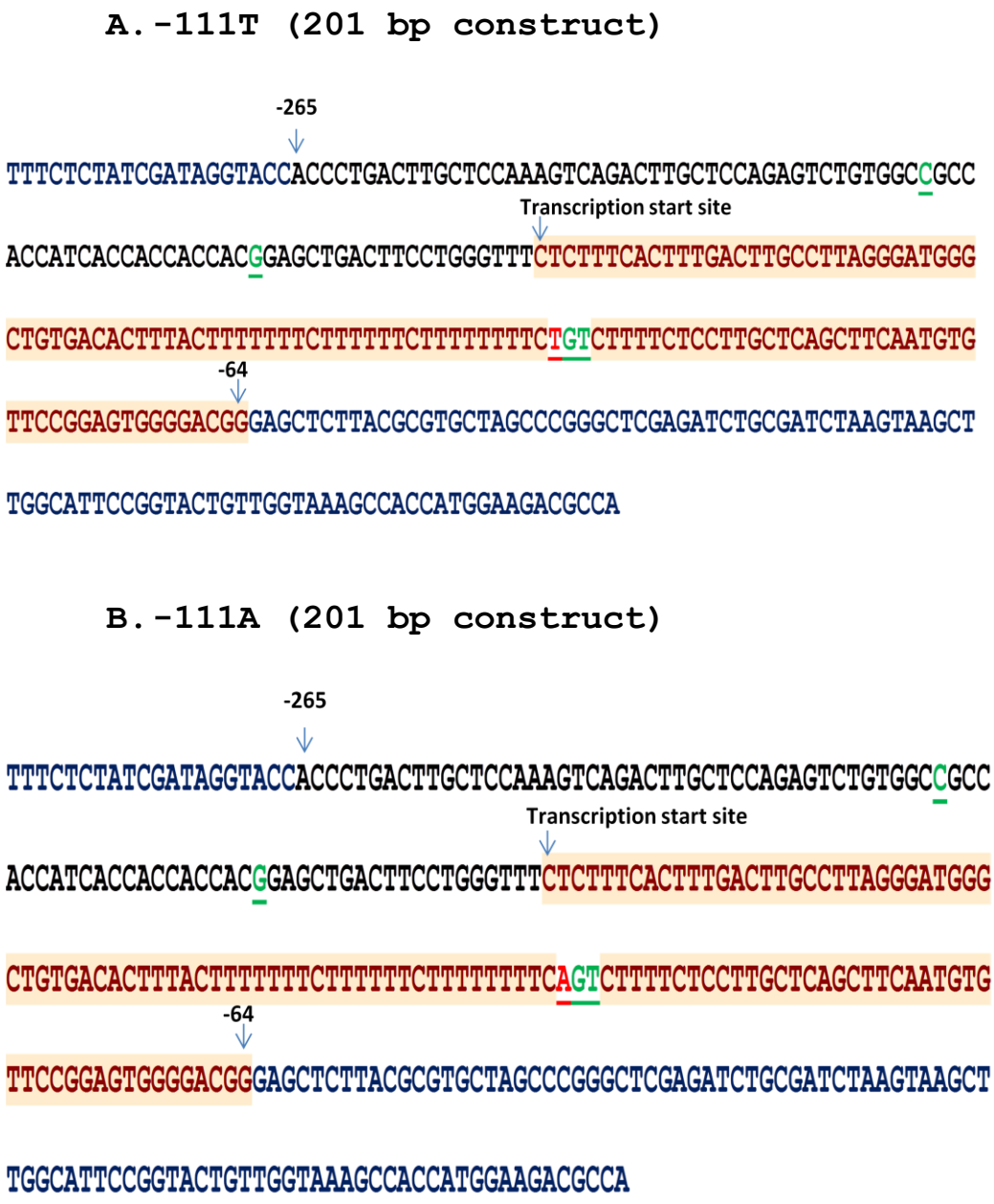


**Figure 6.9**: **Sequences of IL12RB1 -111 promoter inserts (-265/-64, black/orange)-pGL3 basic (blue) with variation, red coloured, only at -111 (A/T).** Other SNPs, green coloured, were found wild-type. The sequence (A) represents *mutant allele* (-111T), (B) indicating *wild* allele (-111A). The transcription start site indicated by an arrow is located at position -180.

### *6.3.7.2 Reporter activity of the 293 bp construct in HepG2 cells*

Relative activity data of the promoter constructs plasmid are presented as a fold increase, represent the mean of three independent experiments ± standard deviation, and compared to the transfected pGL3-basic vector (control) which showed minimal reporter activity.

Functional analysis of IL12RB1 reporter constructs of wild haplotype (-2C/-111A) and mutant haplotype (-2T/-111T) revealed higher luciferase activity of both haplotypes (3.1±0.27 and 2.7±0.22, respectively) than pGL3 basic vector (2.2±0.33), (Figure 6.10). The difference among the 3 groups was highly significant ($P=1.1x10^{-6}$) and the difference in reporter gene activity between reporter haplotypes and empty vector was statistically significant too ($P=6.7x10^{-7}$ for -2C/-111A construct and P=0.0013 for -2T/-111T). In addition, the difference between the two IL12RB1 reporter constructs was also found to be significant (P=0.014) with 13% reduction of reporter activity noted in the mutant haplotype construct (fold change= 1.15).

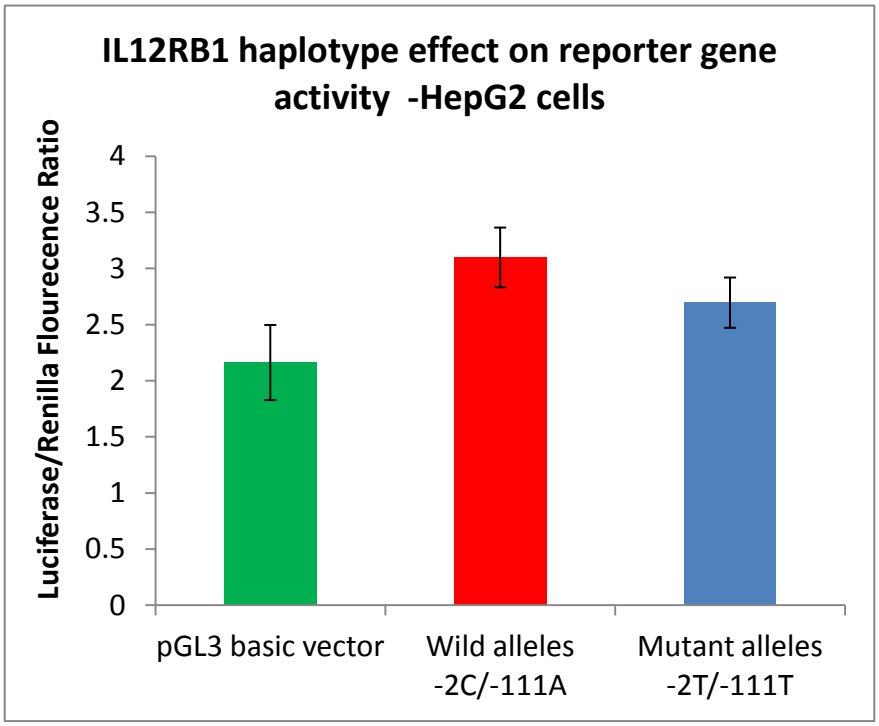


**Figure 6.10**: **Relative luciferase activities of -2T/-111T and -2C/-111A promoter constructs of IL12RB1 gene transfected in HepG2 cells.** The results represent the mean of three independent experiments ± SD.

### *6.3.7.3 Reporter activity of the short construct (201 bp) in HepG2 cells*

Figure 6.11 below is illustrating the IL12RB1 gene promoter activity of the short construct (201 bp) which include only a single polymorphism at position -111. Transfection of this construct in HepG2 cells have shown increased promoter activity of construct carrying wild type (A) allele (3.82±0.43) compared to both mutant (T) allele construct (1.83±0.16) and basic pGL3 vector (2.16±0.33). Highly significant reporter activity difference between the groups was noted ($P=4.9x10^{-12}$). Functional activity of plasmid carrying the wild-type allele (-111A) was doubled with 2.1 fold difference compared to mutant construct and the difference was strongly significant ($P= 5.1x10^{-9}$). On the other hand, the difference between mutant construct and basic vector was not significant. (P=0.107).

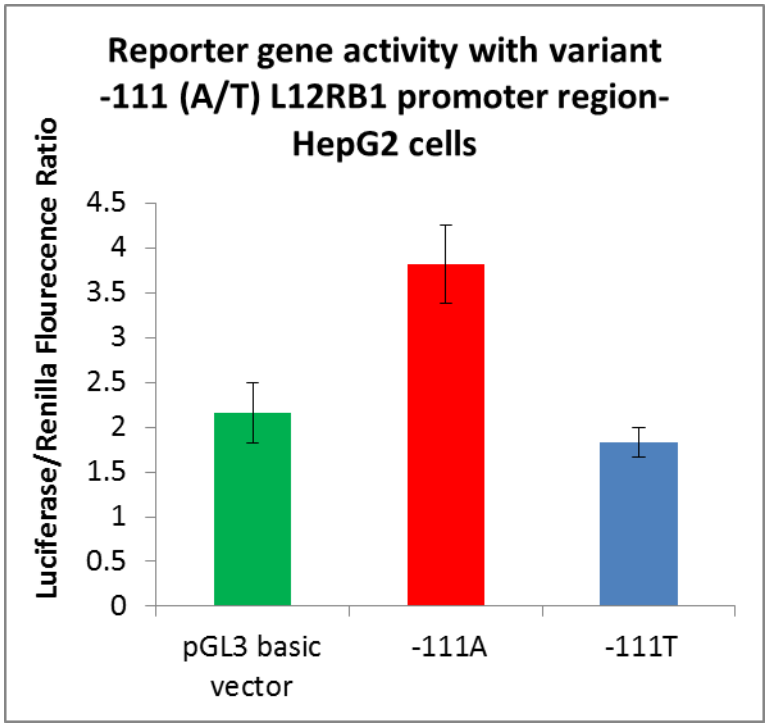


**Figure 6.11**: **Relative luciferase activities of -111T and versus -111A promoter constructs of IL12RB1 gene transfected in HepG2 cells.** The results represent the mean of three independent experiments ± SD.

### *6.3.7.4 Reporter activity of haplotype constructs (293 bp) in Jurkat cells*

Using Jurkat cells as a transfection host provided an overall lower promoter activity of the three tested plasmids compared to HepG2 cells, however, the difference between groups was still significantly high ($P=3.8x10^{-9}$) . As shown in Figure 6.12, activity levels were 2.5± 0.21 for wild-type haplotype -2C/-111A, 2.1± 0.18 for mutant haplotype -2T/-111T and 1.6± 0.17 for control vector. Comparison of functional promoter activity of wild-type and mutant haplotypes versus pGL3 basic control have shown statistical significant differences for both constructs ($P= 7.1x10^{-9}$ for wild-type construct versus basic control and P= 0.00016 for mutant construct versus control plasmid). The activity of -2T/-111T mutant haplotype construct was found 20% lower than wild-type construct -2C/-111A and the difference was also statistically significant (P=0.00013).

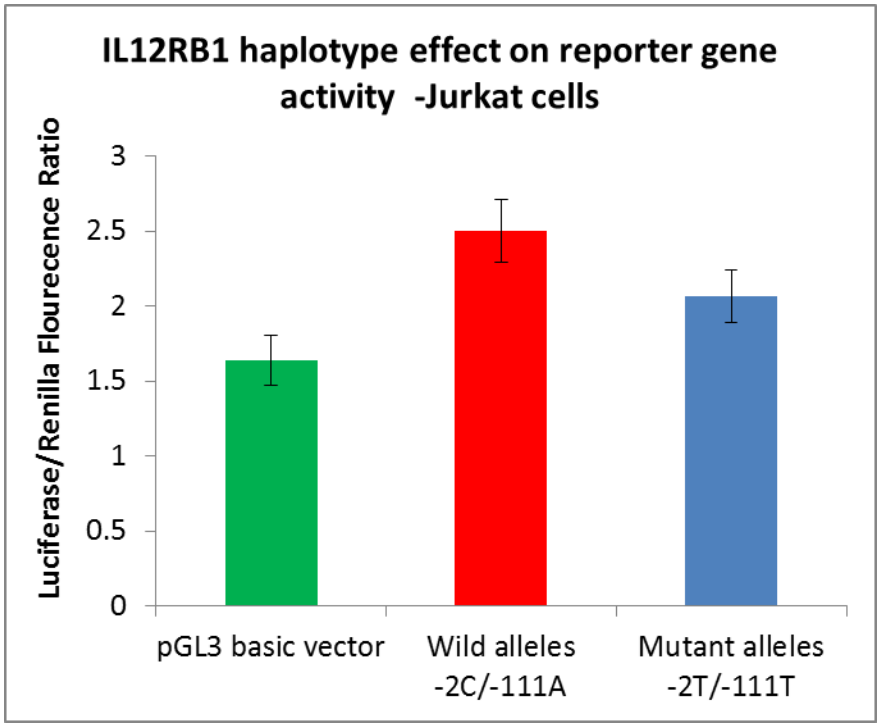


**Figure 6.12**: **Relative luciferase activities of -2T/-111T and -2C/-111A promoter constructs of IL12RB1 gene transfected in Jurkat cells.** The results represent the mean of three independent experiments ± SD.

### *6.3.7.5 Reporter activity of 201 bp construct in Jurkat cells*

Dual luciferase assay performed on harvested cells related to constructs carrying only a single SNP at position -111 has shown considerable significant difference among the 3 tested groups ($P=1.4x10^{-13}$). The reporter activity of mutant construct with allele (T) was 1.21 fold lower than construct carrying wild-type allele (A). This difference in functional activity was statistically significant ($P=2.5x10^{-7}$). However, both wild and mutant reporter constructs have shown higher gene activity (7.22± 0.94 and 4.30± 0.43, respectively) with significant differences ($P=5.1x10^{-9}$ for wild-type (-111A) construct versus control and $P=1.03x10^{-6}$ for -111T construct versus control) when compared to pGL3 basic vector (2.27± 0.29) (Figure 6.13).

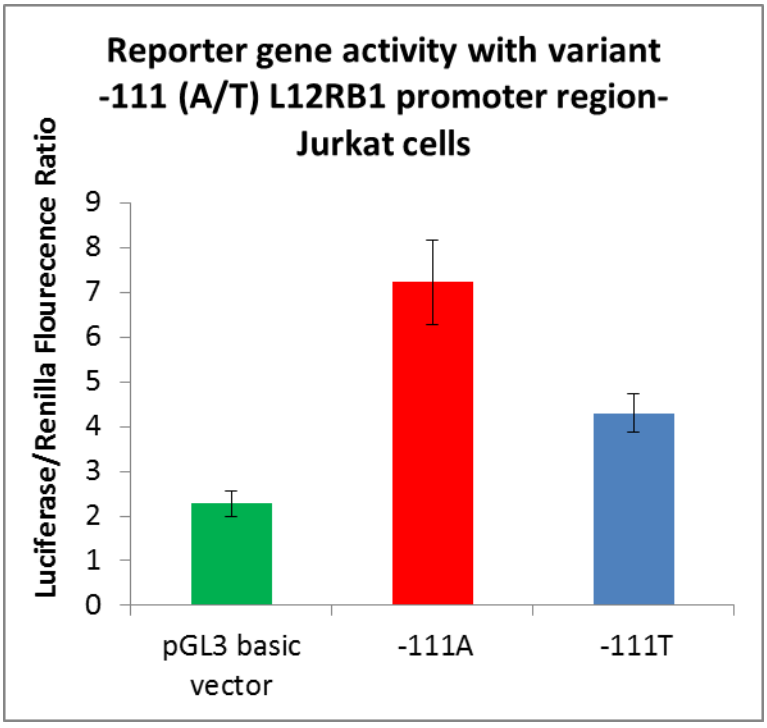


**Figure 6.13**: **Relative luciferase activities of -111T and versus -111A promoter constructs of IL12RB1 gene transfected in Jurkat cells.** The results represent the mean of three independent experiments ± SD.

## 6.4 Discussion

The exome sequencing study conducted by Boston group (Daly, M, Goldstein J and Daly AK, unpublished) on 66 co-amoxiclav DILIGEN cases has helped in identification of a number of novel genetic markers for this form of DILI. Four novel variants were selected for further study on the basis of their location in genes that appeared biologically plausible as risk factors. All the selected variants resulted in nonsynonymous missense mutations that result in amino acid changes. The majority of the most significant variants detected in the exome study were very rare. Three of variants investigated in this project (those in IL12RB1, FMO5 and TPH1 were very rare but 1 common polymorphism (in SHMT1) was also examined. Association with co-amoxiclav hepatotoxicity were confirmed only for the rare variants located in IL12RB1 and TPH1 genes. A possible contribution of the 2 significant variants to flucloxacillin DILI susceptibility were also examined but no association detected which indicates specificity towards co-amoxiclav and possibly two different underlying mechanisms for toxicity. No effect on co-amoxiclav hepatotoxicity could be demonstrated for the FMO5 and SHMT1 variants. It is possible that this could be due either to failure of validating the exome sequencing findings or that the original positive findings were chance observations.

The confirmed association finding for the TPH1 variant with DILI in this project is consistent with the results reported in certain animal studies (Jang et al., 2012; Lesurtel et al., 2006). TPH1 gene is known by its high expression in liver, therefore, mice lacking TPH1 enzyme show a reduced level of liver regeneration compared to healthy species (Lesurtel et al., 2006). In a recent study, 5HT was found involved in reducing the level of bile salts which in turn helped in the protection of mouse liver from cholestatic hepatic toxicity. Mice with TPH1 deficiency show higher levels of bile salts and hepatocellular enzymes three days after bile duct ligation (Jang et al., 2012). This relationship between bile acids and 5HT suggests a role for TPH1 on drug-induced liver injury.

The positive genotyping results obtained with the coding SNP rs117511121 were encouraging to further study its linkage with other promoter variants that could possibly affect the gene transcriptional activity. This project was able to detect a linkage ($r^2$=0.7) between rs117511121 and both promoter polymorphisms located at

positions -2 (rs436857) and -111 (rs393548) which were previously reported as risk factors for certain immunological dysfunctions such as atopic dermatitis, asthma and allergic reactions (Takahashi et al., 2005). Based on these results, we decided to see whether the risk of IL12RB1 on co-amoxiclav DILI susceptibility is related to a particular haplotype rather than its relation with certain multiple independent loci. To investigate this, one more SNP (rs17852635, C/T) located at position +11058 was genotyped and the results have shown a strong association between homozygous mutant (+11058TT) cases and co-amoxiclav hepatotoxicity (P=0.0073). When haplotypes involved the coding SNP (data not shown), the differences in frequency distributions of IL12RB1 haplotypes among cases and controls were statistically significant (P=0.034) with an increased risk of 3.3 times to develop co-amoxiclav DILI in cases carrying the rare mutant haplotype (T-T-T), however, when calculating the risk of coding SNP separately, the risk was found much higher ($P=9.7x10^{-5}$, OR=9.62) than that related to rare haplotype. Furthermore, haplotypes reanalysis involving only the common markers at positions -2 and +11058 with dropping the rare allele (rs117511121) showed lower association (P=0.047) and lower effect of the mutant haplotype (-2T/+11058T) (OR=1.4) compared to wild haplotype (-2C/+11058C). These findings indicate that the tested markers in IL12RB1 are affecting DILI development independently rather than augmenting each other in a particular haplotype and the main effect belongs to the SNP previously detected in the exome study.

In view of the significant association of the IL12RB1 with co-amoxiclav hepatotoxicity, we aimed to confirm the findings of Takahashi et al. (2005) and to extend those findings by looking for novel mutations with possible effects on IL12RB1 gene activity through sequencing of its promoter region, and to examine a new construct that was not studied before. Takahashi et al. (2005) have investigated the effect of -2 and -111 variants separately through studying several constructs located in the promoter region between -2947 and +64. Luciferase dual reporter assay was used and the results indicated that the highest transcriptional activity was related to -265/-65 construct. In addition, a significant decrease in luciferase reporter gene activity ($P<0.01$) associated with the -111T mutant construct was reported.

In our experiment, the functional significance of both promoter polymorphisms (-2 and -111) was tested through measuring the relative transcriptional activity changes among different promoter constructs transfected in two different cell lines. Similar to findings previously reported by Takahashi et al. (2005), the reporter gene activity of the shorter constructs (201 bp, -265/-65) carrying only -111 A/T polymorphism have shown a large decrease in gene transcriptional activity. The level of gene activity reduction reported by Takahashi was 33% while the reductions seen in this project were larger in constructs transfected in Jurkat cells (40%) with reductions also observed when HepG2 cell lines were used (50%). On the other hand, the second construct (-64/+64) used by Takahashi et al (2005) to examine the effect of -2 polymorphism on IL12RB1 transcriptional activity showed limited non-significant influence. The remaining 5 IL12RB1 promoter constructs investigated by Takahashi et al. (2005) were -2947/-65, -1668/-65, -1361/-65, -762/-65 and -104/-65 which were containing -111 variants only. Among them, only 2 constructs (-762/-65 and -104/-65) showed significant lower reporter gene activity in cases carrying the mutant allele -111T compared to the wild –type -111A.

The experiment we performed on the other construct (293 bp, -265/+64) which included -2 and -111 SNPs together to evaluate their combination effect on IL12RB1 transcriptional activity showed significant reduction in promoter activity in patients positive for both SNPs (-2T/-111T) in constructs hosted in Jurkat cells (20%) as well as in HepG2 (13%), however, these reductions were lower than the ones seen in fragments carrying -111 single SNP only. These findings are consistent with the genotyping results of the Japanese study which found that association significancy of IL12RB1 mutant haplotype (-2T/-111T) with atopic dermatitis patients was much less (P=0.035) than significancy obtained when each SNP was tested separately (P=0.0011 for -2T and P=0.00088 for -111T) and similar results were obtained in the case of childhood asthma. This reduction of promoter activity in the longer construct may suggest an existence of a silencer, known as cis-regulatory element, among the sequence -65 to +64 that may intervene with the rate of transcription (Riethoven, 2010). The reporter gene assays used in this project have shown clear evidence that -111 mutant allele could affect IL12RB1 gene function. This finding in addition to the associations obtained in our study between 3 SNPs (rs117511121, rs436857 and rs17852635) in IL12RB1and co-amoxiclav hepatotoxicity, provided a strong

evidence for the involvement of immunological pathway in DILI development. IL12 has a crucial role in regulating T helper cells differentiation and signalling which primarily depends on its binding with both subunits of IL12 receptors, IL12RB1 and IL12RB2 (Chua et al., 1994) (Brightbill et al., 1999). The lack of IL12RB1 expression is known to induce immunodeficiency of patients with an increased risk to develop severe mycobacterial and Salmonella infections (de Jong et al., 1998) (Altare et al., 1998).

# Chapter 7. General Discussion

## 7.1 General discussion

This chapter will summarise and integrate the main findings from the previous chapters of this thesis describing the results and consider what further studies are needed in the area of the pharmacogenetics of drug-induced liver injury.

The work described in this thesis has focused on two antimicrobial drugs, flucloxacillin and co-amoxiclav. These are the most common causes of idiosyncratic liver toxicity in the UK (Andrews and Daly, 2008; Chalasani et al., 2008; Daly et al., 2009; Koek et al., 1994; Lucena et al., 2011; Shin et al., 2013), from recent reports worldwide, co-amoxiclav is a major cause of DILI in many countries (Bjornsson et al., 2013; Hautekeete et al., 1999; Lucena et al., 2011; O'Donohue et al., 2000). Concentrating on common causes of DILI has the advantage that larger numbers of cases can be recruited and studied. However, it appears increasingly that genetic risk factors for DILI are drug-specific and finding common risk factors has been generally unsuccessful. However, one genetic risk factor described in this thesis may extend to more than one drug. In particular, it was possible to replicate the recently reported association of PTPN22 rs2476601 with co-amoxiclav DILI and also demonstrate that it showed an association with flucloxacillin DILI, though this association was not so strong and is less convincing than that seen for co-amoxiclav-related disease. It is possible that this association could extend to other types of DILI that are HLA-associated and further studies on this would be interesting.

Though its function is different to that of the PTPN22 gene product, IL12RB1 also has an important role in T cell responses. In particular, the cytokine interleukin-12 promotes cell-mediated immunity to intracellular pathogens by inducing Th1 T cell responses and interferon-gamma production. This involves the cytokine binding to IL12RB1/IL12RB2 heterodimeric receptor complexes on T cells and natural killer cells. The reason why an apparent decreased function polymorphism in the IL12RB1 gene is linked to an inappropriate T cell response after co-amoxiclav administration is still unclear but it was not possible to show a similar effect in cases of flucloxacillin DILI. One possible explanation might be that IL12RB1 may be more relevant to immune responses involving HLA class II molecules where CD4-positive T cells act as T helper cells. Co-amoxiclav DILI is associated with specific HLA

class I and II alleles but flucloxacillin DILI only shows a class I association which is not associated with CD4-positive T cells but CD8 (Daly et al., 2009). It would be interesting to see if DILI due to other drugs where a HLA class II association has been shown to be important such as for lumiracoxib and lapatinib also show an association with IL12RB1 genotype.

As discussed in detail in Chapter 5 and 6, the positive findings in relation to PTPN22 and IL12RB1 for co-amoxiclav DILI result in a combined odds ratio for disease development in co-amoxiclav cases positive for rs2476601 (PTPN22), rs117511121 (IL12RB1), the class I HLA-A*02:01 and the class II HLA-DRB1*15:01 as high as 17 if an additive model is assumed. This is considerably lower than the odds ratio of 80 seen for HLA B*57:01 for flucloxacillin DILI but it is still progress in understanding susceptibility to this rare disease. Individuals positive for all four risk factors will be very rare though each of the at risk alleles is itself fairly frequent in the population.

The other positive association observed in the current studies was for a rare nonsynonymous variant in the TPH1 (tryptophan hydroxylase) gene with co-amoxiclav DILI only. The basis for this association is much less clear than for the two immune-related genes discussed above. However, the finding that TPH1 knockout mice are more susceptible to severe cholestatic liver injury and death after bile duct ligation (Jang et al., 2012) is of potential relevance to the association. Further studies on humans are needed to explore the relationship of this gene with DILI development to confirm our positive findings.

Attempts to confirm previously reported candidate gene associations for co-amoxiclav DILI were unsuccessful. The inconsistency between our findings and those reported earlier may be due to the current study including considerably more cases than those reported previously so providing better statistical power with less chance of seeing false positive findings but it remains possible that the genes studied might play a role in DILI due to other drugs not included in this study.

The results of the GWAS and exome sequencing studies that were a basis for most of the work described in this thesis have been slightly disappointing. The GWAS provided very clear data showing HLA associations for both co-amoxiclav and

flucloxacillin DILI but not much beyond this, probably because insufficient cases could be studied to see smaller effects. There are a very large number of published GWAS studies relating to many different diseases (see http://www.genome.gov/gwastudies/) but most of these involve use of at least 1000 cases and sometimes up to 100,000 cases have been included. Therefore, ongoing attempts to greatly increase the number of DILI cases available for genetic studies need to continue. International collaboration worldwide will be important. Currently, a further GWAS involving a total of 500 co-amoxiclav DILI cases is being analyzed (A. Daly, personal communication). This includes the cases described in this thesis and will have more than two times as many cases as the previous GWAS on this drug (Lucena et al., 2011). A larger GWAS on flucloxacillin DILI involving 197 cases is also being analyzed currently (Daly A, personal communication). Both these new GWAS will also cover rare exome variants.

This new GWAS data may give interesting new associations but this is not certain. The next step in understanding DILI genetics may need to be the use of whole genome sequencing. The data already obtained from whole exome sequencing has provided some interesting but quite limited information. For complex diseases generally, exome sequencing studies have not extended the findings from previous GWAS very much. For example, a recent exome sequencing study in type 2 diabetes involving 2000 cases failed to detect any significant associations after correction for multiple testing (Lohmueller et al., 2013). DILI is likely to be a complex genetic disease with involvement of a large number of genetic risk factors, similar to type 2 diabetes. Exome sequencing to date appears to be more successful if used for monogenic diseases where single nonsynonymous mutations are often important causes of these diseases. Increasingly, exome sequencing is used in clinical genetics for diagnostic purposes in such diseases (Rabbani et al., 2012).

Moving to whole genome sequencing where non-coding regions are also studied is likely to result in increased sensitivity to detect genes associated with complex diseases such as DILI where variants outside coding regions may make an important contribution to risk. Its use is increasing but most published studies up to the present involve small numbers of cases only. For example, a recent whole genome sequencing study on the rare Mendelian disorder retinitis pigmentosum where

defects in different genes may produce a similar phenotype involved only 16 patients (Nishiguchi et al., 2013). Using small numbers may be due both to the high cost per sample of performing such sequencing and the difficulties of dealing with the large amount of data generated (Puckelwartz et al., 2014).

One area of genetics that has not yet been studied in DILI is epigenetics. It is possible that modification of DNA or histones by processes such as methylation or deacetylation may affect risk of DILI. This would involve a contribution either from environmental factors causing this modification or possibly some modifications could be inherited (Zeybel et al., 2012). Techniques to assess epigenetic modification at the genome level are now available (reviewed Rivera and Ren (2013)) but for DILI it is likely to be important to obtain DNA from liver tissue not white blood cells for these studies and this may not be feasible. Use of white blood cell DNA as a surrogate for the tissue of interest is possible (Li et al., 2010) but it is not certain that this will give reliable results (Mill and Heijmans, 2013).

In summary, the studies described in this thesis have demonstrated the relevance of one genetic risk factor to both flucloxacillin and co-amoxiclav DILI and confirmed another for co-amoxiclav DILI only. The use of larger patient cohorts than previously has enabled demonstration of a lack of association for a number of genetic risk factors suggested previously. It was also possible to confirm that the patient cohorts used in these genetic studies showed a strong similarity in clinical characteristics to those reported previously in epidemiological surveys including the following: the average age of patients at time of DILI incidents is commonly over 60 years, cholestatic and mixed reactions were the most common liver injury patterns, females tended to develop co-amoxiclav DILI at a younger age, males were more susceptible to jaundice, longer courses of flucloxacillin associated with higher risk of DILI mainly in older individuals, the majority of the flucloxacillin DILI cases are female and the co-amoxiclav cases are males.

Overall, the results reported in this thesis make a small contribution to increasing understanding of the genetic basis of DILI. In spite of some progress during the last 5 years approximately, no test suitable for use before prescribing drugs that might cause DILI is currently available. This is different to the situation for some other serious adverse drug reactions, particularly abacavir hypersensitivity where HLA

testing before prescription is now routine and considered cost-effective (Hughes et al., 2004) but also skin rash induced by epileptic drugs (Yip et al., 2012). These are exceptions and for other serious drug-related toxicities such as statin-induced myopathy, cardiotoxicity and renal injury, tests are not available either.

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