Pharmacogenetics of Sulfonamide Hypersensitivity in Humans and Dogs

By

Jennifer M. Reinhart

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Comparative Biomedical Sciences)

at the

UNIVERSITY OF WISCONSIN-MADISON

2017

Date of final oral examination: 8/22/2017

The dissertation is approved by the following members of the Final Oral Committee:

Lauren A. Trepanier, Professor, Medical Sciences
Marulasiddappa Suresh, Professor, Pathobiological Sciences
Christofer A. Bradfield, Professor, Oncology
John P. Svaren, Professor, Comparative Biosciences
Alison Motsinger-Reif, Associate Professor, Statistics, North Carolina State University

Table of Contents

List of Tables

List of Figures

Abstract

Sulfonamide hypersensitivity (HS) is an uncommon but potentially severe drug reaction that affects humans and dogs. In immunocompetent humans, this condition may be heritable, and in dogs, certain breeds appear predisposed. Thus, in both species, risk for sulfonamide HS may have a genetic basis. The goal of this thesis is to understand genetic risk for sulfonamide HS in humans and dogs.

We performed a genome-wide association study (GWAS) in 91 sulfonamide-HS and 184 sulfonamide-tolerant human patients. No associations were identified in the population overall. However, a possible association with a single locus was identified (*COL12A1*) in a subset of patients with severe symptoms. The functional significance of this gene deserves further study. We also investigated the mechanisms of the lymphocyte toxicity assay, an *in vitro* marker for sulfonamide HS in which mononuclear cells of HS patients die more readily when exposed to reactive sulfonamide metabolites. Patients whose cells showed higher cytotoxicity differentially expressed 96 transcripts including a drug transporter (*ABCC5*), a transmembrane protein that mediates cell death (*TMEM123*), and a mitochondrial iron transporter (*SLC25A37*). However, there was no differential transcription between cells of HS vs. tolerant patients and the assay did not differentiate between the two patient groups.

In dogs, we studied a previously identified genetic risk factor in a sulfonamide-detoxifying enzyme gene (*CYB5R3* 729A>G). We found that the variant allele is overrepresented in Doberman Pinschers, a breed with an apparent predisposition to sulfonamide HS. We also

characterized the effects of *CYB5R3* 729G on gene expression and function. Canine livers of the GG genotype showed no differences in mRNA expression or total enzyme activity. It is possible that this variant is linked to other loci that are mechanistically important for sulfonamide HS.

These studies demonstrate the complex nature of risk for sulfonamide HS and highlight the need for accurate clinical diagnosis. Future studies should explore the roles of *ABCC5*, *SLC25A37*, and *TMEM123* in drug transport and cell death during exposure to sulfonamide metabolites *in vitro*. Given strong associations for other idiosyncratic drug reactions, the human and dog leukocyte antigen genes should also be evaluated in sulfonamide HS.

Acknowledgements

First and most importantly, I would like to thank my major professor, Dr. Lauren Trepanier, for her endless support, faith, expertise, and guidance. I've learned so much about research from Lauren – she has taught me that success comes from perseverance, a good sense of humor, and, sometimes, a small piece of chocolate. I would also like to thank my fellow members of the Trepanier lab: Dr. Amos Wong, Ros Luethcke, and particularly Joanne Ekena, for her amazing advice and technical skills. Thanks also to the members of my thesis committee – Drs. Alison Motsinger-Reif, John Svaren, Chris Bradfield, and Marulasiddappa Suresh – whose expertise has greatly elevated the quality of my research. I would like to thank the Comparative Biomedical Sciences training grant committee and the National Institutes of Health for funding my tuition and stipend throughout my graduate studies (T32 OD010423) as well as the National Institutes of Health (R01 GM100784) and American Kennel Club Canine Health Foundation (2017 Clinician-Scientist Fellowship) for research funding. I would also like to thank my residency mentors, Drs. Tom Schermerhorn and Kate KuKanich, who encouraged me to do the PhD in the first place. Finally, thanks to my husband, Mike Dungar, whose love and encouragement got me through the tough spots.

Chapter 1: Introduction

Jennifer M. Reinhart

Sulfonamide antibiotics have a wide range of indications in both human and canine medicine. Unfortunately, their clinical use can be limited by the development of sulfonamide hypersensitivity (HS), an uncommon but potentially severe adverse drug reaction. The occurrence of sulfonamide HS is difficult to predict because these reactions are idiosyncratic and occur at normal dosages. Thus, identifying predictive risk factors for this syndrome is of the upmost importance. In the molecular age of biological research, our ability to detect genetic influence over pharmacologic traits is becoming ever more precise. The fields of pharmacogenetics and pharmacogenomics have identified a host of genetic variants associated with adverse drug reactions including sulfonamide HS. These variants impact the responses of the body to the drug and the pathogenesis of the adverse reaction. Therefore, a better understanding of the mechanisms underlying sulfonamide HS as well as how genetic risk factors modulate these mechanisms will further improve our ability to detect hypersensitivity to sulfonamide antibiotics and prevent this idiosyncratic reaction.

The purpose of this review is to summarize the current understanding of the clinical characteristics, pathogenesis, and risk factors for sulfonamide HS in humans and dogs. It begins with a basic description of the use of sulfonamide antibiotics in clinical practice as well as the potential manifestations of an adverse reaction to these drugs with a focus on delayed-type HS. A discussion of various theories regarding the metabolic and immunologic aspects of the reaction pathogenesis will follow. Risk factors for susceptible populations will then be described

including a known *in vitro* marker of sulfonamide HS, the lymphocyte toxicity assay. Finally, previous studies that have identified genetic variants associated with sulfonamide HS will be critically evaluated, setting the stage for the genetic and mechanistic studies described in the following chapters (**Chapters 2-4**).

Background

Sulfonamide antibiotics are the oldest of the major classes of antibiotics in therapeutic use today and were the first antimicrobial drugs to be used for systemic illness. Synthesized by German physician Paul Gerhard Domagk, the first sulfonamide antibiotic, sulfamidochrysoidine, trade name Prontosil, was introduced in 1935.[1] With its release, mortality from common infections caused by Gram-positive bacteria (particularly *Streptococcus spp*.), such as pneumonia and meningitis, reduced dramatically. Soon after, it was discovered that Prontosil was actually a prodrug and the active form, sulfanilamide, became the progenitor drug for all modern sulfonamide antibiotics including sulfamethoxazole (SMX), sulfadiazine (SDZ), and sulfadimethoxine (SDM).[2]

Today, a wide variety of drug classes contain the sulfonamide moiety ($NHSO_2$) in addition to the antibiotics; these include but are not limited to carbonic anhydrase inhibitors (acetazolamide), thiazide and loop diuretics (hydrochlorothiazide, furosemide), non-steroidal anti-inflammatory drugs (celecoxib), and anticonvulsants (zonisamide). Structurally, the sulfonamide antibiotics can be differentiated from the non-antimicrobial sulfonamide drugs by the presence of an arylamine ring.[3] The sulfonamide scaffold links this ring to an R-group, which

is unique to each antibiotic molecule and modulates the antimicrobial spectrum of the drug (**Figure 1.1**). Non-antibiotic sulfonamides contain the sulfonamide group but lack the arylamine ring. An important exception to this rule is sulfasalazine, an anti-inflammatory drug. Once it reaches the colon, orally administered sulfasalazine is cleaved by bacterial enzymes to yield 5-aminosalicylic acid and sulfapyridine, which contains an arylamine moiety.[4] Thus, from a biochemical and metabolic standpoint, sulfasalazine should be grouped with the sulfonamide antibiotics rather than the non-antibiotics.

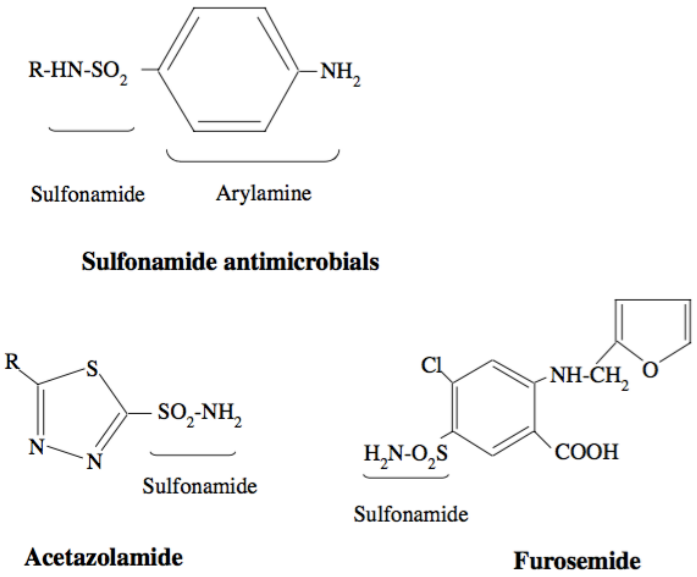

Figure 1.1 – The sulfonamide antibiotic structure containing an arylamine ring, compared to two non-antibiotic sulfonamide drugs. Reproduced from Trepanier 2004.[44]

Sulfonamides exert their antibiotic effect by interfering with folic acid synthesis. Whereas higher organisms can use exogenous sources of folic acid, bacteria are almost entirely dependent on endogenous production for downstream pathways such as nucleic acid synthesis. Thus, the detrimental effects of folic acid deprivation are largely directed at the pathogen, while the host is relatively unaffected. Sulfonamide antibiotics competitively inhibit dihydropteroate synthetase, an enzyme that functions fairly early in folic acid synthesis pathway (**Figure 1.2**). As

a monotherapy, sulfonamide antibiotics are considered bacteriostatic. However, these drugs can be combined with inhibitors of dihydrofolate reductase such as trimethoprim (TMP) or oremetoprim (OMP) in a 5:1 ratio. Sequential blockade of bacterial folic acid synthesis produces a synergistic, bactericidal effect. Most modern sulfonamide products are prepared in a combinatorial form and are known as potentiated sulfonamide antibiotics. These include human formulations such Septra (TMP-SMX), Bactrim (TMP-SMX), and Ditrim (TMP-SDX), and drugs approved for veterinary use: Tribrissen (TMP-SDZ) and Primor (OMP-SDM).[5]

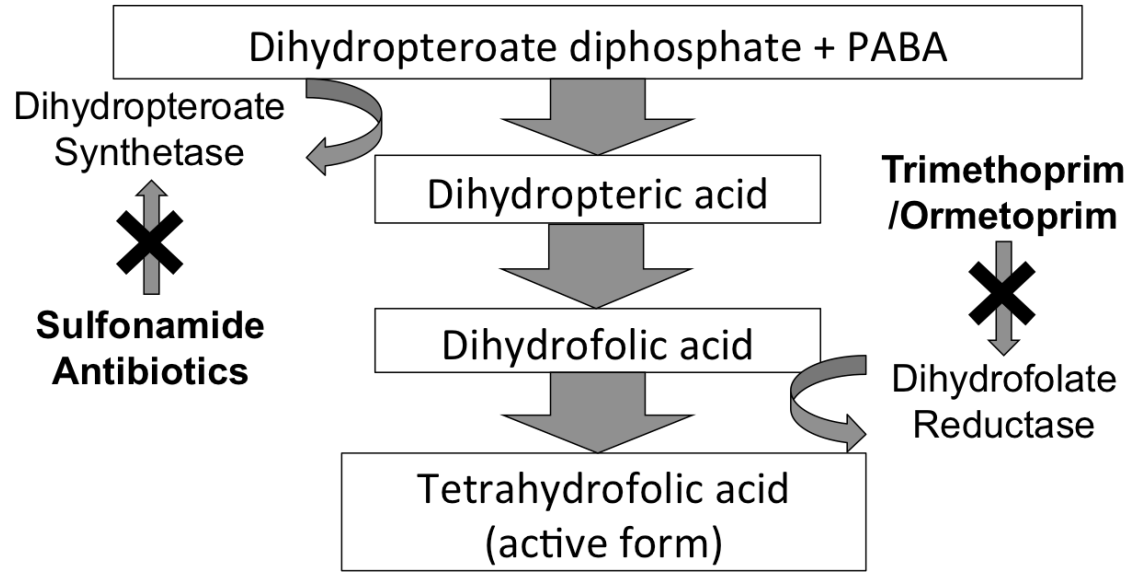

Figure 1.2 – Folic acid synthesis pathway and sites of antibiotic action.

SMX is considered to be the prototypical sulfonamide antibiotic drug and its pharmacokinetics in humans is well established. SMX is rapidly and well absorbed by the oral route with peak blood concentrations occurring 1-4 hours after dosing. The average half-life is 10 hours, making it suitable for twice daily dosing. SMX is moderately protein bound (70%) and distributes well to the ear, respiratory tract and genitourinary tract.[6] SMX concentrates in the urine and the kidney is the primary route of elimination. Approximately 70% is excreted as N_4-

acetyl-SMX and 30% excreted unchanged, although trace levels of other metabolites are detectable.[6]

One of the sulfonamide antibiotic approved for use in dogs in the United States is SDM (Primor [OTM-SDM], Albon [SDM]).[7,8] SDM is well absorbed from the gastrointestinal tract and is widely distributed to various body tissues.[5] In dogs, the drug is moderately protein bound (~75%) with an average half-life of 12 hours.[9-11] As in humans, renal excretion is the major route of elimination for sulfonamides,[5] however, most SDM is excreted unchanged in the urine of dogs.[12] This is because, unlike humans, dogs lack a functional gene for the N-acetyltransferase enzymes (*NAT*) and thus cannot acetylate sulfonamide antibiotic drugs prior to excretion.[13]

Indications for Potentiated Sulfonamide Antibiotics

Microbial susceptibility is relatively similar among potentiated sulfonamide drugs. The spectrum includes Gram-positive isolates such as *Staphylococcus* and *Streptococcus spp.* as well as Gram-negative enterobacteriaceae (e.g. *E. coli, Klebsiella spp., Shigella spp.*).[5,7] Potentiated sulfonamides also have good activity against *Corynebacterium*, *Chlamydiophila*, *Nocardia*, and some anaerobes.[5] Additionally, potentiated sulfonamides have antiprotozoal effects against *Pneumocystis jivoveci*,[14,15] *Toxoplasma gondii*,[16] *Neospora caninum*,[17] and intestinal coccidial organisms.[5] Of note, *Pseudomonas*, *Enterococcus*, and *Bacteriodes spp.* are intrinsically resistant to these drugs and resistance can be acquired by normally susceptible species. Mechanisms of resistance include impaired entry of the drug through the bacterial cell wall and/or drug-resistant dihydropteroate synthetase or dihydrofolate reductase enzyme isoforms.[5] *In vitro* resistance to

combined trimethoprim/sulfonamide preparations apparently develops more slowly than resistance to sulfonamide antibiotics alone.[6,7]

Indications for treatment with potentiated sulfonamide antibiotics are similar between humans and dogs. Sulfonamides concentrate in the urine and are very useful for treatment of urinary tract infections. Short-course, high-dose TMP-SMX (160/800 mg twice daily for 3 days) is considered a first-line therapy for acute bacterial cystitis in women.[18] TMP-SMX is also considered first-line therapy for treatment of uncomplicated canine bacterial cystitis and a similar short-course, high dose protocol has been shown to be effective in female dogs (15 mg/kg twice daily for 3 days).[19,20] Potentiated sulfonamides can also be used to treat prostatitis in male dogs because they are one of only a few antibiotic classes that can penetrate the blood-prostate barrier.[21]

TMP-SMX is indicated for some respiratory tract infections in humans, particularly acute bacterial exacerbations of chronic bronchitis.[22] Although not a first-line drug, TMP-SMX can also be used in certain cases of canine respiratory disease, especially when involving streptococcal species or other susceptible organisms indicated by sensitivity testing.[23] Potentiated sulfonamides are considered a first-line drug for canine superficial bacterial folliculitis,[24] which is an important indication because dermatologic disease is the most common reason antimicrobials are prescribed in small animal medicine.[25] Finally, as the rate of resistance increases to more commonly used antibiotics, such as beta-lactams, potentiated sulfonamides are becoming important alternate therapies, particularly for methicillin-resistant *Staphylococcus*

aureus (humans) and *pseudintermedius* (dogs).[26-28] TMP-SMX has also been used successfully to treat carbapenemase-producing *Klebsiella pneumoniae* in humans.[29]

In both humans and dogs, potentiated sulfonamides are important drugs in the prevention and treatment of opportunistic infections in immunosuppressed patients. TMP-SMX is the drug of choice for prevention of *Pneumocystis jiroveci* pneumonia in human patients with AIDS, cancer, systemic lupus erythematosus, and primary immunodeficiency syndromes as well as post-transplant patients undergoing immunosuppressive therapy.[14,15,30-32] In dogs, prophylactic TMP-SDZ has been shown to reduce morbidity associated with chemotherapy-induced immunosuppression in cancer patients.[33] Potentiated sulfonamides can also be useful in the treatment of infections with atypical organisms such as *Burkholderia spp*. in humans with cystic fibrosis[34] and in dogs on immunosuppressive drugs[35] as well as fast-growing *Mycobacterium spp*. in both species.[36,37]

Adverse Drug Reactions to Sulfonamide Antibiotics

Despite their wide range of applications, use of potentiated sulfonamide antibiotics is limited by the occurrence of adverse drug reactions (ADRs). The ADR with the potential for the most severe consequences is the idiosyncratic, delayed-type hypersensitivity reaction, which is the primary topic of this review. However, other types of ADRs to sulfonamide antibiotics are possible in both humans and dogs. In general, ADRs are divided into dose-dependent (Type A) and idiosyncratic (Type B) reactions. Dose-dependent reactions are relatively predictable and will occur in a large proportion of the dosed population, given a high enough dose. Dose-dependent reactions can be further sub-divided into on-target and off-target effects. On-target

effects are simply an exaggerated manifestation of the intended response to the drug, e.g. hypotension in response to an anti-hypertensive drug. Off-target, dose-dependent reactions affect body systems or processes other than the intended target of the drug, but their incidence is still related to the dose administered, e.g. bone marrow suppression during chemotherapy.[38]

In contrast, idiosyncratic reactions occur independently of the dose given. That is, ***drug exposure* rather than the *drug dose* is the risk factor for the ADR**. This makes idiosyncratic reactions less predictable. The incidence of idiosyncratic reactions is also generally lower than that of dose-dependent reactions; however, idiosyncratic reactions can be more severe in patients that are affected. Most often, idiosyncratic reactions involve a direct or indirect immune response to the drug. These are termed hypersensitivity (HS) reactions or drug allergy and can be sub-divided by the standard Coombs HS classification system (**Table 1.1**).[39] Occasionally, idiosyncratic ADR manifestations occur without immune stimulation, which are termed drug pseudoallergy. A final, essential differentiating feature of idiosyncratic reactions is that, whereas dose-dependent reactions can sometimes be managed with dose reduction, successful treatment of idiosyncratic ADRs mandates immediate cessation of drug therapy.[38]

	Hypersensitivity Reaction Type					
	I	II	III	IV		
	Acute	Cytotoxic	Immune-complex	Delayed		
Subtype				a	b	c
Immune reactant	IgE	IgG	IgG	Th1 cells	Th2 cells	Cytotoxic T cells
Antigen	Soluble	Cell/matrix-associated	Soluble	Soluble	Soluble	Cell-associated
Effector mechanism	Mast cell activation	Opsonization/ phagocytosis	Opsonization/ phagocytosis	Macrophage activation	Eosinophil activation	Cytotoxicity

Table 1.1 – Coombs classification of immune-mediated hypersensitivity reactions.[39]

Dose-Dependent Adverse Drug Reactions to Potentiated Sulfonamides

There are several potential dose-dependent ADRs of sulfonamide antibiotics. As with many antibiotics, gastrointestinal upset may occur in both humans and dogs and headaches are possible in human patients.[40,41] Less commonly, other transient neurologic symptoms such as tremors, disorientation, and gait disturbances are possible.[3,40,42,43] Acute kidney injury secondary to renal tubular necrosis is yet another possible ADR to sulfonamide antibiotics. In the past, this was primarily due to drug precipitation and tubular crystalluria, particularly from SDZ.[3] However, newer sulfonamides such as SMX and particularly SDM have higher solubility in normal urine pH and so are less likely to precipitate and cause tubular obstruction.[3,7] The acetyl metabolites of sulfonamides are more likely to precipitate than the native drugs, so dogs, as poor acetylators, may be at lower risk for crystalluria.[5,44] Dose-dependent, intrinsic, renal dysfunction without crystalluria has also been reported in humans.[3,45] These reactions are presumably caused by direct nephrotoxicity of the drug or drug metabolite and must be differentiated from interstitial nephritis, which can present as a part of idiosyncratic sulfonamide HS.

Although bacteria are much more susceptible to the anti-folate effects of sulfonamide antibiotics, folate deficiency can occur in mammalian patients undergoing therapy, particularly when sequential blockade of folic acid synthesis is present with the use of combination products (e.g. TMP-SMX). In humans, megaloblastic anemia is the most common manifestation, but granulopoeisis and thrombopoeisis can also be affected by folate deficiency leading to neutropenia and thrombocytopenia.[3,46-49] The risk for these ADRs is higher in patients on high-doses of TMP-SMX and those with comorbidities that predispose to folate deficiency.[3,47] Cytopenias associated with folate deficiency also are possible in dogs taking potentiated

sulfonamides, however, the anemias are usually normocytic rather than megaloblastic.[44] In both species, dose-reduction and supplementation with folinic acid (leucovorin) rather than folic acid is recommended since folinic acid does not require dihydrofolate reductase, the target enzyme of trimethoprim, for metabolism to the active form of folate, tetrahydrofolate.[47,50] These dose-dependent effects on the bone marrow may complicate or be confused with the immune-mediated destruction of blood cells and blood progenitor cells that can occur in the sulfonamide hypersensitivity syndrome.

Another potential hematologic sulfonamide ADR that rarely occurs is methemoglobinemia.[42,51,52] The pathogenesis is unclear, but may be explained by a shared common pathway between sulfonamide-hydroxylamine metabolites and methemoglobin, the cytochrome b_5 reductase system. At high doses, the hydroxylamine metabolite may saturate this pathway and prevent reduction of methemoglobin back to hemoglobin, thus diminishing the oxygen-carrying capacity of the patient.[3,51]

Some rare, dose-dependent ADRs of sulfonamide antibiotics may result from shared mechanisms of action with other non-antibiotic sulfonamide drugs. Similar to glipizide and other sulfonylurea drugs, TMP-SMX augments insulin release from pancreatic beta cells[53] and can lead to hypoglycemia in some patients.[40] Both hyponatremia and hyperkalemia have been reported during therapy with sulfonamide antibiotics, which may block the epithelial sodium transporter in the distal tubule, similar to the action of the potassium-sparing diuretic, amiloride.[42,45,54] Early sulfonamide antibiotics were known inhibitors of carbonic anhydrase, which led to the development of a non-antimicrobial drug class with this intended mechanism.[3]

Modern potentiated sulfonamides such as TMP-SMX are much less likely to have this effect, but carbonic anhydrase inhibition may rarely occur, leading to bicarbonate wasting and renal tubular acidosis.[55] Thus far, these biochemical ADRs have not been reported for sulfonamide antibiotics in dogs. However, a case report did describe renal tubular acidosis in a dog associated with zonisamide, a sulfonamide-containing anticonvulsant.[56] Thus, it is possible that other dose-dependent reactions due to structural similarity between sulfonamide antibiotics and other sulfonamide drug classes could occur in canine patients.

Finally, sulfonamide antibiotics have dose-dependent anti-thyroid effects. Sulfonamides reversibly inhibit thyroid peroxidase, a key enzyme in thyroid hormone synthesis.[57] In dogs, potentiated sulfonamides reliably decrease circulating total and free thyroid to subnormal concentrations,[58] and clinical hypothyroidism has been well documented including goitrous disease and hypothyroid crises.[59-62] In contrast, humans appear to be relatively resistant to this particular sulfonamide ADR.[3] In one study, thyroid hormone concentrations in human patients taking sulfonamide antibiotics did show a mild decrease relative to pre-treatment measurements, but remained within the reference range.[63] To date, hypothyroidism due to thyroid peroxidase inhibition by sulfonamide antibiotics has not been reported in humans. However, rarely, idiosyncratic sulfonamide hypersensitivity can lead to lymphoplasmacytic thyroiditis and hormone deficiency.[64]

Idiosyncratic Adverse Drug Reactions to Potentiated Sulfonamides

Clinically, delayed-type reactions are the most important idiosyncratic ADRs to sulfonamide antibiotics. However, there are two other idiosyncratic syndromes that are worth

addressing first. Immediate hypersensitivity reactions to sulfonamides have been reported developing minutes to hours after initial exposure. Symptoms can include pruritus, urticaria, and edema, as well as anaphylactic signs such as dypnea, hypotension, and tachycardia.[65-70] Drug-specific IgE antibodies have been identified in some of these patients confirming a type I hypersensitivity.[71] Although most delayed-type reactions to potentiated sulfonamides are due to the sulfonamide component, rare cases of trimethoprim hypersensitivity have been reported. These have included hepatopathy, aseptic meningitis, and toxic epidermal necrolysis.[3,72,73]

Delayed-type Sulfonamide Hypersensitivity in Humans: Delayed-type idiosyncratic sulfonamide hypersensitivity reactions (which will be referred to as sulfonamide HS, for sake of simplicity) generally occur 5-14 days after initial exposure to the drug, although can present more quickly in cases of re-exposure.[3,74] The manifestations generally follow a cell-mediated or type IV hypersensitivity reaction (e.g. cutaneous and hepatic involvement); however, some signs are more consistent with antibody-mediated disease (e.g. hemolytic anemia, thrombocytopenia, polyarthropathy) so may be considered type II or III hypersensitivity syndromes.

In humans, the most common symptom is a maculopapular rash or fixed drug eruption, which may be accompanied by fever.[3,42,75-77] In a recent investigation in our laboratory including 99 patients with sulfonamide HS identified through medical records search at the Marshfield Clinic, 95% of patients had a rash; 18% had documented fever.[78] More severe, multi-systemic organ dysfunction can also occur.[3] Although these manifestations are less common than a simple rash, they may carry a higher morbidity and risk for mortality and thus are of significant interest.

Rarely, severe dermatologic lesions beyond macropapular rash can occur as a manifestation of sulfonamide HS.[3] These presentations are termed severe cutaneous adverse drug reactions (SCAR) and include Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug rash with eosinophilia and systemic symptoms (DRESS). SJS and TEN represent a spectrum of the same disease characterized by epidermal detachment and necrosis with potential mucocutaneous involvement. Disease with less than 10% of body surface area affected is termed SJS and disease with more than 30% is termed TEN. Patients with 10-30% body surface area affected are said to have SJS-TEN overlap. DRESS, also termed drug-induced hypersensitivity syndrome (DIHS), generally does not feature skin detachment or mucocutaneous lesions. Instead it manifests with internal organ dysfunction, specifically, fever and eosinophilia, but also can include blood dyscrasias, hepatopathy, and other immune-mediated disease.[79] These syndromes are quite rare; a study reported an SJS-TEN incidence of only 5.37 cases per 10 million defined daily drug doses for modern sulfonamide antibiotics.[80] However, among antibiotics, potentiated sulfonamides are one of the most commonly cited causative agents of SCAR, accounting for 6-9% of all cases.[81-83] Importantly, these syndromes carry a high rate of mortality, approximately 30% for TEN.[84] Thus, patients should be monitored for sulfonamide-induced SCAR, regardless of the low incidence.

Drug-induced liver injury (DILI) has been described as a delayed reaction in patients administered TMP-SMX or other potentiated sulfonamide antibiotics.[3,74,76,85] Most commonly, sulfonamide-associated DILI presents as asymptomatic hepatocellular injury, evidenced by elevated serum transaminase activities, but cholestatic change with alkaline phosphatase elevation and hyperbilirubinemia may also be present.[74,85] Rarely, life-threatening hepatic failure

can occur.[74,85,86] Biopsy usually reveals acute hepatic necrosis with surrounding mononuclear and eosinophilic infiltrates, although neutrophilic inflammation and evidence of cholestasis (e.g. bile casts) have also been described.[74]

A variety of blood dyscrasias have been described as ADRs to sulfonamide antibiotics. As previously discussed, dose-dependent cytopenias can result in drug-induced folate deficiency.[47] However, immunogenic destruction of both circulating cells and precursors of all three hematopoietic cell lines can also occur as a part of the idiosyncratic sulfonamide HS. Leukopenia, primarily characterized by neutropenia, accounts for about 40% of the blood dyscrasias in humans associated with sulfonamide antibiotics.[76,87] Severity may range from mild, subclinical neutropenia to agranulocytosis with high risk for sepsis and death.[47,87] Leukopenia associated with HS syndromes is thought to be due to peripheral destruction of neutrophils, although suppression of granulopoeisis in the bone marrow may also occur.[47] Thrombocytopenia is also a reported component of sulfonamide HS. In our recent study in humans, thrombocytopenia was the most common blood dyscrasia identified, affecting 11% of HS (vs. 5% and 4% for neutropenia and anemia, respectively)[77] and TMP-SMX is the most common cause of drug-induced thrombocytopenia in children.[88] Similar to neutropenia, peripheral immune-mediated destruction is thought to be the primary mechanism of sulfonamide-associated thrombocytopenia with some patients developing drug-dependent platelet-reactive antibodies.[3,47,88] Red blood cells appear to be the cell line least commonly affected by sulfonamide HS.[47,77,87] However, both marrow-directed aplastic anemia and peripheral immune-mediated hemolytic anemia have both been reported.[47,87,89] In fact, TMP-SMX use has been identified as a general risk factor for immune-mediated hemolytic anemia in human patients.[89]

These drug-induced cytopenias can occur singularly or in combination with higher risk for mortality in those patients with bi- and tricytopenias.[87] One final blood cell abnormality that should be mentioned is eosinophilia. Eosinophilia is actually the most common hematologic change documented in sulfonamide HS and often manifests alongside rash and fever as a part of the DRESS syndrome.[3]

Rarely, sulfonamide HS manifests as disease in other organ systems of human patients. For example, serum sickness and polyarthritis have been reported in both children and adults taking potentiated sulfonamide antibiotics.[90,91] Additionally, TMP-SMX is one of the leading causes of drug-induced aseptic meningitis.[92] This condition can be particularly difficult to diagnose since many patients present with a fever, but no rash, along with a wide range of neurologic signs ranging from head and neck ache to hypotension and seizures.[93] Interstitial nephritis leading to acute kidney injury may also present as a part of the HS syndrome.[3] However, compared to acute kidney injury from dose-dependent, intrinsic renal insufficiency, these cases are quite rare.[94] Other reported components of the sulfonamide HS syndrome include thyroiditis,[64] pancreatitis,[95] uveitis,[96] myocarditis,[97] and pneumonitis.[3,98]

Sulfonamide Hypersensitivity in Dogs: Aside from humans, dogs are the most commonly reported species to exhibit delayed-type idiosyncratic sulfonamide HS[44] and, therefore, are of interest from a comparative medicine as well as a veterinary point of view. Similar to humans, canine sulfonamide HS is a relatively rare event. The true incidence in dogs is unknown, but one study in dermatology patients reported an estimated incidence of 0.25%.[99] The timeframe is also similar with clinical signs occurring a minimum of 5 days and an average of 12 days after

initiation of antimicrobial therapy.[100] Although cutaneous and mucocutaneous lesions can occur, systemic manifestations predominate in canine sulfonamide HS. In one study, fever and thrombocytopenia were the most common clinical signs, affecting 55% and 54% of dogs, respectively.[100] Thrombocytopenia and other blood dyscrasias including neutropenia, aplastic anemia, and Coombs-positive hemolytic anemia have also been identified by investigators.[100-103] A particularly severe manifestation of sulfonamide HS in dogs is hepatopathy. This is generally characterized by increases in hepatocellular enzyme activities (ALT, AST), but cholestatic involvement (increased ALP activity, hyperbilirubinemia) is not uncommon.[100,104] Synthetic liver failure such as hypoglycemia and coagulopathies are also possible.[104] The primary histopathologic finding is acute hepatic necrosis, but cholestasis and lymphoplasmacytic infiltrates have been reported.[100,104]

Polyarthropathy is another manifestation of sulfonamide HS, which appears to be more common in dogs than in humans. Distal joints tend to be most commonly affected and fever often accompanies signs of joint swelling and pain.[102,105] Joint fluid analysis usually reveals neutrophilic non-septic inflammation.[100,102] Interestingly, sulfonamide-associated polyarthritis affects large breed dogs much more commonly than small breed dogs.[44] Doberman Pinschers in particular are overrepresented.[102,106-108] It is unclear why this breed is affected so, but this predisposition could reflect antibiotic prescribing patterns or represent altered physiomechanical forces on the joints of large dogs. Alternately, a hereditary risk could be present. Other reported manifestations of sulfonamide HS in dogs include lymphadenopathy, uveitis, retinitis, proteinuria, edema, pneumonitis, pancreatitis, and neurologic complications.[100,102]

One final ADR to sulfonamide antibiotics in dogs deserves special mention. Keratoconjunctivitis sicca (KCS) or "dry eye" is an inflammatory condition of the ocular surface usually caused by pathology of the lacrimal gland and decreased tear production.[109] In contrast to other sulfonamide-related morbidities, KCS is a fairly common occurrence in dogs, with a reported incidence of 15% in one study.[110] Sulfonamide-associated KCS also appears to be more common in small breeds[110] and is delayed in onset relative to other manifestations, occurring months after initiation of antimicrobial therapy.[111] There is also evidence that some sulfonamide antibiotics may be directly toxic to the lacrimal gland mediated by a pyrimidine or pyridine ring in the R-group structure.[112,113] In these ways, sulfonamide-associated KCS may be a separate reaction with a distinct pathogenesis, rather than a part of the classic idiosyncratic hypersensitivity. However, KCS can occur in combination with other classic signs of sulfonamide HS,[100] so the classification of this ADR remains unclear.

Diagnosis, Treatment, Outcome, and Prevention of Recurrence: Diagnosis of sulfonamide HS is primarily based on the clinical course of the disease. This may be challenging since clinical signs can overlap with many conditions including other immune-mediated diseases, ADRs to other drugs the patient may be taking, or even the infection for which the potentiated sulfonamide was prescribed. Appropriate timing of clinical signs relative to initiation of antimicrobial therapy is key to diagnosis. In a patient naïve to sulfonamide antibiotics, clinical signs of the HS reaction generally do not occur less than 5 days after starting the drug.[3] However, patients who have been previously exposed and sensitized to sulfonamides may exhibit symptoms more quickly.[74] Time to recovery is also an important component to diagnosis. In patients with mild reactions (e.g. rash +/- fever), symptoms usually improve within a few days after withdrawal of the drug. This can

be interpreted as supportive evidence for the drug as a causative agent. However, more severe manifestations may require additional time or therapies to resolve, so time to recovery may not be useful as a diagnostic criterion in these cases. In some reports, testing for immunologic involvement (e.g. drug-specific antibodies or T cells, skin patch testing) has provided supplementary evidence to support a diagnosis of sulfonamide HS.[114-118] These results must be interpreted with caution, because patients who are clinically tolerant to sulfonamide antibiotics can also have positive results on some of these tests.[118,119] In the end, **the clinician must *weigh the evidence* in support of or against a diagnosis of sulfonamide HS and proceed according to their best judgment**.

For research purposes, diagnostic criteria have been codified into adverse drug reaction scoring systems to assist investigators when dealing with large populations of patients.[120] After resolution of clinical signs, the diagnosis of a HS reaction may be confirmed with a drug rechallenge and the patient observed for recurrence. Rechallenge trials have been performed with success in both human and canine patients.[102,121,122] These studies may be particularly useful when there is a high likelihood that the potentiated sulfonamide will be needed again and antibiotic choices are limited, as in a patient with a resistant bacterial infection or in an immunosuppressed patient in need of long-term prophylaxis. Because of the possibility of recurrence of severe clinical signs, rechallenge trials should only be attempted in patients with mild symptoms and the benefits and risks must be weighed carefully.

In all cases, the first step in treatment of sulfonamide HS is withdrawal of the offending drug. As with any idiosyncratic drug reaction, dose-reduction is inappropriate and will prolong

the course of disease. The remainder of treatment is based on disease severity and which clinical manifestations are present. For example, a maculopapular rash usually resolves within a few days of cessation of sulfonamide therapy without any other intervention. In contrast, patients with SJS/TEN require intensive management, often in burn wards, to maintain adequate fluid balance and prevent secondary infections.[79] Patients with drug-induced liver injury may benefit from antioxidant therapy and other supportive care, whereas those with polyarthropathy will likely require analgesics. Of note, folinic acid supplementation may be considered when treating patients with sulfonamide-associated blood dyscrasias because of the possible involvement of dose-dependent anti-folate effects of the drug.[50] In some cases, immunomodulatory therapy has proposed because of the immunologic nature of hypersensitivity reactions. Such treatments may include glucocorticoids, secondary immunosuppressants (e.g. cyclosporine), and human intravenous immunoglobulin (hIVIG).[79]

Similar to treatment of this reaction, outcome of sulfonamide HS appears to be dependent on clinical presentation and the severity of disease. For example, in one study, human patients with sulfonamide-induced blood dyscrasias had an overall mortality rate of 17%, but this increased to 52% in patients with pancytopenias.[87] In contrast, in humans treated with TMP-SMX for *Pneumocystis* pneumonia, mortality rate was not significantly different between patients with sulfonamide-associated DILI and those without.[85] This suggests that the hepatotoxicity component of sulfonamide HS may not influence outcome in most patients. SJS/TEN is the most severe manifestation of sulfonamide HS and carries an overall mortality rate of approximately 30%.[84] Scoring systems have been developed for SJS/TEN that identify additional risk factors for poor outcome including age, heart rate, presence of malignancy,

biochemical values, and total body surface area affected.[84] In dogs, the overall mortality rate for sulfonamide HS has been reported as 21%.[100] Both hepatopathy and thrombocytopenia have been identified as negative prognostic indicators for survival.[100]

For most patients, recurrence of sulfonamide HS can be prevented by avoiding sulfonamide antibiotics in the future. However, for some, TMP-SMX is an important component of long-term therapy, in particular, HIV-positive patients undergoing primary or secondary prophylaxis for *Pneumocystis jiroveci* infection. To address this need, desensitization protocols have been developed in an attempt to produce immune-tolerance to the drug.[123-126] Specific protocols vary widely and have described in both HIV-positive[123,124 126] and HIV-negative patients.[127,128] All protocols involved increasing doses of TMP-SMX over several steps with dosing intervals that range from 15 minutes to daily; total desensitization time ranges from a few hours to two weeks.[123-125,128] Both inpatient and outpatient procedures have been described; route of administration may be injectable, oral, or a combination of the two.[123,125,128] Some protocols include rechallenge with TMP alone prior to desensitization, to ensure the ADR was due to the sulfonamide component of the drug.[124] Overall success rates for desensitization protocols have been reported as 70-90%[123-125,128,129] but, given the difficulty in obtaining a definitive diagnosis of sulfonamide HS and the possibility of false positives, these results must be interpreted with caution. Nevertheless, metaanalysis of these studies do support the use of desensitization with dose escalation rather than rechallenge of HS patients beginning at full therapeutic doses.[130]

Proposed Pathogenesis of Sulfonamide HS

Although the pathogenesis of sulfonamide HS is incompletely understood, investigations over the past 30 years have uncovered much about this process. Development of sulfonamide HS requires multiple metabolic and immunologic steps, culminating in an immune-mediated attack against target tissue, leading to clinical signs and patient morbidity.[3] Immune involvement is indirectly evidenced by the delayed time to manifestation of clinical signs (5 day minimum), which represents a period of sensitization of the immune system to the drug as antigen.[3,74] Similarly, the more rapid onset of symptoms sometimes seen on drug re-exposure likely represents reactivation of immunologic memory.[74] In addition to this clinical evidence, a significant body of *in vivo* and *in vitro* work exists that supports the central role of the immune system in sulfonamide HS.

Immunopathogenesis

Several models have been proposed to explain the immune mechanisms underlying drug hypersensitivities.[131] Evidence regarding the pathogenesis of sulfonamide HS best fits the pro-hapten-carrier model, although other mechanisms may further augment the immune response. According to the pro-hapten-carrier model, most drugs are too small to stimulate immune cells on their own (pro-haptens), but may be converted to reactive metabolites (haptens) by metabolic enzymes and covalently bind endogenous proteins to form drug-protein adducts. These adducts are processed and presented on major histocompatibility complex (MHC) molecules by antigen presenting cells (APCs) as part of normal immunosurveillance. However, because drug binding changes the chemical structure and antigenicity of endogenous protein, T cells can recognize the novel antigen as foreign, become activated, and elicit an immune response.[132]

Most requisite steps to stimulate an immune response via the pro-hapten-carrier model have been demonstrated for sulfonamide antibiotics. Naturally occurring drug-protein adducts have been detected in both humans[133] and dogs[119] exposed to sulfonamide antibiotics. Adduct formation is metabolism-dependent and the nitroso form of the drug (e.g. sulfamethoxazole-nitroso, SMX-NO) is the proximate metabolite.[134,135] By itself, SMX-NO is quite unstable,[136] but in a biological system, SMX-NO binds cysteine residues on a variety of intracellular,[137,138] cell-surface,[139,140] and plasma proteins.[119,133] The ability of APCs to internalize these SMX-drug adducts has been demonstrated for both soluble[139] and membrane-bound[134] antigen. Dendritic cells stimulated with SMX-NO upregulate CD40, a marker of dendritic cell maturation.[141] Furthermore, the immune response to SMX-NO is apparently MHC-restricted and can be invoked both MHC-I and MHC-II molecules.[136,139,141] Taken together, these data suggest that processing and presentation of drug-adducts as antigen is requisite for effector cell activation, although other theories for this interaction have been proposed (see "p-i concept" below).[131] Interestingly, patients who are tolerant to SMX may generate drug-protein adducts without displaying clinical signs,[119] which provides further evidence that downstream immune activation is requisite for the drug reaction.

The classic presentation of sulfonamide HS is that of a type IV hypersensitivity reaction and, as such, the primary effector cells are T cells. Multiple investigators have identified drug-specific T cells in dermal lesions and peripheral circulation of HS patients.[115,142-146] Both $CD4^+$ and $CD8^+$ T cells have been isolated,[115,143-146] however, the type of skin reaction may be determined by the predominant T cell phenotype, with a $CD4^+$ phenotype associated with

maculopapular reactions and a $CD8^+$ phenotype associated with severe bullous reactions.[144,145,147] When stimulated with the drug, SMX-specific T cells show an activation pattern with down-regulation of the T cell receptor[114] and increased RANTES production,[148] as well as secretion of both Th1 and Th2 cytokines.[115,142-146] Of those lesions predominated by cytotoxic T cells, perforin/granulozyme activity, rather than the fas/fas-ligand pathway, appears to mediate cell death.[115,144,145]

Drug-specific antibodies have also been identified in humans[133,149] and dogs[119] with sulfonamide HS. Patients who are tolerant to sulfonamide antibiotics are capable of antibody generation,[116,119,140] so it is possible that this is a clinically irrelevant epiphenomenon. Alternatively, antibodies may play a role in disease pathogenesis in clinical patients. For example, drug-specific antiplatelet antibodies have been documented in children with TMP-SMX-associated thrombocytopenia.[150] These antibodies are more likely to be of importance in patients with symptoms consistent with a Type II (cytopenias) or Type III (polyarthropathy, glomerulopathy, retinitis) HS reaction. However, SMX-stimulated T cells can secrete a cytokine profile consistent with a Th2 phenotype (e.g. IL-4, IL-5, IL-10), which stimulates humoral immunity.[142,151] This can be classified as a Type IVb HS reaction.[147] In particular, IL-5 secretion seems particularly prominent, which may help explain the eosinophilia commonly reported in patients with sulfonamide HS.[3]

Although the pro-hapten-carrier model is the most widely accepted explanation for the stimulation of an immune response by sulfonamide antibiotics, other processes may contribute to this interaction.[131] Not only do SMX drug-adducts provide a novel antigen for presentation by

APCs, SMX metabolites can also directly increase the production CD40 by dendritic cells.[141] Similarly, bystander cells previously exposed to SMX can elicit surface-expression of both MHC-II and co-stimulatory factors (CD40, CD80, CD86).[131] In this way, SMX and its metabolites act as a "danger signal" that further enhances APC function, independent of drug-adducts acting as antigen. Another theory that does not require antigen processing and presentation of SMX-adducted proteins is the pharmacologic interaction hypothesis or "p-i concept."[132] This theory is based on the observations that some human SMX-specific T cells, once generated, can respond to SMX alone without metabolism to SMX-NO and adduct formation.[142,152] Furthermore, these T cells can respond to SMX-NO directly when APC antigen processing has been chemically blocked.[142,152] Rather than presentation on an MHC molecule covalently bound to a peptide fragment, the drug interacts directly with the T cell receptor in the variable regions.[131,152,153] The drug may also interact non-covalently with the MHC molecule or the peptide it is presenting, which allows the formation of an immunologic synapse and co-stimulation of the T cell.[132] *In silico* modeling has been used to identify possible binding site for SMX on these molecules,[153] but most biological evidence for the "p-i concept" thus far is indirect.

Sulfonamide Biotransformation

In the pro-hapten-carrier model, the parent drug (SMX) must be converted to the proximate metabolite, SMX-NO. This occurs in a two-step process: (1) hydroxylation of SMX to the hydroxylamine metabolite (SMX-HA) and (2) oxidation of SMX-HA to its nitroso derivative (SMX-NO; **Figure 1.3**). SMX-HA can be generated in the liver by CYP2C8 and CYP2C9,[141,154,155] and subsequently delivered to target tissues via circulation. Importantly,

hydroxylation occurs on the amino group of the arylamine ring present in sulfonamide antibiotics, but not other sulfonamide drugs. This explains the lack of cross-reactivity between sulfonamide antibiotics and non-antibiotic drugs as well as the failure of non-antibiotic sulfonamides to elicit a sulfonamide HS reaction.[3,44,156] SMX may also be converted to SMX-HA by myeloperoxidase in dendritic cells.[141] Local metabolite generation may help explain the predominance of cutaneous ADRs in sulfonamide HS, given the large number of dendritic cells present in skin.

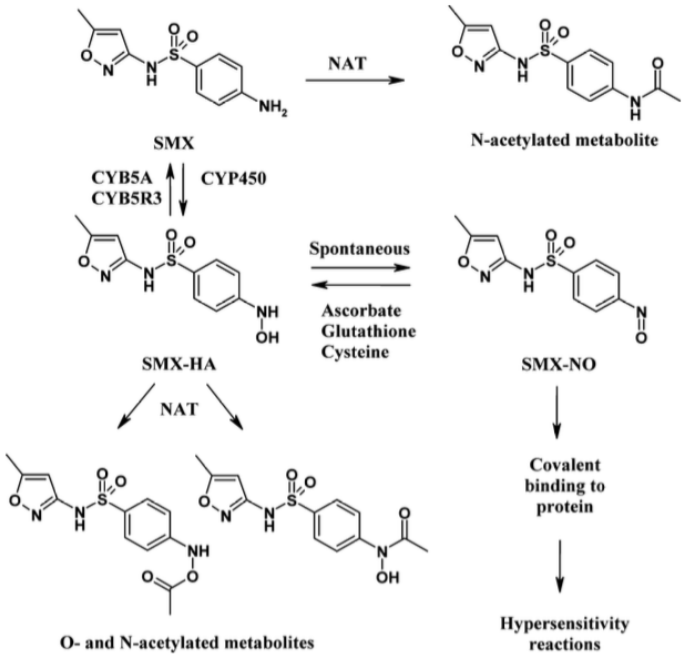

Figure 1.3 – Proposed schema for bioactivation of sulfonamide antibiotics, represented by sulfamethoxazole (SMX) to the reactive, nitroso metabolite (SMX-NO). SMX-NO adducts endogenous protein to form a novel antigen, which can elicit an immune response. Reproduced from Funk-Keenan et al 2012.[157]

The hydroxylamine is a minor sulfonamide metabolite with only ~2% of SMX administered excreted as SMX-HA in the urine.[158-160] However, urinary recovery may underestimate the total amount of SMX-HA produced because SMX-HA can undergo spontaneous oxidation to SMX-NO.[161,162] SMX-NO itself is difficult to quantify because it is unstable in solution, with alternate metabolites appearing within minutes of dissolution. These

may include nitro-SMX, homodimers, reduction back to SMX-HA, or haptenization with endogenous protein.[136]

Detoxification of Sulfonamide Metabolites

Not all patients who are exposed to sulfonamide antibiotics develop drug-protein adducts.[119] Additionally, most of the drug is excreted in the urine in a form other than SMX-HA or SMX-NO.[10-12,158-160] These findings suggest the presence of other metabolic pathways that subvert or reverse the SMX to SMX-HA to SMX-NO route that contributes to the pathogenesis of sulfonamide HS.

In humans, the major urinary metabolite of SMX is N_4-acetyl-SMX, accounting for ~70% of all drug excreted in the urine.[6] SMX conversion to N_4-acetyl-SMX is catalyzed by the *N*-acetyltransferases (NATs), which are phase II detoxification enzymes (**Figure 1.3**). NAT covalently binds an acetyl group to the arylamine moiety of SMX, preventing direct hydroxylation by CYP2C8/9 and facilitating excretion by active tubular secretion. NAT also acetylates the arylhydroxylamine group on SMX-HA to form *N*-acectoxy-SMX and prevent further oxidation to SMX-NO.[3] In humans, there are two isoforms of the N-acetyltransferase enzyme: NAT1 and NAT2 encoded for by the *NAT1* and *NAT2* genes, respectively. SMX is a substrate for both isoforms, but tissue distribution of these enzymes varies substantially.[3,163] In humans, NAT1 is expressed in a wide range of tissues including liver, lung, digestive and urinary tracts, mammary tissue, and, interestingly, leukocytes. In contrast, NAT2 expression is limited to liver and gut, suggesting a more specific role in xenobiotic detoxification.[163,164] This differential

isoform distribution may have implications for SMX metabolite generation and target tissues in sulfonamide HS.

The hydroxylation of SMX to SMX-HA by cytochrome P450 enzymes is the first step in generation of reactive metabolites in sulfonamide HS. However, this process can be reversed through SMX-HA reduction by another important detoxification enzyme pathway: cytochrome b_5 (cyt b_5) and cytochrome b_5 reductase (**Figure 1.3**).[165,166] SMX-HA reduction by this system is an NADH-dependent process:[165] the FAD-containing reductase enzyme facilitates electron transfer to cyt b_5, which acts as an electron carrier to SMX-HA, yielding the less reactive parent compound.[167] SMX may go on to be acetylated by NAT or excreted directly by the kidney. Cyt b_5 and cyt b_5 reductase, encoded for by *CYB5A* and *CYB5R3*, respectively, are primarily expressed as microsomal enzymes in the liver,[166] although some mitochondrial SMX-HA reduction activity may also occur.[168] Activity is significantly reduced with removal of either cyt b_5 and cyt b_5 reductase, suggesting that both are requisite for SMX-HA reduction; optimal stoichiometry of the components has been determined.[166] In the liver, these proteins are membrane-bound, but soluble form of both are expressed in red blood cells via alternate splicing.[169] In the red cell, their primary function is to maintain hemoglobin in a reduced state; however, the contribution of soluble cyt b_5 and cyt b_5 reductase, also known as methemoglobin reductase, to total body SMX-HA reduction capacity is unknown.

The spontaneous degradation of SMX-HA to SMX-NO occurs in oxidizing conditions, and redox cycling between these intermediates may actually exacerbate oxidation status of the environment.[170] This process can be reversed in the presence of antioxidants. Both glutathione

and cysteine have been shown to mediate reduction SMX-NO, which may be reduced to SMX-HA or back to the parent SMX compound via a sulfinamide intermediate.[161,171] Glutathione, cysteine, and ascorbate can also prevent SMX-HA auto-oxidation to SMX-NO.[154,161,165,171,172] In clinical patients, antioxidant deficiency leads to impaired SMX-NO reduction[173,174] and may be associated with sulfonamide HS.[175] However, prophylactic supplementation with the glutathione precursor N-acetylcysteine has not been shown to be effective in reducing the risk of sulfonamide HS.[176]

Species Differences in Sulfonamide HS Pathogenesis

In dogs, much of sulfonamide biotransformation is known or thought to be similar to that in humans. For example, SMX-HA generation has been documented in canine microsomes.[107] However, a few differences do exist. In humans, the primary urinary metabolite is N_4-acetyl-SMX whereas, in dogs, most of the drug is excreted unchanged.[12] This is because dogs and other canid species lack *NAT1* and *NAT2* genes for *N*-acetyltransferase and, therefore, do not express the enzymes responsible for generation of the acetylated metabolite.[13] This detoxification deficiency may predispose dogs, as a species, to sulfonamide HS and help explain why they are the only naturally-occurring, non-human model of this ADR.[44] Interestingly, SMX-HA reduction activity, catalyzed by the cyt b_5/cyt b_5 reductase system, is ~4x higher in dogs vs. humans.[165] This may represent compensation of another detoxification pathway for the *N*-acetylation deficit in this species.

Population Risk Factors for Sulfonamide HS

Idiosyncratic drug reactions are inherently difficult to predict. Without the risk factor of dose, it is harder for clinicians to anticipate the occurrence of the ADR and implement appropriate monitoring or potential preventative measures in individual patients. Thus, identifying risk factors for idiosyncratic ADRs is an important area of pharmacologic research. In general, risk can be classified as heritable (genetic) factors or as acquired (environmental) factors. Epigenetics can also affect the response of an individual to a drug and may be considered a distinct set of risk factors since epigenetic regulation of expression is initially inherited, but is altered throughout life in response to various environmental pressures.

In humans, a major risk factor for sulfonamide HS is immunosuppression. In immunosuppressed patients, particularly those with HIV, the incidence of sulfonamide HS has been reported as 20-57%.[32,76,176,177] This is in contrast to the general, immunocompetent population where the incidence is only ~3%.[75] Because of this large difference, immunosuppressed vs. immunocompetent patients are usually considered separate populations with distinct risk factors for the development of sulfonamide HS. To the author's knowledge, no studies have reported differences in incidence of sulfonamide HS between immunosuppressed vs. immunocompetent dogs. Therefore, risk for canine sulfonamide HS shall be considered for the species as a whole.

Risk Factors in Immunosuppressed Patients

A variety of factors have been identified that may help explain the higher incidence of sulfonamide HS in immunosuppressed patients. Antibiotic prescribing patterns generally differ

between immunosuppressed and immunocompetent patients, with the former much more likely to be taking antibiotics. TMP/SMX is the drug of choice for prophylaxis and treatment of *Pneumocystis* pneumonia, a common comorbidity in HIV and cystic fibrosis patients, both of whom have increased rates of sulfonamide HS.[142,177,178] However, this fact by itself cannot explain the higher percentage of individuals exposed to the drug that experience the ADR.

Altered drug metabolism in diseases affecting the immune system may contribute to this higher incidence of drug HS. HIV-positive patients excrete lower amounts of SMX-HA in the urine compared to HIV-negative patients and those with severe HS reactions may have even lower urinary concentrations.[160,179] This may suggest more conversion of SMX-HA to SMX-NO and adduct formation, which is indirectly supported by evidence of a deficiency in cellular antioxidant systems in HIV patients.[180] Sulfonamide HS has also been associated with a slow acetylator phenotype in HIV patients.[158,181,182] This phenotype, determined by NAT2 activity, decreases the amount drug detoxified by the acetylation pathway and may leave more drug available for metabolism to SMX-HA, subsequent degradation to SMX-NO, and adduct formation. Acetylation phenotype is primarily genetically determined, but may also be adversely affected by retroviral infection.[183-185]

Immunologic changes induced by disease may also alter susceptibility to sulfonamide HS. T cells from cystic fibrosis patients with sulfonamide HS show a different antigen reactivity pattern and cytokine profile from those that are sulfonamide-tolerant.[186] Untreated HIV patients have higher levels of interferon-γ, which enhances SMX-adduct antigen presentation by dendritic cells.[187] However, the best studied risk factor for sulfonamide HS in HIV patients is the severity

of the HIV infection. Lower $CD4^+$ T cell counts, percentages, or both have been consistently identified in HIV patients with sulfonamide HS vs. those tolerant to the drug.[129,177,188] Higher $CD8^+$ T cell concentrations may also be a marker.[178,189]

The evidence presented suggests that much of the additional risk for sulfonamide HS in immunosuppressed patients, particularly in HIV patients, is attributable to the disease itself, its severity, or the biological changes associated with that disease. Thus, risk factors for sulfonamide HS in immunosuppressed patients appear to be primarily acquired risk factors.

Risk Factors in Immunocompetent Patients

The risk factors contributing to the development of sulfonamide HS in immunocompetent patients are even less well understood than those for immunosuppressed patients. Immunocompetent patients are oftentimes systemically healthy when antibiotics are initially prescribed, with urinary tract and bronchial infections being some of the more common indications for TMP-SMX therapy.[18,22] Thus, these patients lack many of the metabolic and immunologic changes thought to be involved in the pathogenesis of the ADR in HIV-positive and other immunosuppressed individuals. Instead, some evidence suggests that sulfonamide HS may be heritable among the general population.[190-192] This includes one report in which individuals in three generations of a family were affected.[192] The apparent heritability of sulfonamide HS and the lack of identifiable acquired risk factors in immunocompetent patients suggests that genetic factors may be in play when assessing risk in the general population.

Risk Factors in Dogs

Some of the most common indications for sulfonamide antibiotics in dogs include lower urinary tract infections and superficial folliculitis.[19,20,24] Therefore, dogs with sulfonamide HS are also often systemically healthy at the time of initiation of therapy and lack the acquired risk factors seen in immunosuppressed humans. However, breed may contribute to risk for sulfonamide HS. Multiple reports suggest that Doberman Pinschers are predisposed to sulfonamide HS.[106-108,193,194] In the largest case series of canine sulfonamide HS to date, Trepanier et al. found that Samoyed and Miniature Schnauzers were overrepresented compared to the general hospital population, although it is unknown whether antibiotic prescribing patterns could have affected these results.[100] Breed may also impact which clinical signs manifest, with large breed dogs potentially predisposed to sulfonamide-associated polyarthropathy.[100,102,106-108] Given the high degree of relatedness between dogs within a breed, this evidence suggests that genetic risk factors may be present for dogs with sulfonamide HS, as well as humans.

Lymphocyte Toxicity Assay (LTA)

Given the lack of well-defined risk factors and the difficulty in obtaining a definitive diagnosis, a clinical or biologic marker for sulfonamide HS is greatly needed. Such a tool could aid in both confirming the diagnosis of HS reactions and identifying at-risk patients. The lymphocyte toxicity assay (LTA) has been proposed as this marker and has been associated with sulfonamide HS in both immunocompetent and HIV-infected patients. Results of this *in vitro* cytotoxicity assay show that peripheral blood mononuclear cells (PBMCs) from HS patients are more susceptible to apoptosis in the presence of sulfonamide metabolites when compared to cells from sulfonamide-tolerant patients.[122,162,195,196]

In the LTA, PBMCs are isolated from the subject of interest and incubated with various concentrations (~25-1000 μM) SMX metabolites for ~2 hours. The drug is then removed by washing and the cells are allowed to "recover" overnight followed by assessment of cell death. In all patients, a dose dependent cytotoxicity is observed, but the percentage of cell death is greater in HS patients, particularly at the higher metabolite concentrations.[107,161,162,196-202] The parent drug itself appears to be only minimally cytotoxic,[107,161,162,190,196-202] which has led to the hypothesis that those patients whose cells exhibit more cell death *in vitro* (a "positive" LTA phenotype) may have a defect in detoxification pathways for the drug metabolites.[190,203]

In the earliest reports of the LTA, the responsible metabolites were unknown and were generated by pre-incubating the antibiotic with hepatic microsomes.[191,204,205] Later studies demonstrated that the hydroxylamine metabolite (SMX-HA) is the proximate metabolite responsible for *in vitro* cytotoxicity and incubation with purified SMX-HA has since become standard practice for the LTA.[162,178,195,199,201-203,206] SMX-HA induced cytotoxicity can be diminished in the presence of glutathione, which may prevent oxidation of SMX-HA to SMX-NO.[162,195] Additionally, substitution of SMX-NO for SMX-HA in the LTA also causes enhanced cell death, suggesting a role for SMX-NO in the observed cytotoxicity.[162,202]

In the LTA, the events leading from SMX-HA exposure up to detectable cell death are poorly understood. It is known that a "recovery period" (usually 16-19 hours) is necessary to elicit maximal cytotoxicity. Although some acute cell death is detectable directly following drug exposure (~2 hours), cell death continues after the drug is removed and the largest difference in

% cytotoxicity between drug-exposed cells and vehicle-controls is delayed by several hours.[202] Whether this delay is due to further metabolism of xenobiotics or activation of cell death pathways is not known. However, cell death during the acute and delayed phases of the LTA are mechanistically distinct. The acute phase is primarily characterized by cell necrosis whereas the delayed phase, which is quantitatively more important, is characterized by apoptosis.[199] All PBMCs show dose-dependent cytotoxicity in the LTA; however, the extent of cell death varies with lymphocyte sub-population. $CD8^+$ T cells are the most susceptible, $CD4^+$ T cells the least, and B cells have an intermediate phenotype.[199]

Three primary methods for detecting cell death in the LTA have been reported. The earliest reports of the assay investigating sulfonamide toxicity *in vitro* used trypan blue dye exclusion, which detects dead PBMCs when the dye is able to enter the cell through a ruptured cell membrane.[191,205] However, trypan blue dye exclusion requires manual cell counting, which introduces inter-operator variability and the high potential for user error. Thus, the LTA was adapted to incorporate an automated detection system using the MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).[207] In this form of the assay, cell viability is measured when MTT is converted by mitochondrial dehydrogenases to formazan, which is detected colorometrically[208] and cytotoxicity is derived from cell viability. To date, the majority of studies investigating the LTA as a marker for sulfonamide HS have used the MTT method of detection.[122,195,196,201,206,207] This method assumes a one-to-one relationship between mitochondrial enzyme function and cell viability. However, it is possible for an exogenous compound to disrupt enzyme function without causing true cell death, so a third methodology was introduced, which addresses the limitations of both prior detection systems. YO-PRO-1 is a

DNA-intercalating fluorophore that is impermeant to the cell membrane. Similar to trypan blue, only cells with permeable membranes will stain with YO-PRO, however, detection and quantification of the fluorescent signal can be automated.[209] The LTA using YO-PRO detection has been used to investigate sulfonamide HS in HIV-infected patients,[202] but has yet to be reported for immunocompetent subjects.

The LTA has been evaluated as a means to distinguish HS from tolerant patients in both retrospective and prospective studies.[122,206] LTA results appear to be a durable marker in immunocompetent patients, with increased cytotoxicity still noted years after the HS event.[4,148] In addition, this apparent defect can be demonstrated in family members of HS patients who have never been administered potentiated sulfonamides,[190,191,203] which suggests a heritable defect. Given these findings, the cytotoxicity phenomenon is unlikely to be a residual effect of the HS event itself; rather, it appears that the defect is intrinsic to individual patients.

In the general population, the reported specificity of the LTA for predicting sulfonamide HS is quite high (78-99%); however, sensitivity varies greatly (41-100%).[148,190,206,210] Additionally, the performance characteristics of the LTA appear to vary with the severity of clinical signs. In patients with only an exanthemous rash, sensitivity and specificity are quite poor (41% and 78%, respectively), but are vastly improved when the LTA is performed in patients with systemic organ involvement (92% and 99%, respectively).[148] This disparity in test performance may represent true biological differences between cells of individuals that manifest sulfonamide HS in different ways. Alternately, the poor sensitivity and specificity in patients with mild symptoms could be due to misdiagnosis and misattribution of a rash to drug exposure.

Use of the LTA in dogs has only been reported in one study. Using MTT for detection, Cribb et al.[107] investigated SMX-HA induced cytotoxicity in PBMCs from 10 mixed-breed dogs and 15 Doberman Pinschers, one of whom had documented sulfonamide HS. Overall, the Doberman Pinscher PBMCs showed more cell death compared to the mixed breed dogs. Doberman Pinschers appear to be overrepresented for sulfonamide HS, so it was proposed that the observed *in vitro* susceptibility to sulfonamide metabolites might help explain this clinical predisposition. However, to date, no studies have been published that compare results between tolerant vs. HS dogs. Our laboratory has attempted to validate a YO-PRO-based canine assay, but high background fluorescence has complicated detection (Reinhart, unpublished). Thus, the LTA cannot be considered a marker of sulfonamide HS in dogs at this time.

Enhanced cytotoxicity to sulfonamide metabolites in the LTA has been interpreted as a "detoxification defect" in HS but not tolerant patients.[162,190,211] However, it is unclear whether this *in vitro* "defect" translates to *in vivo* effects that may be part of the pathogenesis of sulfonamide HS. The concentration of SMX-HA used in the LTA (up to 1 mM in some reports) is much higher than concentrations that would be generated in a patient administered standard therapeutic doses of SMX.[155,212] Thus, direct lymphocyte toxicity is unlikely in HS individuals. However, failure to detoxify SMX metabolites in the LTA may indicate similar problems during antibiotic therapy, predisposing to adduct formation and immunogenicity. Alternately, this defect could represent a cellular dysfunction of lymphocytes as immune responders to SMX-adducts as antigen, an intriguing possibility since $CD8^+$ T cells, which are most susceptible to LTA-associated cytotoxicity, are also the primary effector cells of delayed-type HS reactions. These

theories are largely speculative and the association between LTA results and clinical status could simply be an epiphenomenon. A better understanding of the mechanisms underlying the enhanced cytotoxicity of SMX-HA in HS patients is needed.

Pharmacogenetics of Sulfonamide HS in Immunocompetent Patients

Identifying genetic risk factors for sulfonamide HS is of paramount importance because of the unpredictable and sometimes severe manifestations of idiosyncratic drug reactions. Although polymorphisms in certain candidate genes have been associated with sulfonamide HS in HIV-infected patients (*NAT1*,[213] *NAT2*,[214,215] *GCLC*,[216] *GSTM1*,[217] *GSTP1*[218]), acquired risk is probably more important in this population since the underlying HIV infection may modulate metabolic or immunologic phenotype normally determined genetically.[184,219] On the other hand, genetic risk factors may be quite relevant for immunocompetent patients with sulfonamide HS, given the potentially heritable nature of this condition.[190-192] Several candidate genes have been evaluated in this population.

NAT2 is the best-studied candidate gene for risk of sulfonamide HS in immunocompetent humans. The large variation in total *N*-acetylation capacity is due primarily to variable NAT2 activity across the population. As such, multiple low-function polymorphisms in *NAT2* have been correlated to a "slow acetylator" phenotype.[163] Several studies have compared the prevalence of these polymorphisms between sulfonamide HS vs. TOL or healthy patients, but results have been contradictory. Wolkenstein et al.[220] examined four known *NAT2* polymorphisms in sulfonamide HS patients and found that significantly more HS patients had a slow acetylator genotype (17/18) vs. controls (10/20). However, this difference was only present

when all loci were combined and was not attributable to a single variant. Furthermore, control subjects were healthy volunteers with unknown clinical status, instead of patients known to be tolerant to sulfonamide antibiotics. In two similar studies, Zielinska et al.[221,222] compared *NAT2* allele frequencies between young patients who were sulfonamide HS vs. TOL. Low function alleles were associated with HS in both infants (2 to 12 months)[221] and children (3 months to 17 years)[222]. However, in the infant study, HS was associated with the *NAT2**5A allele whereas it was associated with *NAT2**6A and *7B in children. Finally, our laboratory investigated *NAT2* genotypes in 99 HS vs. 99 TOL patients.[78] Despite full resequencing of the coding region, we did not identify any difference in allele frequencies for known variants or in predicted acetylator phenotype between the two groups. The large sample size and rigorous genotyping methodology of this study may call previous findings in to question.

Based on the proposed pathogenesis of sulfonamide HS, other metabolic enzyme genes have been evaluated for genetic associations. The *CYP2C9*2/*3* variants have been associated with increased production of SMX-HA in human cell lines;[223] however, allele frequencies have only been investigated in HIV-positive patients with sulfonamide HS, and were not a risk factor.[215] In a large case-control study performed by our group, we investigated the sulfamethoxazole detoxification genes, *CYB5A* and *CYB5R3*, but the polymorphism frequencies were not significantly different between sulfonamide HS and tolerant patients.[78] Finally, an increased risk for cutaneous drug eruption in general has been documented for patients with *GSTM1* and *GSTT1* null genotypes, but this study population was mixed, with sulfonamide HS patients representing only 4/36 affected patients.[117]

Some of the strongest genetic associations for idiosyncratic adverse drug reactions have been found for HLA variants. Presumably, this is because the molecular conformation of HLA groove determines the ease with which drugs or drug-adducts can be presented as antigen. For example, HS reactions to the antiviral drug, abacavir, are strongly associated with the MHC-I allele, HLA-B*5701, and pre-therapy genotyping has been shown to significantly reduce the incidence of abacavir HS reactions in clinical practice.[224] HLA alleles have also been investigated as genetic risk factors for sulfonamide HS. Ozkaya-Bayizit et al.[225] found an association between the HLA-A30 B16 Cw6 haplotype and sulfonamide-induced skin eruptions in a Turkish population. In a more recent study in Thailand, three HLA alleles (HLA-B*1502, HLA-C*0602, HLA-C*0801) were found to increase risk for sulfonamide-associated SJS/TEN by 3-10-fold, with carriage of multiple alleles having an additive effect on risk.[226] It should be noted that the population for this latter study included primarily HIV-positive patients and only included those with the most severe manifestations of the sulfonamide HS. Thus these results may not be applicable to the average immunocompetent patient taking sulfonamide antibiotics. Furthermore, these two studies performed in separate geographic regions yielded different associations, which is not unexpected since HLA allele distribution varies significantly among ethnic groups.

Genetic risk factors for sulfonamide HS have also been investigated in dogs. Since dogs lack *NAT1* and *NAT2* genes, this may confer a species-level predisposition to sulfonamide HS but cannot account for individual or breed risk.[13] Our laboratory has also investigated *CYB5A* and *CYB5R3* as potential candidate genes in dogs.[157] We identified a single nucleotide polymorphism (SNP) in the *CYB5R3* gene, 729A>G, which was overrepresented in dogs with

sulfonamide HS. This variant is a synonymous coding SNP, but is predicted to alter the conformation of the mRNA transcript to a more stable form.[157] Therefore, it is possible that *CYB5R3* 729G creates a more difficult to translate mRNA structure, which leads to decreased enzyme synthesis and detoxification activity. Other candidate genes of interest are those encoding dog leukocyte antigens (DLAs), which are analogous to the HLAs in humans. Although DLAs has not been specifically investigated in sulfonamide hypersensitivity, DLA alleles have been associated immune-mediated hemolytic anemia,[227] diabetes mellitus,[228] inflammatory bowel disease,[229] and other immune-mediated canine diseases in dogs.[230]

Sulfonamide HS is relatively uncommon in immunocompetent humans and dogs. However, severe, potentially fatal, reactions are possible and concern for these reactions limit the clinical utility of potentiated sulfonamide antibiotics in both species. Thus, identifying risk factors has the potential to improve the use and safety of these drugs in practice. Given the apparent heritability of sulfonamide HS, genetic risk factors are of great interest; known variants need further investigation and modern methodologies can be used to search for novel variants. Although it is fairly well established that drug bioactivation and an immunologic response are requisite for this ADR to occur, much of the pathogenesis of sulfonamide HS is yet to be understood. Of particular interest is the lymphocyte toxicity assay – interrogation of the mechanisms underlying this *in vitro* marker may lead to the discovery of new pathways influencing clinical phenotype. Such investigations may suggest improved treatment or prevention strategies for sulfonamide HS in humans and dogs.

The overarching goal of this thesis is to ***better understand the genetic factors and molecular mechanisms underlying sulfonamide HS in immunocompetent humans and dogs***. This is accomplished through three specific aims:

1) To identify genes associated with human sulfonamide HS, using a genome-wide association study (GWAS) design with sulfonamide tolerant patients as controls (**Chapter 2**).
2) To determine the mechanisms for the enhanced *in vitro* SMX-HA induced cytotoxicity in HS human patients by comparing $CD8^+$ T cell transcriptomes of HS vs. tolerant patients (**Chapter 3**).
3) To further investigate the importance of the *CYB5R3* 729A>G SNP in dogs (**Chapter 4**).
    a. To determine whether *CYB5R3* 729A>G is associated with sulfonamide hypersensitivity in a larger population of dogs, and whether this SNP is overrepresented in Doberman Pinschers relative to other dog breeds.
    b. To characterize the effect of the canine *CYB5R3* 729A>G variant on transcription, translation, and cytochrome b_5 reductase activity.

References

1. Otten H. Domagk and the development of the sulphonamides. J Antimicrob Chemoth. 1986;17:689–696.
2. Mohr KI. History of antibiotics research. Curr Top in Microbiol. 2016;398:237–272.
3. Cribb AE, Lee BL, Trepanier LA, Spielberg SP. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. Adverse Drug React Toxicol Rev. 1996;15(1):9–50.
4. Zawodniak A, Lochmatter P, Beeler A, Pichler WJ. Cross-reactivity in drug hypersensitivity reactions to sulfasalazine and sulfamethoxazole. Int Arch Allergy

Immunol. 2010;153(2):152–156.

5. Papich MG, Riviere JE. Sulfonamides and Potentiated Sulfonamides. In: Riviere JE, Papich MG, editors. Veterinary Pharmacology Therapeutics. 9 ed. Ames, IA; pp. 835–864.

6. Bactrim [package insert]. Philadelphia, PA: AR Scientific, Inc.; 2013.

7. Primor [package insert]. Parsippany-Troy Hills, NJ: Zoetis, Inc.; 2013.

8. Albon [package insert]. Parsippany-Troy Hills, NJ: Zoetis, Inc.; 2013.

9. Boothe DM. Antimicrobial Drugs. In: Boothe DM, editor. Small Animal Clinical Pharmacology & Therapeutics. 2nd ed. St. Louis, MO; pp. 189–269.

10. Riffat S, Nawaz M, Zia-ur-Rehman. Pharmacokinetics of sulphadimidine in normal and febrile dogs. J Vet Pharmacol Ther. 1982;5(2):131–135.

11. Yagi N, Agata I, Kawamura T, Tanaka Y, Sakamoto M, Itoh M, et al. Fundamental pharmacokinetic behavior of sulfadimethoxine, sulfamethoxazole and their biotransformed products in dogs. Chem Pharm Bull. 1981;29(12):3741–3747.

12. Bridges JW, Kibby MR, Walker SR, Williams RT. Species differences in the metabolism of sulphadimethoxine. Biochem J. 1968;109(5):851–856.

13. Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. Cytosolic arylamine N-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. Biochem Pharmacol. 1997;54(1):73–80.

14. Rodriguez M, Sifri CD, Fishman JA. Failure of low-dose atovaquone prophylaxis against Pneumocystis jiroveci infection in transplant recipients. Clin Infect Dis. 2004;38(8):e76–78.

15. Thomas M, Rupali P, Woodhouse A, Ellis-Pegler R. Good outcome with trimethoprim 10 mg/kg/day-sulfamethoxazole 50 mg/kg/day for Pneumocystis jirovecii pneumonia in HIV infected patients. Scand J Infect Dis. 2009;41:862–868.

16. Wei H-X, Wei S-S, Lindsay DS, Peng H-J. A systematic review and meta-Analysis of the efficacy of anti-Toxoplasma gondii medicines in humans. PLoS ONE. 2015;10(9):e0138204.

17. Garosi L, Dawson A, Couturier J, Matiasek L, De Stefani A, Davies E, et al. Necrotizing cerebellitis and cerebellar atrophy caused by Neospora caninum infection: magnetic resonance imaging and clinicopathologic findings in seven dogs. J Vet Intern Med. 2010;24(3):571–578.

18. Grigoryan L, Trautner BW, Gupta K. Diagnosis and management of urinary tract Infections in the outpatient setting. J Am Med Assoc. 2014 Oct;312(16):1677–1678.

19. Weese JS, Blondeau JM, Boothe D, Breitschwerdt EB, Guardabassi L, Hillier A, et al. Antimicrobial use guidelines for treatment of urinary tract disease in dogs and cats:

Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases. Vet Med Int. 2011;2011(4):1–9.

20. Clare S, Hartmann FA, Jooss M, Bachar E, Wong YY, Trepanier LA, et al. Short- and long-term cure rates of short-duration trimethoprim-sulfamethoxazole treatment in female dogs with uncomplicated bacterial cystitis. J Vet Intern Med. 2014;28(3):818–826.

21. Olin SJ, Bartges JW. Urinary tract infections: treatment/comparative therapeutics. Vet Clin North Am Small Anim Pract. 2015;45(4):721–746.

22. Korbila IP, Manta KG, Siempos II, Dimopoulos G, Falagas ME. Penicillins vs trimethoprim-based regimens for acute bacterial exacerbations of chronic bronchitis: meta-analysis of randomized controlled trials. Can Fam Physician. 2009;55(1):60–67.

23. Lappin MR, Blondeau J, Boothe D, Breitschwerdt EB, Guardabassi L, Lloyd DH, et al. Antimicrobial use guidelines for treatment of respiratory tract disease in dogs and cats: Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases. J Vet Intern Med. 2017;31(2):279–294.

24. Hillier A, Lloyd DH, Weese JS, Blondeau JM, Boothe D, Breitschwerdt E, et al. Guidelines for the diagnosis and antimicrobial therapy of canine superficial bacterial folliculitis (Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases). Vet Dermatol. 2014;25(3):163–e43.

25. Baker SA, Van-Balen J, Lu B, Hillier A, Hoet AE. Antimicrobial drug use in dogs prior to admission to a veterinary teaching hospital. J Am Vet Med Assoc. 2012;241(2):210–217.

26. Harbarth S, Dach von E, Pagani L, Macedo-Vinas M, Huttner B, Olearo F, et al. Randomized non-inferiority trial to compare trimethoprim/sulfamethoxazole plus rifampicin versus linezolid for the treatment of MRSA infection. J Antimicrob Chemother. 2014;70(1):264–272.

27. Drekonja DM, Traynor LM, DeCarolis DD, Crossley KB, Johnson JR. Treatment of non-life-threatening methicillin-resistant Staphylococcus aureus infections with alternative antimicrobial agents: a 2-year retrospective review. Diagn Microbiol Infect Dis. 2009;63(2):201–207.

28. Morris DO, Loeffler A, Davis MF, Guardabassi L, Weese JS. Recommendations for approaches to meticillin-resistant staphylococcal infections of small animals: diagnosis, therapeutic considerations and preventative measures. Vet Dermatol. 2017;28(3):304–369.

29. Murri R, Fiori B, Spanu T, Mastrorosa I, Giovannenze F, Taccari F, et al. Trimethoprim–sulfamethoxazole therapy for patients with carbapenemase-producing Klebsiella pneumoniae infections: retrospective single-center case series. Infection. 2017;45(2):209–213.

30. Gupta D, Zachariah A, Roppelt H, Patel AM, Gruber BL. Prophylactic antibiotic usage for Pneumocystis jirovecii pneumonia in patients with systemic lupus erythematosus on cyclophosphamide: a survey of US rheumatologists and the review of literature. J Clin Rheumatol. 2008;14(5):267–272.

31. Seger RA. Modern management of chronic granulomatous disease. Br J Haematol. 2008;140(3):255–266.

32. Medina I, Mills J, Leoung G, Hopewell PC, Lee B, Modin G, et al. Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. A controlled trial of trimethoprim-sulfamethoxazole versus trimethoprim-dapsone. N Engl J Med. 1990;323(12):776–782.

33. Chretin JD, Rassnick KM, Shaw NA, Hahn KA, Ogilvie GK, Kristal O, et al. Prophylactic trimethoprim-sulfadiazine during chemotherapy in dogs with lymphoma and osteosarcoma: a double-blind, placebo-controlled study. J Vet Intern Med. 2007;21(1):141–148.

34. Avgeri SG, Matthaiou DK, Dimopoulos G, Grammatikos AP, Falagas ME. Therapeutic options for Burkholderia cepacia infections beyond co-trimoxazole: a systematic review of the clinical evidence. Int J Antimicrob Ag. 2009;33(5):394–404.

35. Banovic F, Koch S, Robson D, Jacob M, Olivry T. Deep pyoderma caused by Burkholderia cepaciacomplex associated with ciclosporin administration in dogs: a case series. Vet Dermatol. 2015;26(4):287–e64.

36. Gonzalez-Santiago TM, Drage LA. Nontuberculous Mycobacteria: skin and soft tissue infections. Dermatol Clin. 2015;33(3):563–577.

37. Greene CE, Gunn-Moore D, R OC, Fyfe JC, Malik R. Mycobacterial Infections. In: Greene CE, editor. Infectious Diseases of the Dog and Cat. 4 ed. St. Louis, MO; 2012. pp. 495–520.

38. Elzagallaai AA, Greff M, Rieder MJ. Adverse drug reactions in children: the double-edged sword of therapeutics. Clin Pharmacol Ther. 2017;101(6):725–735.

39. Coombs RR. Fundamental concepts on allergic reactions to drugs. Br J Dermatol. 1969;81:Suppl3:2–6.

40. Stevens RC, Laizure SC, Williams CL, Stein DS. Pharmacokinetics and adverse effects of 20-mg/kg/day trimethoprim and 100-mg/kg/day sulfamethoxazole in healthy adult subjects. Antimicrob Agents Chemother. 1991;35(9):1884–1890.

41. Bozzette SA, Forthal D, Sattler FR, Kemper C. The tolerance for zidovudine plus thrice weekly or daily trimethoprim-sulfamethoxazole with and without leucovorin for primary prophylaxis in advanced HIV disease. Am J Med. 1995;98:177-182.

42. Ho JM-W, Juurlink DN. Considerations when prescribing trimethoprim-sulfamethoxazole. CMAJ. 2011;183(16):1851–1858.

43. Gray DA, Foo D. Reversible myoclonus, asterixis, and tremor associated with high dose trimethoprim-sulfamethoxazole: a case report. J Spinal Cord Med. 2016;39(1):115–117.

44. Trepanier LA. Idiosyncratic toxicity associated with potentiated sulfonamides in the dog. J Vet Pharmacol Ther. 2004;27(3):129–138.

45. Gentry CA, Nguyen AT. An evaluation of hyperkalemia and serum creatinine elevation associated with different dosage levels of outpatient trimethoprim-sulfamethoxazole with and without concomitant medications. Ann Pharmacother. 2013;47(12):1618–1626.

46. Bernstein LS. Adverse reactions to trimethoprim-sulfamethoxazole, with particular reference to long-term therapy. CMAJ. 1975;112:96–98.

47. Heimpel H, Raghavachar A. Hematological side effects of co-trimoxazole. Infection. 1987;15:S248–253.

48. Bjornson BH, McIntyre AP, Harvey JM, Tauber AI. Studies of the effects of trimethoprim and sulfamethoxazole on human granulopoiesis. Am J Hematol. 1986;23(1):1–7.

49. Tapp H, Savarirayan R. Megaloblastic anaemia and pancytopenia secondary to prophylactic cotrimoxazole therapy. J Paediatr Child Health. 1997;33(2):166–167.

50. Leucovorin [package insert]. London, UK: Bedford Laboratories (Hikma Pharmaceuticals); 2012.

51. Koirala J. Trimethoprim-sulfamethoxazole–induced methemoglobinemia in an HIV-infected patient. Mayo Clin Proc. 2011;79(6):829–830.

52. Damergis JA, Stoker JM, Abadie JL. Methemoglobinemia after sulfametoxazole and trimethoprim. J Am Med Assoc. 1983;249(5):590–591.

53. Kenteu B, Noubiap JJN, Etoa MC, Azabji-Kenfack M, Dehayem M, Sobngwi E. Acute glycaemic effects of co-trimoxazole at prophylactic dose in healthy adults. BMC Endocr Disord. 2016;16(1):62–67.

54. Huntsberry AM, Linnebur SA, Vejar M. Hyponatremia after initiation and rechallenge with trimethoprim-sulfamethoxazole in an older adult. Clin Interv Aging. 2015;10:1091–1096.

55. Murphy JL. Renal tubular acidosis in children treated with trimethoprim-sulfamethoxazole during therapy for acute lymphoid leukemia. Pediatrics. 1992;89(6):1072–1074.

56. Cook AK, Allen AK, Espinosa D, Barr J. Renal tubular acidosis associated with zonisamide therapy in a dog. J Vet Intern Med. 2011;25(6):1454–1457.

57. Doerge DR, Decker CJ. Inhibition of peroxidase-catalyzed reactions by arylamines: mechanism for the anti-thyroid action of sulfamethazine. Chem Res Toxicol. 1994;7:164–169.

58. Frank LA, Hnilica KA, May ER, Sargent SJ, Davis JA. Effects of sulfamethoxazole-trimethoprim on thyroid function in dogs. Am J Vet Res. 2005;66(2):256–259.

59. Gookin JL, Trepanier LA, Bunch SE. Clinical hypothyroidism associated with trimethoprim-sulfadiazine administration in a dog. J Am Vet Med Assoc. 1999;214(7):1028–1031.

60. Taeymans O, O'Marra SK. Imaging diagnosis-acquired goitrous hypothyroidism following treatment with trimethoprim sulfamethoxazole. Vet Radiol Ultrasoun 2009;50(4):442–444.

61. Seelig DM, Whittemore JC, Lappin MR, Myers AM, Avery PR. Goitrous hypothyroidism associated with treatment with trimethoprim-sulfamethoxazole in a young dog. J Am Vet Med Assoc. 2008;232(8):1181–1185.

62. Brenner K, Harkin K, Schermerhorn T. Iatrogenic, sulfonamide-induced hypothyroid crisis in a Labrador Retriever. Aust Vet J. 2009;87(12):503–505.

63. Cohen HN, Beastall GH, Ratcliffe WA, Gray C, Watson ID, Thomson JA. Effects on human thyroid function of sulphonamide and trimethoprim combination drugs. Br Med J. 1980;281:646–647.

64. Gupta A, Eggo MC, Uetrecht JP, Cribb AE, Daneman D, Rieder MJ, et al. Drug-induced hypothyroidism: The thyroid as a target organ in hypersensitivity reactions to anticonvulsants and sulfonamides. Clin Pharmacol Ther. 1992;51(1):56–67.

65. Johnson MP, Goodwin SD, Shands JW. Trimethoprim-sulfamethoxazole anaphylactoid reactions in patients with AIDS: case reports and literature review. Pharmacotherapy. 1990;10(6):413–416.

66. Teker D, Tanir G, Ozmen S, Teke TA, Keles S, Bostancı I. Treatment of brucellosis in a young child with trimethoprim/sulfamethoxazole anaphylaxis. J Infect and Public Heal. King Saud Bin 2014;7(6):553–556.

67. Ghosh JB. Anaphylaxis following oral cotrimoxazole. Indian Pediatr. 1995;32(7):827.

68. Kumar AS. Treatment of anaphylaxis following oral co-trimoxazole. Indian Pediatr. 1996;33(3):249–250.

69. Hemstreet BA, Page RL. Sulfonamide allergies and outcomes related to use of potentially cross-reactive drugs in hospitalized patients. Pharmacotherapy. 2006;26(4):551–557.

70. Persichino J, Sutjita M. Anaphylactic-like reaction from trimethoprim-sulfamethoxazole in a patient with AIDS. Int J STD AIDS. 2016;27(7):595–597.

71. Harle DG, Baldo BA, Wells JV. Drugs as allergens: detection and combining site specificities of IgE antibodies to sulfamethoxazole. Mol Immunol. 1988;25(12):1347–1354.

72. Das G, Bailey MJ, Wickham JEA. Drug points: Toxic epidermal necrolysis and trimethoprim. Br Med J. 1988;296:1604–1605.

73. Nwokolo C, Byrne L, Misch KJ. Toxic epidermal necrolysis occurring during treatment with trimethoprim alone. Br Med J. 1988;296:970.

74. Berg PA, Daniel PT. Co-trimoxazole-induced hepatic injury--an analysis of cases with hypersensitivity-like reactions. Infection. 1987;15:S259–264.

75. Bigby M. Drug-induced cutaneous reactions. A report from the Boston Collaborative Drug Surveillance Program on 15,438 consecutive inpatients, 1975 to 1982. J Am Med Assoc. 1986;256(24):3358–3363.

76. Gordin FM, Simon GL, Wofsy CB, Mills J. Adverse reactions to trimethoprim-sulfamethoxazole in patients with the acquired immunodeficiency syndrome. Ann Intern Med. 1984;100(4):495–499.

77. Reinhart JM, Motsinger-Reif A, Dickey A, Yale S, Trepanier LA. Genome-wide association study in immunocompetent patients with delayed hypersensitivity to sulfonamide antimicrobials. PLoS ONE. 2016;11(6):e0156000.

78. Sacco JC, Abouraya M, Motsinger-Reif A, Yale SH, McCarty CA, Trepanier LA. Evaluation of polymorphisms in the sulfonamide detoxification genes NAT2, CYB5A, and CYB5R3 in patients with sulfonamide hypersensitivity. Pharmacogenet Genomics. 2012;22(10):733–740.

79. Chung W-H, Wang C-W, Dao R-L. Severe cutaneous adverse drug reactions. J Dermatol. 2016;43(7):758–766.

80. Diphoorn J, Cazzaniga S, Gamba C, Schroeder J, Citterio A, Rivolta AL, et al. Incidence, causative factors and mortality rates of Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) in northern Italy: data from the REACT registry. Pharmacoepidemiol Drug Saf. 2015;25(2):196–203.

81. East-Innis AD, Thompson DS. Stevens-Johnson syndrome and toxic epidermal necrolysis at the University Hospital of the West Indies, Jamaica. West Indian Med J. 2013;62(7):589–592.

82. Kelly JP, Kaufman DW, Dunant A, Mockenhaupt M, Roujeau J-C. Seasonal variation of Stevens-Johnson syndrome and toxic epidermal necrolysis associated with trimethoprim-sulfamethoxazole. J Am Acad Dermatol. 2011;64(6):1193–1194.

83. Roujeau JC, Kelly JP, Naldi L, Rzany B, Stern RS, Anderson T, et al. Medication use and the risk of Stevens-Johnson syndrome or toxic epidermal necrolysis. N Engl J Med. 1995;333(24):1600–1607.

84. Bastuji-Garin S, Fouchard N, Bertocchi M, Roujeau JC, Revuz J, Wolkenstein P. SCORTEN: a severity-of-illness score for toxic epidermal necrolysis. J Invest Dermatol. 2000;115(2):149–153.

85. Yang J-J, Huang C-H, Liu C-E, Tang H-J, Yang C-J, Lee Y-C, et al. Multicenter study of trimethoprim/sulfamethoxazole-related hepatotoxicity: incidence and associated factors among HIV-infected patients treated for Pneumocystis jirovecii pneumonia. PLoS ONE. 2014;9(9):e106141.

86. Bell TL, Foster JN, Townsend ML. Trimethoprim-sulfamethoxazole-induced hepatotoxicity in a pediatric patient. Pharmacotherapy. 2010;30(5):539.

87. Keisu M, Wiholm BE, Palmblad J. Trimethoprim-sulphamethoxazole-associated blood dyscrasias. Ten years' experience of the Swedish spontaneous reporting system. J Int Med. 1990;228(4):353–360.

88. Reese JA, Nguyen LP, Buchanan GR, Curtis BR, Terrell DR, Vesely SK, et al. Drug-induced thrombocytopenia in children. Pediatr Blood Cancer. 2013;60(12):1975–1981.

89. Garbe E, Andersohn F, Bronder E, Klimpel A, Thomae M, Schrezenmeier H, et al. Drug induced immune haemolytic anaemia in the Berlin Case-Control Surveillance Study. Br J Haematol. 2011;154(5):644–653.

90. Platt R, Dreis MW, Kennedy DL, Kuritsky JN. Serum sickness-like reactions to amoxicillin, cefaclor, cephalexin, and trimethoprim-sulfamethoxazole. The J Infect Dis. 1988;158(2):474–477.

91. Heckbert SR, Stryker WS, Coltin KL, Manson JE, Platt R. Serum sickness in children after antibiotic exposure: estimates of occurrence and morbidity in a health maintenance organization population. Am J Epidemiol. 1990;132(2):336–342.

92. Morís G, Garcia-Monco JC. The challenge of drug-induced aseptic meningitis revisited. J Am Med Assoc Intern Med. 2014;174(9):1511–1512.

93. Bruner KE, Coop CA, White KM. Trimethoprim-sulfamethoxazole-induced aseptic meningitis-not just another sulfa allergy. Ann Allergy Asthma Immunol. 2014;113(5):520–526.

94. Fraser TN, Avellaneda AA, Graviss EA, Musher DM. Acute kidney injury associated with trimethoprim/sulfamethoxazole. J Antimicrob Chemother. 2012;67(5):1271–1277.

95. Brett AS, Shaw SV. Simultaneous pancreatitis and hepatitis associated with trimethoprim-sulfamethoxazole. Am J Gastroenterol. 1999;924:267–268.

96. Tilden ME, Rosenbaum JT, Fraunfelder FT. Systemic sulfonamides as a cause of bilateral, anterior uveitis. Arch Ophthalmol. 1991;109(1):67–69.

97. Jeremic I, Vujasinovic-Stupar N, Terzic T, Damjanov N, Nikolic M, Bonaci-Nikolic B. Fatal sulfasalazine-induced eosinophilic myocarditis in a patient with periodic fever syndrome. Med Princ Pract. 2015;24(2):195–197.

98. Jamous F, Ayaz SZ, Choate J. Acute fibrinous organising pneumonia: a manifestation of trimethoprim-sulfamethoxazole pulmonary toxicity. BMJ Case Rep. 2014. doi:10.1136/bcr2014205017.

99. Noli C, Koeman JP, Willemse T. A retrospective evaluation of adverse reactions to trimethoprim-sulphonamide combinations in dogs and cats. Vet Quart. 1995;17(4):123–128.

100. Trepanier LA, Danhof R, Toll J, Watrous D. Clinical findings in 40 dogs with hypersensitivity associated with administration of potentiated sulfonamides. J Vet Intern Med. 2003;17(5):647–652.

101. Sullivan PS, Arrington K, West R, McDonald TP. Thrombocytopenia associated with administration of trimethoprim/sulfadiazine in a dog. J Am Vet Med Assoc. 1992;201(11):1741–1744.

102. Giger U, Werner LL, Millichamp NJ, Gorman NT. Sulfadiazine-induced allergy in six Doberman pinschers. J Am Vet Med Assoc. 1985;186(5):479–484.

103. Fox LE, Ford S, Alleman AR, Homer BL, Harvey JW. Aplastic anemia associated with prolonged high-dose trimethoprim-sulfadiazine administration in two dogs. Vet Clin Pathol. 1993;22(3):89–92.

104. Twedt DC, Diehl KJ, Lappin MR, Getzy DM. Association of hepatic necrosis with trimethoprim sulfonamide administration in 4 dogs. J Vet Intern Med. 1997;11(1):20–23.

106. Harvey RG. Possible sulphadiazine-trimethoprim induced polyarthritis. Vet Rec. 1987;120(22):537–538.

107. Whur P. Possible trimethoprim-sulphonamide induced polyarthritis. Vet Rec. 1987;121(4):91–92.

108. Gray A. Trimethoprim-sulphonamide hypersensitivity in dogs. Vet Rec. 1990;127(23):579–580.

109. Williams DL. Immunopathogenesis of keratoconjunctivitis sicca in the dog. Vet Clin N Am–Small. 2008;38(2):251–268.

110. Berger SL, Scagliotti RH, Lund EM. A quantitative study of the effects of Tribrissen on canine tear production. J Am Anim Hosp Assoc. 1995;31(3):236–241.

111. Morgan RV, Bachrach A. Keratoconjunctivitis sicca associated with sulfonamide therapy in dogs. J Am Vet Med Assoc. 1982;180(4):432–434.

112. Slatter DH, Blogg JR. Keratoconjunctivitis sicca in dogs associated with sulphonamide administration. Aust Vet J. 1978 Sep;54(9):444–6.

113. Slatter DH. Keratoconjunctivitis sicca in the dog produced by oral phenazopyridine hydrochloride. J Sm Anim Pract. 1973 Dec;14(12):749–71.

114. Schnyder B, Burkhart C, Schnyder-Frutig K, Greyerz von S, Naisbitt DJ, Pirmohamed M, et al. Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. J Immunol. 2000;164(12):6647–6654.

115. Schnyder B, Frutig K, Mauri-Hellweg D, Limat A, Yawalkar N, Pichler WJ. T-cell-mediated cytotoxicity against keratinocytes in sulfamethoxazol-induced skin reaction. Clin Exp Allergy. 1998;28(11):1412–1417.

116. Daftarian MP, Filion LG, Cameron W, Conway B, Roy R, Tropper F, et al. Immune response to sulfamethoxazole in patients with AIDS. Clin Diagn Lab Immunol. 1995;2(2):199–204.

117. Ateş NA, Tursen U, Tamer L, Kanik A, Derici E, Ercan B, et al. Glutathione S-transferase polymorphisms in patients with drug eruption. Arch Dermatol Res. 2004;295(10):429–433.

118. Ozkaya-Bayazit E, Bayazit H, Ozarmagan G. Topical provocation in 27 cases of cotrimoxazole-induced fixed drug eruption. Contact Derm. 1999;41(4):185–189.

119. Lavergne SN, Danhof RS, Volkman EM, Trepanier LA. Association of drug-serum protein adducts and anti-drug antibodies in dogs with sulphonamide hypersensitivity: a naturally occurring model of idiosyncratic drug toxicity. Clin Exp Allergy. 2006;36(7):907–915.

120. Naranjo CA, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts EA, et al. A method for estimating the probability of adverse drug reactions. Clin Pharmacol Ther. 1981;30(2):239–245.

121. Bousquet P-J, Gaeta F, Bousquet-Rouanet L, Lefrant J-Y, Demoly P, Romano A. Provocation tests in diagnosing drug hypersensitivity. Curr Pharm Design. 2008;14:2792–2802.

122. Carr A, Penny R, Cooper DA. Efficacy and safety of rechallenge with low-dose trimethoprim-sulphamethoxazole in previously hypersensitive HIV-infected patients. AIDS. 1993;7(1):65–71.

123. Gluckstein D, Ruskin J. Rapid oral desensitization to trimethoprim-sulfamethoxazole (TMP-SMZ): use in prophylaxis for Pneumocystis carinii pneumonia in patients with AIDS who were previously intolerant to TMP-SMZ. Clin Infect Dis. 1995;20(4):849–853.

124. Bonfanti P, Pusterla L, Parazzini F, Libanore M, Cagni AE, Franzetti M, et al. The effectiveness of desensitization versus rechallenge treatment in HIV-positive patients with previous hypersensitivity to TMP-SMX: a randomized multicentric study. Biomed Pharmacother. 2000;54(1):45–49.

125. Nguyen M-T, Weiss PJ, Wallace MR. Two-day oral desensitization to trimethoprim-sulfamethoxazole in HIV-infected patients. AIDS. 1995;9(6):573–575.

126. Straatmann A, Bahia F, Pedral-Sampaio D, Brites C. A randomized, pilot trial comparing full versus escalating dose regimens for the desensitization of AIDS patients allergic to sulfonamides. Braz J Infect Dis. 2002;6(6):276–280.

127. D'Amelio CM, Del Pozo JL, Vega O, Madamba R, Gastaminza G. Successful

desensitization in a child with delayed cotrimoxazole hypersensitivity: A case report. Pediatr Allergy Immunol. 2016;27(3):320–321.

128. Pyle RC, Butterfield JH, Volcheck GW, Podjasek JC, Rank MA, Li JTC, et al. Successful outpatient graded administration of trimethoprim-sulfamethoxazole in patients without HIV and with a history of sulfonamide adverse drug reaction. J Allergy Clin Immunol Pract. 201;2(1):52–58.

129. Ryan C, Madalon M, Wortham DW, Graziano FM. Sulfa hypersensitivity in patients with HIV infection: onset, treatment, critical review of the literature. WMJ. 1998;97(5):23–27.

130. Lin D, Li WK, Rieder MJ. Cotrimoxazole for prophylaxis or treatment of opportunistic infections of HIV/AIDS in patients with previous history of hypersensitivity to cotrimoxazole. Cochrane Database Syst Rev. 2007;(2):CD005646.

131. Pichler WJ, Naisbitt DJ, Park BK. Immune pathomechanism of drug hypersensitivity reactions. J Allergy Clin Immunol. 2011;127(3):S74–81.

132. Pirmohamed M, Ostrov DA, Park BK. New genetic findings lead the way to a better understanding of fundamental mechanisms of drug hypersensitivity. J Allergy Clin Immunol. 2015;136(2):236–244.

133. Gruchalla RS, Pesenko RD, Do TT, Skiest DJ. Sulfonamide-induced reactions in desensitized patients with AIDS--the role of covalent protein haptenation by sulfamethoxazole. J Allergy and Clin Immunol. 1998;101(3):371–378.

134. Manchanda T, Hess D, Dale L, Ferguson SG, Rieder MJ. Haptenation of sulfonamide reactive metabolites to cellular proteins. Mol Pharmacol. 2002;62(5):1011–1026.

135. Naisbitt DJ, Gordon SF, Pirmohamed M, Burkhart C, Cribb AE, Pichler WJ, et al. Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. Br J Pharmacol. 2001;133(2):295–305.

136. Naisbitt DJ, Farrell J, Gordon SF, Maggs JL, Burkhart C, Pichler WJ, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. Mol Pharmacol. 2002;62(3):628–637.

137. Eyanagi R, Toda A, Imoto M, Uchiyama H, Ishii Y, Kuroki H, et al. Covalent binding of nitroso-sulfonamides to glutathione S-transferase in guinea pigs with delayed type hypersensitivity. Int Immunopharmacol. 2012;12(4):694–700.

138. Lavergne SN, Volkman EM, Maki JE, Yoder AR, Trepanier LA. Evaluation of the clinical, immunologic, and biochemical effects of nitroso sulfamethoxazole administration to dogs: a pilot study. 2005;208(1):63–72.

139. Roychowdhury S, Vyas PM, Svensson CK. Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. Drug Metab Dispos.

2007;35(4):676–681.

140. Wong YY, Rakasz EG, Gasper DJ, Friedrich TC, Trepanier LA. Immunogenicity of trimethoprim/sulfamethoxazole in a macaque model of HIV infection. Toxicology. 2016;368-369:10–18.

141. Sanderson JP, Naisbitt DJ, Farrell J, Ashby CA, Tucker MJ, Rieder MJ, et al. Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. J Immunol. 2007;178(9):5533–5542.

142. Elsheikh A, Castrejon L, Lavergne SN, Whitaker P, Monshi M, Callan H, et al. Enhanced antigenicity leads to altered immunogenicity in sulfamethoxazole-hypersensitive patients with cystic fibrosis. J Allergy Clin Immunol. 2011;127(6):1543–1551.

143. Hertl M, Jugert F, Merk HF. CD8+ dermal T cells from a sulphamethoxazole-induced bullous exanthem proliferate in response to drug-modified liver microsomes. Br J Dermatol. 1995;132(2):215–220.

144. Nassif A, Bensussan A, Dorothée G, Mami-Chouaib F, Bachot N, Bagot M, et al. Drug specific cytotoxic T-cells in the skin lesions of a patient with toxic epidermal necrolysis. J Invest Dermatol. 2002;118(4):728–733.

145. Nassif A, Bensussan A, Boumsell L, Deniaud A, Moslehi H, Wolkenstein P, et al. Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. J Allergy Clin Immunol. 2004;114(5):1209–1215.

146. Mauri-Hellweg D, Bettens F, Mauri D, Brander C, Hunziker T, Pichler WJ. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. J Immunol. 1995;155(1):462–472.

147. Meth MJ, Sperber KE. Phenotypic diversity in delayed drug hypersensitivity: an immunologic explanation. Mt Sinai J Med. 2006;73(5):769–776.

148. Neuman MG, Shear NH, Malkiewicz IM, Taeri M, Shapiro LE, Krivoy N, et al. Immunopathogenesis of hypersensitivity syndrome reactions to sulfonamides. Transl Res. 2007;149(5):243–253.

149. O'Neil CE, Reed MA, Lopez M, Hyslop N, Gutierrez E, Salvaggio J. Evaluation of immune parameters in HIV+ subjects reporting adverse reactions to sulfamethoxazole. Int Arch Allergy Appl Immunol. 1991;94:246–247.

150. Hayashi M, Strouse JJ, Veltri MA, Curtis BR, Takemoto CM. Immune thrombocytopenia due to Trimethoprim-Sulfamethoxazole; under-recognized adverse drug reaction in children? Pediatr Blood Cancer. 2015;62(5):922–923.

151. Hopkins JE, Naisbitt DJ, Humphreys N, Dearman RJ, Kimber I, Park BK. Exposure of mice to the nitroso metabolite of sulfamethoxazole stimulates interleukin 5 production by CD4+ T-cells. Toxicology. 2005;206(2):221–231.

152. von Greyerz S, Zanni MP, Frutig K, Schnyder B, Burkhart C, Pichler WJ. Interaction of sulfonamide derivatives with the TCR of sulfamethoxazole-specific human alpha beta+ T cell clones. J Immunol. 1999;162(1):595–602.

153. Watkins S, Pichler WJ. Sulfamethoxazole Induces a Switch Mechanism in T Cell Receptors Containing TCRVβ20-1, Altering pHLA Recognition. PLoS ONE. 2013;8(10):e76211–23.

154. Cribb AE, Spielberg SP, Griffin GP. N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. Drug Metab Dispos. 1995;23(3):406–414.

155. Cribb AE, Spielberg SP. Sulfamethoxazole is metabolized to the hydroxylamine in humans. Clin Pharmacol Ther. 1992;51:522–526.

156. Lehmann DF. The metabolic rationale for a lack of cross-reactivity between sulfonamide antimicrobials and other sulfonamide-containing drugs. Drug Metab Lett. 2012;6(2):129–133.

157. Funk-Keenan J, Sacco J, Wong YYA, Rasmussen S, Motsinger-Reif A, Trepanier LA. Evaluation of polymorphisms in the sulfonamide detoxification genes CYB5A and CYB5R3 in dogs with sulfonamide hypersensitivity. J Vet Intern Med. 2012;26(5):1126–1133.

158. Lee BL, Wong D, Benowitz NL. Altered patterns of drug metabolism in patients with acquired immunodeficiency syndrome. Clin Pharmacol Ther. 1993;53(5):529–535.

159. van der Ven AJ, Mantel MA, Vree TB, Koopmans PP, van der Meer JW. Formation and elimination of sulphamethoxazole hydroxylamine after oral administration of sulphamethoxazole. Br J Clin Pharmacol. 1994;38(2):147–150.

160. Ven AJ, Vree TB, Ewijk Beneken Kolmer EW, Koopmans PP, Meer JW. Urinary recovery and kinetics of sulphamethoxazole and its metabolites in HIV-seropositive patients and healthy volunteers after a single oral dose of sulphamethoxazole. Br J Clin Pharmacol. 1995;39(6):621–625.

161. Cribb AE, Miller M, Leeder JS, Hill J, Spielberg SP. Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. Implications for idiosyncratic toxicity. Drug Metab Dispos. 1991;19(5):900–906.

162. Rieder MJ, Krause R, Bird IA. Time-course of toxicity of reactive sulfonamide metabolites. Toxicology. 1995;95:141–146.

163. Sim E, Walters K, Boukouvala S. Arylamine N-acetyltransferases: from structure to function. Drug Metab Rev. 2008;40(3):479–510.

164. Cribb AE, Grant DM, Miller MA, Spielberg SP. Expression of monomorphic arylamine N-acetyltransferase (NAT1) in human leukocytes. J Pharmacol Exp Ther. 1991;259(3):1241–1246.

165. Trepanier LA, Miller JL. NADH-dependent reduction of sulphamethoxazole hydroxylamine in dog and human liver microsomes. Xenobiotica. 2000;30(12):1111–1121.

166. Kurian JR, Bajad SU, Miller JL, Chin NA, Trepanier LA. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. J Pharmacol Exp Ther. 2004;311(3):1171–1178.

167. Elahian F, Sepehrizadeh Z, Moghimi B, Mirzaei SA. Human cytochrome b5 reductase: structure, function, and potential applications. Crit Rev Biotechnol. 2012;34(2):134–143.

168. Ott G, Plitzko B, Krischkowski C, Reichmann D, Bittner F, Mendel RR, et al. Reduction of Sulfamethoxazole Hydroxylamine (SMX-HA) by the Mitochondrial Amidoxime Reducing Component (mARC). Chem Res Toxicol. 2014;27(10):1687-1695.

169. Leroux A, Mota Vieira L, Kahn A. Transcriptional and translational mechanisms of cytochrome b5 reductase isoenzyme generation in humans. Biochem J. 2001;355(Pt 2):529–535.

170. Lavergne SN, Kurian JR, Bajad SU, Maki JE, Yoder AR, Guzinski MV, et al. Roles of endogenous ascorbate and glutathione in the cellular reduction and cytotoxicity of sulfamethoxazole-nitroso. Toxicology. 200;222:25–36.

171. Naisbitt DJ, Hough SJ, Gill HJ, Pirmohamed M, Kitteringham NR, Park BK. Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity. Br J Pharmacol. 1999;126(6):1393–1407.

172. Cribb AE, Miller M, Tesoro A, Spielberg SP. Peroxidase-dependent oxidation of sulfonamides by monocytes and neutrophils from humans and dogs. Mol Pharmacol. 1990;38(5):744–751.

173. Naisbitt DJ, Vilar FJ, Stalford AC. Plasma cysteine deficiency and decreased reduction of nitrososulfamethoxazole with HIV infection. AIDS Res Hum Retroviruses. 2000;16(18):1929–1938.

174. Trepanier LA, Yoder AR, Bajad S, Beckwith MD, Bellehumeur JL, Graziano FM. Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. J Acquir Immune Defic Syndr Hum Retrovirol. 2004;36(5):1041–1050.

175. Lehmann DF, Holohan PD, Blair DC. Comparisons of oxidative metabolism and reductive capacity in sulfonamide-tolerant and -intolerant patients with human immunodeficiency virus. J Clin Pharmacol. 1996;36(12):1149–1153.

176. Walmsley SL, Khorasheh S, Singer J, Djurdjev O. A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in Pneumocystis carinii pneumonia prophylaxis. J Acquir Immune Defic Syndr Hum Retrovirol. 1998;19(5):498–505.

177. Hennessy S, Strom BL, Berlin JA, Brennan PJ. Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary Pneumocystis carinii pneumonia prophylaxis. J Gen Intern Med. 1995;10(7):380–386.

178. Carr A, Swanson C, Penny R, Cooper DA. Clinical and laboratory markers of hypersensitivity to trimethoprim-sulfamethoxazole in patients with Pneumocystis carinii pneumonia and AIDS. J Infect Dis. 1993;167(1):180–185.

178. Lee BL, Delahunty T, Safrin S. The hydroxylamine of sulfamethoxazole and adverse reactions in patients with acquired immunodeficiency syndrome. Clin Pharmacol Ther. 1994;56:184–189.

180. Treitinger A, Spada C, Verdi JC, Miranda AFB, Oliveira OV, Silveira MVS, et al. Decreased antioxidant defense in individuals infected by the human immunodeficiency virus. European J Clin Invest. 2000;30(5):454–459.

181. Carr A, Gross AS, Hoskins JM, Penny R, Cooper DA. Acetylation phenotype and cutaneous hypersensitivity to trimethoprim-sulphamethoxazole in HIV-infected patients. AIDS. 1994;8(3):333–337.

182. Kaufmann GR, Wenk M, Taeschner W, Peterli B, Gyr K, Meyer UA, et al. N-acetyltransferase 2 polymorphism in patients infected with human immunodeficiency virus. Clin Pharmacol Ther. 1996;60(1):62–67.

183. Wolkenstein P, Loriot MA, Aractingi S, Cabelguenne A, Beaune P, Chosidow O. Prospective evaluation of detoxification pathways as markers of cutaneous adverse reactions to sulphonamides in AIDS. Pharmacogenetics. 2000;10(9):821–828.

184. O'Neil WM, MacArthur RD, Farrough MJ, Doll MA, Fretland AJ, Hein DW, et al. Acetylator phenotype and genotype in HIV-infected patients with and without sulfonamide hypersensitivity. J Clin Pharmacol. 2002;42(6):613–619.

185. Quirino T, Bonfanti P, Arcidiacono M, Pusterla L, Cagni A, Scaglione F, et al. Acetylator phenotype prevalence in HIV-infected patients without previous trimethoprim-sulfamethoxazole hypersensitivity. Biomed Pharmacother. 1999;53:286–287.

186. Elsheikh A, Lavergne SN, Castrejon JL, Farrell J, Wang H, Sathish J, et al. Drug antigenicity, immunogenicity, and costimulatory signaling: evidence for formation of a functional antigen through immune cell metabolism. J Immunol. 2010;185(11):6448–6460.

187. Lavergne SN, Wang H, Callan HE, Park BK, Naisbitt DJ. “Danger” conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. J Pharmacol Exp Ther. 2009;331(2):372–381.

188. Rabaud C, Charreau I, Izard S, Raffi F, Meiffredy V, Leport C, et al. Adverse reactions to cotrimoxazole in HIV-infected patients: predictive factors and subsequent HIV disease progression. Scand J Infect Dis. 2001;33(10):759–764.

189. Eliaszewicz M, Flahault A, Roujeau JC, Fillet AM. Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. J Am Acad Dermatol. 2002;47:40–46.

190. Wolkenstein P, Charue D, Laurent P, Revuz J, Roujeau JC, Bagot M. Metabolic predisposition to cutaneous adverse drug reactions. Role in toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. Arch Dermatol. 1995;131(5):544–551.

191. Shear NH, Spielberg SP, Grant DM, Tang BK, Kalow W. Differences in metabolism of sulfonamides predisposing to idiosyncratic toxicity. Ann Intern Med. 1986;105(2):179–184.

192. Mohanasundaram J, Rajaram S, Mohanasundaram S. Familial tendency in hypersensitivity reactions to co-trimoxazole. J Indian Med Assoc. 1998;96(1):21–22.

193. Cribb AE. Idiosyncratic reactions to sulfonamides in dogs. J Am Vet Med Assoc. 1989;195(11):1612–1614.

194. Werner LL, Bright JM. Drug-induced immune hypersensitivity disorders in two dogs treated with trimethoprim sulfadiazine: case reports and drug challenge studies. J Am Anim Hosp Assoc. 1983;19:783–790.

195. Rieder MJ, Uetrecht J, Shear NH, Spielberg SP. Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. J Pharmacol Exp Ther. 1988;244(2):724–728.

196. Rieder MJ, Uetrecht J, Shear NH, Cannon M, Miller M, Spielberg SP. Diagnosis of sulfonamide hypersensitivity reactions by in-vitro rechallenge with hydroxylamine metabolites. Ann Intern Med. 1989;110(4):286–289.

197. Leeder JS, Dosch HM, Spielberg SP. Cellular toxicity of sulfamethoxazole reactive metabolites--I. Inhibition of intracellular esterase activity prior to cell death. Biochem Pharmacol. 1991;41(4):567–574.

198. Summan M, Cribb AE. Novel non-labile covalent binding of sulfamethoxazole reactive metabolites to cultured human lymphoid cells. Chem Biol Interact. 2002;142:155–173.

199. Hess DA, Sisson ME, Suria H, Wijsman J, Puvanesasingham R, Madrenas J, et al. Cytotoxicity of sulfonamide reactive metabolites: apoptosis and selective toxicity of CD8(+) cells by the hydroxylamine of sulfamethoxazole. FASEB J. 1999;13(13):1688–1698.

200. Carr A, Tindall B, Penny R, Cooper DA. In vitro cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. Clin Exp Immunol. 1993;94(1):21–25.

201. Reilly TP, Bellevue FH, Woster PM, Svensson CK. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. Biochem Pharmacol. 1998;55(6):803–810.

202. Reilly TP, MacArthur RD, Farrough MJ, Crane LR, Woster PM, Svensson CK. Is hydroxylamine-induced cytotoxicity a valid marker for hypersensitivity reactions to sulfamethoxazole in human immunodeficiency virus-infected individuals? J Pharmacol Exp Ther. 1999;291(3):1356–1364.

203. Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. Clin Biochem. 2000;33(7):517–524.

204. Spielberg SP. In vitro analysis of idiosyncratic drug reactions. Clin Biochem. 1986;19(2):142–144.

205. Shear NH, Spielberg SP. In vitro evaluation of a toxic metabolite of sulfadiazine. Can J Physiol Pharmacol. 1985;63(11):1370–1372.

206. Elzagallaai AA, Jahedmotlagh Z, Del Pozzo-Magaña BR, Knowles SR, Prasad AN, Shear NH, et al. Predictive value of the lymphocyte toxicity assay in the diagnosis of drug hypersensitivity syndrome. Mol Diagn Ther. 2010;14(5):317–322.

207. Leeder JS, Cannon M, Nakhooda A, Spielberg SP. Drug metabolite toxicity assessed in human lymphocytes with a purified, reconstituted cytochrome P-450 system. J Pharmacol Exp Ther. 1988;245(3):956–962.

208. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63.

209. Tabatabaei AR, Thies RL, Farrell K, Abbott FS. A rapid in vitro assay for evaluation of metabolism-dependent cytotoxicity of antiepileptic drugs on isolated human lymphocytes. Fundam Appl Toxicol. 1997;37(2):181–189.

210. Riley RJ, Cribb AE, Spielberg SP. Glutathione transferase mu deficiency is not a marker for predisposition to sulphonamide toxicity. Biochem Pharmacol. 1991;42(3):696–698.

211. Westphal GA, Reich K, Schulz TG, Neumann C, Hallier E, Schnuch A. N-acetyltransferase 1 and 2 polymorphisms in para-substituted arylamine-induced contact allergy. Br J Dermatol. 2000;142(6):1121–1127.

212. Watson ID, Cohen HN, Stewart MJ, McIntosh SJ, Shenkin A, Thomson JA. Comparative pharmacokinetics of co-trifamole and co-trimoxazole to “steady state” in normal subjects. Br J Clin Pharmacol. 1982;14(3):437–443.

213. Wang D, Para MF, Koletar SL, Sadee W. Human N-acetyltransferase 1 *10 and *11 alleles increase protein expression through distinct mechanisms and associate with sulfamethoxazole-induced hypersensitivity. Pharmacogenet Genomics. 2011;21(10):652–664.

214. Deloménie C, Grant DM, Mathelier-Fusade P, Jacomet C, Leynadier F, Jacqz-Aigrain E, et al. N-acetylation genotype and risk of severe reactions to sulphonamides in AIDS patients. Br J Clin Pharmacol. 1994;38(6):581–582.

215. Pirmohamed M, Alfirevic A, Vilar J, Stalford A, Wilkins EG, Sim E, et al. Association

analysis of drug metabolizing enzyme gene polymorphisms in HIV-positive patients with co-trimoxazole hypersensitivity. Pharmacogenetics. 2000;10(8):705–713.

216. Wang D, Curtis A, Papp AC, Koletar SL, Para MF. Polymorphism in glutamate cysteine ligase catalytic subunit (GCLC) is associated with sulfamethoxazole-induced hypersensitivity in HIV/AIDS patients. BMC Med Genomics. 2012;5(1):32.

217. Deloménie C, Mathelier-Fusade P, Longuemaux S, Rozenbaum W, Leynadier F, Krishnamoorthy R, et al. Glutathione S-transferase (GSTM1) null genotype and sulphonamide intolerance in acquired immunodeficiency syndrome. Pharmacogenetics. 1997;7(6):519–520.

218. Wolkenstein P, Loriot M-A, Flahault A, Cadilhac M, Caumes E, Eliaszewicz M, et al. Association analysis of drug metabolizing enzyme gene polymorphisms in AIDS patients with cutaneous reactions to sulfonamides. J Invest Dermatol. 2005;125(5):1080–1082.

219. O'Neil WM, Drobitch RK, MacArthur RD, Farrough MJ, Doll MA, Fretland AJ, et al. Acetylator phenotype and genotype in patients infected with HIV: discordance between methods for phenotype determination and genotype. Pharmacogenetics. 2000;10(2):171–182.

220. Wolkenstein P, Carrière V, Charue D, Bastuji-Garin S, Revuz J, Roujeau JC, et al. A slow acetylator genotype is a risk factor for sulphonamide-induced toxic epidermal necrolysis and Stevens-Johnson syndrome. Pharmacogenetics. 1995;5(4):255–28.

221. Zielińska E, Niewiarowski W, Bodalski J, Rebowski G, Skretkowicz J, Mianowska K, et al. Genotyping of the arylamine N-acetyltransferase polymorphism in the prediction of idiosyncratic reactions to trimethoprim-sulfamethoxazole in infants. Pharm World Sci. 1998;20(3):123–130.

222. Zielińska E, Niewiarowski W, Bodalski J. The arylamine N-acetyltransferase (NAT2) polymorphism and the risk of adverse reactions to co-trimoxazole in children. E J Clin Pharmacol. 1998;54:779–785.

223. Gill HJ, Tjia JF, Kitteringham NR, Pirmohamed M, Back DJ, Park BK. The effect of genetic polymorphisms in CYP2C9 on sulphamethoxazole N-hydroxylation. Pharmacogenetics. 1999;9(1):43–53.

224. Yip VLM, Alfirevic A, Pirmohamed M. Genetics of Immune-Mediated Adverse Drug Reactions: a Comprehensive and Clinical Review. Clinic Rev Allerg Immunol. 2014 ;48(2-3):165–175.

225. Ozkaya-Bayazit E, Bayazit H, Ozarmagan G. Drug related clinical pattern in fixed drug eruption. Eur J Dermatol. 2000;10(4):288–291.

226. Kongpan T, Mahasirimongkol S, Konyoung P, Kanjanawart S, Chumworathayi P, Wichukchinda N, et al. Candidate HLA genes for prediction of co-trimoxazole-induced severe cutaneous reactions. Pharmacogenet Genomics. 2015;25(8):402–411.

227. Threlfall AJ, Boag AM, Soutter F, Glanemann B, Syme HM, Catchpole B. Analysis of DLA-DQB1 and polymorphisms in CTLA4 in Cocker spaniels affected with immune-mediated haemolytic anaemia. Canine Genetics and Epidemiology; 2015;2:8.

228. Kennedy LJ, Davison LJ, Barnes A, Short AD, Fretwell N, Jones CA, et al. Identification of susceptibility and protective major histocompatibility complex haplotypes in canine diabetes mellitus. Tissue Antigens. 2006;68(6):467–476.

229. Peiravan A, Allenspach K, Boag AM, Soutter F, Holder A, Catchpole B, et al. Single nucleotide polymorphisms in major histocompatibility class II haplotypes are associated with potential resistance to inflammatory bowel disease in German shepherd dogs. Vet Immunol Immunopathol. 2016;182:101–105.

230. Friedenberg SG, Buhrman G, Chdid L, Olby NJ, Olivry T, Guillaumin J, et al. Evaluation of a DLA-79 allele associated with multiple immune-mediated diseases in dogs. Immunogenetics. 2015;68(3):205–217.

Chapter 2: Genome-wide association study in immunocompetent patients with delayed hypersensitivity to sulfonamide antimicrobials

Jennifer M. Reinhart, Alison Motsinger-Reif, Allison Dickey, Steven Yale,

and Lauren A. Trepanier

Published as: Reinhart JM, Motsinger-Reif A, Dickey A, Yale S, Trepanier LA. Genome-Wide Association Study in Immunocompetent Patients with Delayed Hypersensitivity to Sulfonamide Antimicrobials. PloS One. 201611(6):e0156000.

Abstract

Background – Hypersensitivity (HS) reactions to sulfonamide antibiotics occur uncommonly, but with potentially severe clinical manifestations. A familial predisposition to sulfonamide HS is suspected, but robust predictive genetic risk factors have yet to be identified. Strongly linked genetic polymorphisms have been used clinically as screening tests for other HS reactions prior to administration of high-risk drugs.

Objective – The purpose of this study was to evaluate for genetic risk of sulfonamide HS in the immunocompetent population using genome-wide association.

Methods – Ninety-one patients with symptoms after trimethoprim-sulfamethoxazole (TMP-SMX) attributable to "probable" drug HS based on medical record review and the Naranjo Adverse Drug Reaction Probability Scale, and 184 age- and sex-matched patients who tolerated a therapeutic course of TMP-SMX, were included in a genome-wide association study using both common and rare variant techniques. Additionally, two subgroups of HS patients with a more refined clinical phenotype (fever and rash; or fever, rash and eosinophilia) were evaluated separately.

Results – For the full dataset, no single nucleotide polymorphisms were suggestive of or reached genome-wide significance in the common variant analysis, nor was any genetic locus significant

in the rare variant analysis. A single, possible gene locus association (*COL12A1*) was identified in the rare variant analysis for patients with both fever and rash, but the sample size was very small in this subgroup (n=16), and this may be a false positive finding. No other significant associations were found for the subgroups.

Conclusions – No convincing genetic risk factors for sulfonamide HS were identified in this population. These negative findings may be due to challenges in accurately confirming the phenotype in exanthematous drug eruptions, or to unidentified gene-environment interactions influencing sulfonamide HS.

Introduction

Potentiated sulfonamides, such as sulfamethoxazole (SMX) in combination with trimethoprim (TMP), are effective antibiotics for the treatment of urinary infections, bronchitis, pneumonia, and methicillin-resistant *S. aureus* (MRSA) infections.[1-4] TMP-SMX is also the drug of choice for the prevention of opportunistic protozoal infections, such as toxoplasmosis and *Pneumocystis jiroveci* pneumonia, in immunocompromised patients, particularly in those with AIDS.[5-8]

Despite this broad-spectrum of activity, clinical use of TMP-SMX is limited by the development of idiosyncratic, delayed-type hypersensitivity (HS) reactions including fever and cutaneous drug eruptions and, less commonly, multi-organ dysfunction.[9] In fact, TMP-SMX is the leading cause of cutaneous hypersensitivity reactions, and the most common culprit in severe bullous skin eruptions such as Stevens-Johnson syndrome and toxic epidermal necrolysis, which carry up to 30% mortality.[10-14] Hypersensitivity reactions occur in approximately 3% of TMP-

SMX-treated patients in the general population.[15,16] Both clinical reports and *ex vivo* drug challenge studies have suggested that this syndrome may be familial; thus a genetic basis for sulfonamide HS has been proposed.[17,18]

Pharmacogenetic risk for sulfonamide HS has only been evaluated in a limited number of studies. Unfortunately, most of these studies were in HIV-positive patients, for whom acquired, rather than genetic, risk is more likely.[19-23] Genetic studies have been sparse in immunocompetent patients and have focused predominately on enzymes responsible for SMX biotransformation or on glutathione pathways, which can neutralize reactive drug metabolites. Two reports suggested that "slow" acetylator *NAT2* genotypes were associated with sulfonamide HS.[24,25] However, in a larger study in our laboratory, we found no such association despite full resequencing of *NAT2* coding region.[26] In that same study we also investigated the sulfamethoxazole detoxification genes, *CYB5A* and *CYB5R3*, but polymorphism frequencies were low and were not significantly different between sulfonamide HS and tolerant patients.[26]

*CYP2C9*2/*3* variant alleles have been associated with decreased production of the reactive, SMX-hydroxylamine (SMX-HA) metabolite in human liver microsomes;[27] however, allele frequency variation has not been investigated in immunocompetent patients with sulfonamide HS.[20] Further, an increased risk for cutaneous drug eruptions overall was reported for patients with *GSTM1* and *GSTT1* null genotypes, but this study population was mixed, with sulfonamide HS patients representing only 4/36 affected patients.[28] Finally, an association between an HLA-A30 haplotype and sulfonamide-induced skin eruptions was found in a Turkish population, but HLA genotypes have yet to be studied in other immunocompetent populations.[12]

Beyond these few candidate-gene studies, no other drug biotransformation, redox, or immunoregulatory genes have been evaluated for an association with sulfonamide HS. Genome-wide association studies (GWAS) have successfully identified genetic targets associated with a variety of other adverse drug reactions when a candidate gene approach has proved insufficient.[29-32] Therefore, the purpose of this study was to screen for genetic markers of sulfonamide HS in an immunocompetent population, compared to drug tolerant controls, using a GWAS design.

Methods

Patient Identification

Patients with delayed sulfonamide HS and patients tolerant of a course of sulfonamide antibiotics were identified through the Marshfield Clinic Research Foundation's Personalized Medicine Research Project (PMRP), a cohort of over 20,000 patients who receive their medical care at the Marshfield Clinic, Marshfield, WI. These patients have previously provided informed consent for the use of their samples for biomedical research, linked to de-identified medical records data.[33] Medical records were searched electronically for a history of TMP-SMX administration or for a diagnosis of sulfonamide HS. Marshfield Clinic Research Foundation staff individually reviewed medical records, using a structured abstraction form to identify patients with sulfonamide HS. Each case was adjudicated to ensure consistency and accuracy. The abstraction form included the following eligibility criteria: (1) administration of TMP-SMX for at least 5 days prior to the adverse event;[9] (2) documentation of one or more new clinical signs after starting TMP-SMX, including fever with or without eosinophilia, skin rash, increases

in liver enzyme activities, hyperbilirubinemia, blood dyscrasias (anemia, leukopenia or thrombocytopenia), pneumonitis, myocarditis, aseptic meningitis, polyarthritis, acute interstitial nephritis, toxic epidermal necrolysis, or Stevens-Johnson syndrome;[9] (3) lack of other clinical explanation for the adverse event; and (4) resolution of clinical signs with discontinuation of TMP-SMX alone. Patients with only gastrointestinal symptoms such as nausea, vomiting or diarrhea,[9] or with acute anaphylactoid reactions,[34,35] were excluded. Because some forms of immunosuppression, in particular AIDS, lead to a high acquired risk of SMX hypersensitivity, apparently independent of genotype,[20] immunocompromised patients, including those with HIV infection or undergoing immunosuppressive chemotherapy, were not eligible. These criteria together were designed to yield a score of 6 or more, or "probable" adverse reaction, using the Naranjo Adverse Drug Reaction scale.[36]

Control patients ("tolerant;" TOL) within the PMRP that were prescribed TMP-SMX were enrolled sequentially from medical records in random order, to provide a 2:1 match with the HS patients for race, gender, and decade of age at sulfonamide treatment. TOL patients must have been prescribed a course of TMP-SMX at a standard therapeutic daily dosage for at least 10 days, with adequate follow-up in the medical record to indicate that the drug was taken and tolerated without adverse event. Clinical and demographic variables, including body weight, dosage, duration of treatment, and reason for TMP-SMX prescription were also abstracted. Patient data were provided to the investigators in a de-identified format, and therefore the study protocol was reviewed and granted exemption from federal regulations by the UW Health Sciences human subjects Minimal Risk Institutional Review Board. One investigator (SY) from

the PRMP did have direct access to patient information as a part of the validation process but the review board was aware of this at the time the exemption was granted.

Genotyping, Quality Control, and Data Pre-analysis

DNA samples from 99 HS and 198 TOL patients from the PMRP were genotyped using one or more of the following platforms: HumanCoreExome, Illumina 660Hg18, Illumina 660Hg19, or Infinium (Illumina; San Diego, CA). Genotype-calls were performed in Genome Studio software (Illumina; San Diego, CA). For the HumanCoreExome, 660Hg18, and 660Hg19 platforms, the data was formatted for use in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/).[37,38] The genotyped Infinium raw data files existed in the A/B format and the single nucleotide polymorphisms (SNPs) were converted to their corresponding alleles to match the PLINK format of the other platforms. SNPs in the Infinium dataset whose genotypes were denoted as either an indel (12,602) or had ambiguous strand designations (1,286) were removed. PLINK formatted files for the HumanCoreExome, 660Hg18, and 660Hg19 platforms are available on dbGaP, along with the raw text files for the Infinium platform.

For each platform, called data were filtered for quality control (QC) in PLINK. No patients were removed from the study due to low genotyping (efficiency < 90%). SNPs were excluded if the genotyping efficiency was < 98% or if the minor allele frequency (MAF) was < 0.01.[39] Trend tests for Hardy-Weinberg equilibrium were performed and variants with extreme deviations were also excluded from analyses. Q-Q plots were used to assess overall quality of the

GWAS data, and the quality of any significant SNPs was evaluated manually after association analysis.

Data from the four platforms were combined to form a single dataset. However, few SNPs were shared among all four platforms. Therefore, to maximize the number of SNPs evaluated, only data from the HumanCoreExome and Infinium platforms were included in the final analysis. This resulted in the exclusion of 10 patients (4 HS, 6 TOL) who were only genotyped on the Illumina Hg660 systems. Imputation was performed on the combined dataset using SHAPEIT (https://mathgen.stats.ox.ac.uk/genetics_software/shapeit/shapeit.html#home) and IMPUTE2 (https://mathgen.stats.ox.ac.uk/impute/impute_v2.html) software. The EUR group of individuals (n=379) in the 1000 Genomes Project reference panel (June 2014 release; 1000genomes.org) was used to phase the haplotypes because the majority of the patients in this study were Caucasian. 420 SNPs in the dataset were either missing in the reference panel or had strand incompatibilities and were removed. The number of genotyped SNPs used in the imputation was 226,516 and the resulting number of imputed SNPs that had an info score > 0.7 and that were used in the downstream analyses was 10,329,316.

Including relatives within the same association study can skew results, so the KING program (http://people.virginia.edu/~wc9c/KING/) was used to assess relatedness between subjects. Patients were removed if a relatedness of the 3rd degree or less was identified, resulting in the exclusion of an additional 10 patients (3 HS, 7 TOL). Further population substructure was assessed using EIGENSOFT software (http://www.hsph.harvard.edu/alkes-price/software/) with a linkage disequilibrium pruned set (62,653) from the genotyped SNPs; principal component

analysis identified two clear outliers (1 HS, 1 TOL) from the population, which were removed. The final dataset included 275 patients: 91 HS and 184 TOL.

Common Variant Analysis

Multivariable logistic regression analysis was performed on the genotyped SNPs using PLINK and using SNP dosages for the imputed SNPs. 2-bit dummy-encoding was used to ensure that no genetic model assumptions were made.[39] The first four principle components, determined in EIGENSOFT, were included as covariates to minimize spurious associations due to underlying population substructure. The population was almost entirely Caucasian (99%), so race was not included as a covariate. Similarly, sex and age were not found to be significant covariates (coefficient p-value < 0.05), so were not included in the final model. Before calculating the genomic inflation factor (λ) for the combined genotyped and imputed common variant results, any SNPs with a MAF < 0.01 were removed along with any imputed SNPs that also existed in the genotyped set. This resulted in 226,505 genotyped SNPs and 8,066,491 imputed SNPs. λ was 1.03 and the p-values were adjusted to correct for it. The Q-Q plot is shown in **Figure 2.1A**. All SNPs were assessed in the final analysis, but those within candidate genes previously hypothesized to be involved in sulfonamide HS pathogenesis (**Table 2.1**) were of particular interest. Based on the number of comparisons including both the genotyped and the imputed SNPs, $p \leq 6.03 \times 10^{-7}$ was considered suggestive of significance and $p \leq 6.03 \times 10^{-9}$ was considered significant at the genome-wide level. The Bonferroni correction was used for multiple comparison corrections at an alpha level of 0.05.

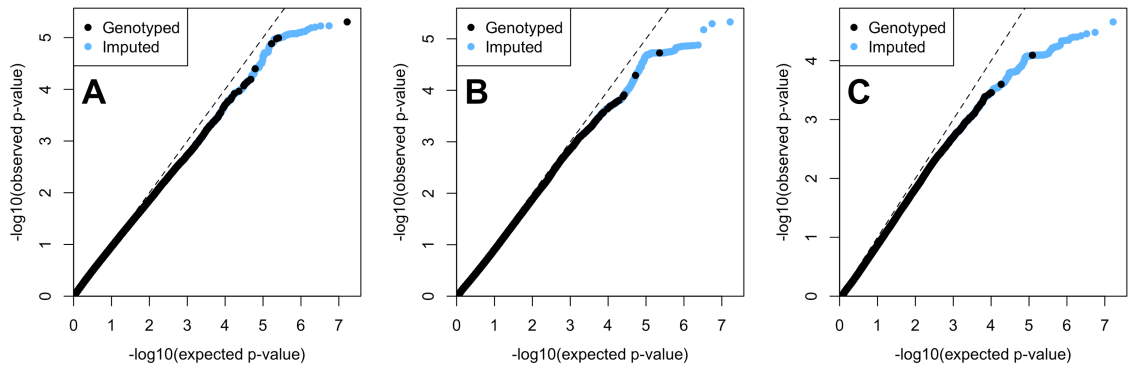

Figure 2.1 – (A) Q-Q plot for common variant analysis of full set of sulfonamide hypersensitive (HS) vs. drug tolerant (TOL) patients (λ=1.03). (B) Q-Q plot for common variant analysis of HS subgroup with rash and fever (FEV-RASH) vs. all TOL patients (λ=0.87). (C) Q-Q plot for common variant analysis of HS subgroup with rash, fever, and eosinophilia (FEV-RASH-EOS) vs. all TOL patients (λ=0.68).

Assuming a multiplicative model of inheritance and a risk-allele frequency of 0.5, an *a priori* power calculation demonstrated that an available sample of 99 HS and 198 TOL patients would yield at least 80% power to detect a genetic risk model with a relative risk of ≥ 3.0, while maintaining an overall family-wise error rate of 10^{-8} (representing a correction at a genome-wide level).

Gene	Protein	Rationale
CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	SMX biotransformation
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8	SMX biotransformation
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	TMP biotransformation
GCLC	Glutamate-cysteine ligase, catalytic subunit	Glutathione pathways
GCLM	Glutamate-cysteine ligase, modifier subunit	Glutathione pathways
GSS	Glutathione synthetase	Glutathione pathways
GSTM1	Glutathione S-transferase mu 1	Glutathione pathways
GSTP1	Glutathione S-transferase pi 1	Glutathione pathways
GSTT1	Glutathione S-transferase tau 1	Glutathione pathways
HLA-A	Major histocompatibility complex, class I, A	Antigen presentation
HLA-B	Major histocompatibility complex, class I, B	Antigen presentation
HLA-C	Major histocompatibility complex, class I, C	Antigen presentation
HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1	Antigen presentation
MARC1	Mitochondrial amidoxime reducing component 1	SMX biotransformation
MARC2	Mitochondrial amidoxime reducing component 2	SMX biotransformation
MPO	Myeloperoxidase	SMX biotransformation
NAT1	N-acetyltransferase 1	SMX biotransformation
NAT2	N-acetyltransferase 2	SMX biotransformation

Table 2.1 – Candidate genes within the GWAS hypothesized to be involved in the pathogenesis of sulfonamide HS, and the mechanistic rationale for inclusion of each candidate gene.

Rare Variant Analysis

The present study was not powered to detect a significant difference in allele frequencies for SNPs with low MAFs.[38] However, associations between low-MAF calls and various diseases have been previously identified, emphasizing the potential importance of these rare variants.[40-42] Therefore, we characterized the influence of rare variants using a combined multivariate and collapsing gene-level approach.[43,44] All SNPs with MAF < 0.03 within an individual gene were collapsed into a single covariate and each gene was included in a multivariate logistical regression model. 17,793 loci were included in this analysis and candidate genes were specifically evaluated. Several rare-variant burden tests (ind, prop, weight) and variance-

component tests (skat, skat-o) were used including those that weighted for possible functional significance of individual SNPs.[45,46] For each locus included in the analysis, p-values from the separate rare-variant tests were combined to yield a single p-value using Lancaster's method.[47] Gene-level associations with model adjusted q-value ≤ 0.05 were considered significant.

Subgroup Analyses

Because definitive diagnosis of sulfonamide HS is difficult and misclassification can occur, HS patients matching a more refined clinical phenotype were included in two separate subgroup analyses. The first subgroup (FEV-RASH) included patients exhibiting both a fever (body temperature > 98.8°F) and cutaneous drug eruption (rash) (n=16). The second subgroup (FEV-RASH-EOS) included patients exhibiting fever, rash, and documented peripheral eosinophilia (eosinophils > 0.5 M/l) on complete blood count (n=8). Each subgroup was compared with the full control group (n=184) in both a common and a rare variant analysis. The genotyped (226,516) and imputed SNPs (10,329,316) that were used as input for the full set common and rare variant analyses were also used as input for the subgroup analyses. After quality filtering the common variant analysis results, 226,444 genotyped and 8,077,026 imputed SNPs remained for subgroup FEV-RASH. λ was 0.87 and the resulting Q-Q plot can be seen in **Figure 2.1B**. 17,766 loci were included in the rare variant analysis. For subgroup FEV-RASH-EOS, 226,415 genotyped and 8,078,605 imputed SNPs remained after the common variant analysis results were quality filtered. λ was 0.68 and the resulting Q-Q plot can be seen in **Figure 2.1C**. 17,778 loci were included in the rare variant analysis in this subgroup. Candidate genes were specifically evaluated in both the common and rare variant analyses.

Results

Patient Population

Ninety-one HS and 184 TOL patients were included in the final analysis. Inclusion and exclusion of subjects are represented in **Figure 2.2**.

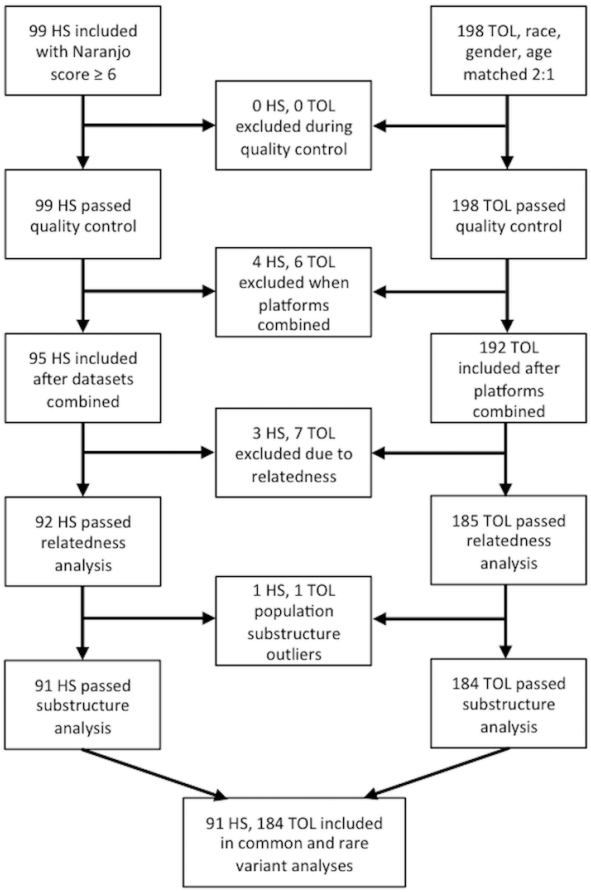

Figure 2.2 – Inclusion and exclusion of recruited subjects.

Patient demographics are summarized in **Table 2.2**. HS patients were predominantly Caucasian (98%) and female (82%), with a median age of sulfonamide treatment of 38 years (range 1–80). Most of the HS patients had exanthematous (95%) or bullous (1%) drug eruptions. Other clinical manifestations included fever (20%), eosinophilia (17%), thrombocytopenia (11%), neutropenia

(5%), anemia (4%), or elevated liver enzymes/hyperbilirubinemia (3%). Matched TOL patients were predominantly Caucasian (99%) and female (83%), with a median age of sulfonamide treatment of 41 years (range 8–88).

	HS (n=91)	**FEV-RASH (n=16)** Subgroup of HS	**FEV-RASH-EOS (n=8)** Subgroup of HS	**TOL (n=184)**
Age at Administration (yr)	40.0 ± 17.2 (1.1-80.4)	40.8 ± 18.7 (1.1-80.4)	46.5 ± 15.0 (24.3-70.1)	41.1 ± 17.3 (8.5-87.7)
Body weight (kg)	77.0 ± 19.2 (13-124)	84.0 ± 16.0 (55-105)	87.0 ± 17.0 (61-97)	85.3 ± 21.4 (27.2-150.0)
Total Daily Dose (mg/kg)	25.7 ± 6.2 (10.3-37.5)	22.9 ± 6.7 (10.3-35.1)	23.0 ± 5.6 (19.8-31.3)	23.5 ± 6.2 (7.0-40.6)
GENDER				
Female	75	10	4	153
Male	16	6	4	31
RACE				
Caucasian	89	15	7	183
Native American	2	1	1	1
DIAGNOSIS				
Urinary Tract Infection	32	5	3	41
Respiratory Tract Infection	55	9	4	101
Other Soft Tissue Infection	2	1	1	38
Unknown/Multiple	2	1	0	4

Table 2.2. Demographic information for sulfonamide hypersensitive (HS) and drug-tolerant (TOL) patients. Continuous data are presented as mean ± standard deviation (range). FEV-RASH = hypersensitive subgroup with fever and rash; FEV-RASH-EOS = hypersensitive subgroup with fever, rash, and eosinophilia. Diagnosis represents the underlying rationale for TMP/SMX prescription. (Note: body weight and therefore total daily dose in mg/kg were not available for 58 patients.)

Common Variant Analysis

The Manhattan plot of the full dataset is shown in **Figure 2.3**. No SNP was suggestive of, or reached, genome-wide significance. Similarly, no SNP in any candidate gene reached significance. The SNP with the lowest p-value was rs160978 ($p = 4.98 \times 10^{-6}$) located in an intergenic region of chromosome 5.

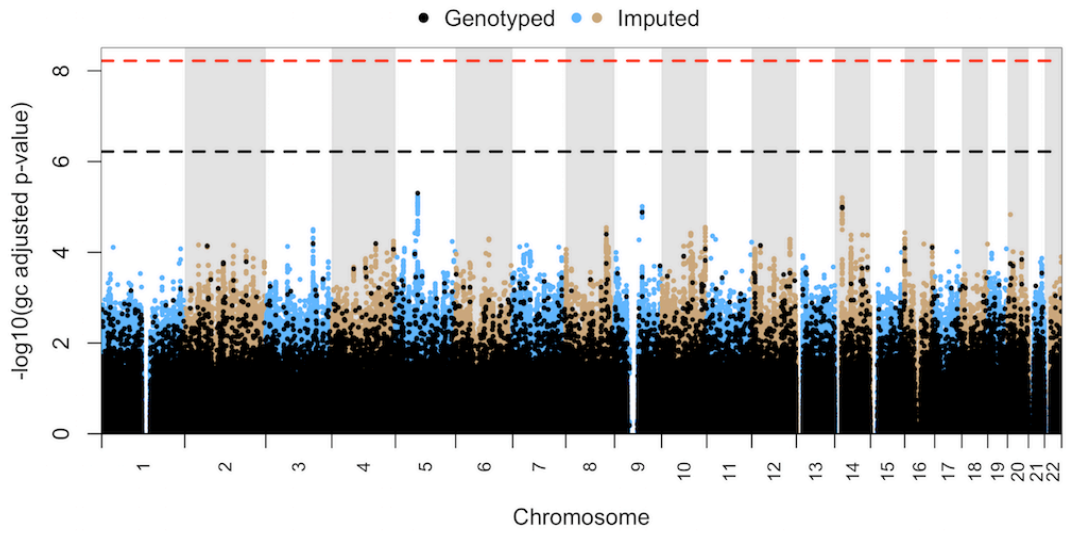

Figure 2.3 – Manhattan plot for common variant analysis of full set of sulfonamide hypersensitive (HS) vs. drug tolerant (TOL) patients. No SNPs were suggestive of ($p \leq 6.03 \times 10^{-7}$) or reached genome-wide significance ($p \leq 6.03 \times 10^{-9}$).

Rare Variant Analysis

Of the 17,793 loci included in this gene-level analysis, there were no statistically significant differences between HS and TOL patients in the full data set. In particular, there were no significant differences for any of the candidate genes. The gene with the lowest q-value was *FNBP1* (q = 0.2558), located on chromosome 9, which codes for formin binding protein 1.

Subgroup Analyses

The 16 patients included in subgroup FEV-RASH were predominantly Caucasian (15/16) and female (10/16) with a median age of 42 years (range 1–70). In addition to fever and rash, 7 had blood dyscrasias (neutropenia, thrombocytopenia, and/or anemia) and 1 had bullous skin eruptions. In the common variant analysis, no SNP was suggestive of or reached genome-wide significance (**Figure 2.4**). However, in the rare variant analysis, there was a statistically

significant difference between the TOL and FEV-RASH groups for the gene *COL12A1* (q = 0.0167) on chromosome 6, which codes for type XII α1 collagen.

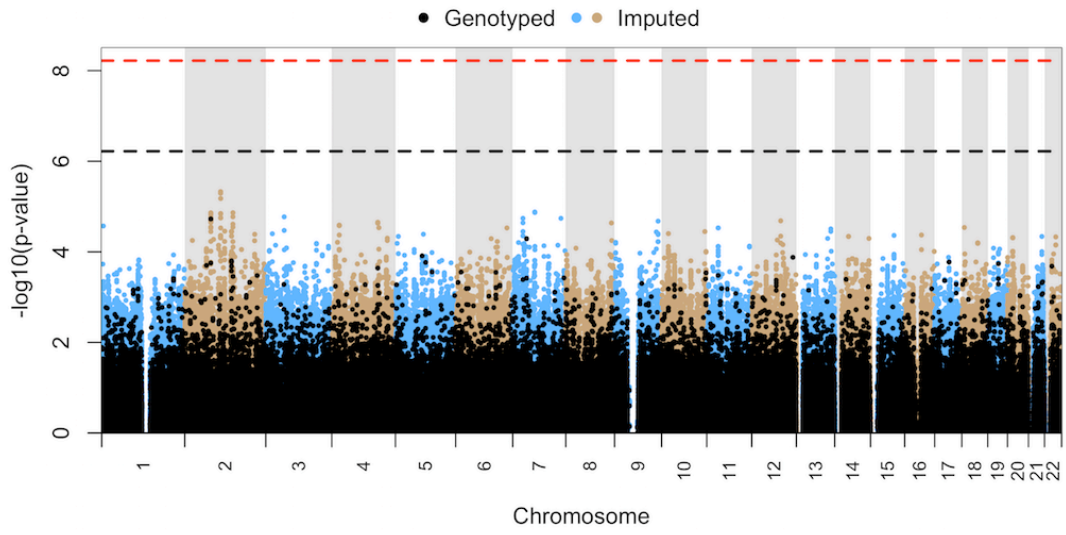

Figure 2.4 – Manhattan plot for common variant analysis of the HS subgroup with rash and fever (FEV-RASH) vs. all TOL patients. No SNPs were suggestive of ($p \leq 6.02 \times 10^{-7}$) or reached genome-wide significance ($p \leq 6.02 \times 10^{-9}$).

The 8 patients in subgroup FEV-RASH-EOS were predominantly Caucasian (7/8) with a median age of 48 years (range 24–70). Males (4/8) and females (4/8) were equally distributed. Six of these patients had blood dyscrasias and 1 exhibited bullous skin eruptions. In the common variant analysis, no SNP was suggestive of, or reached, genome-wide significance (**Figure 2.5**). In the rare variant analysis, there were no significant differences between TOL and FEV-RASH-EOS patients for any locus, including *COL12A1*.

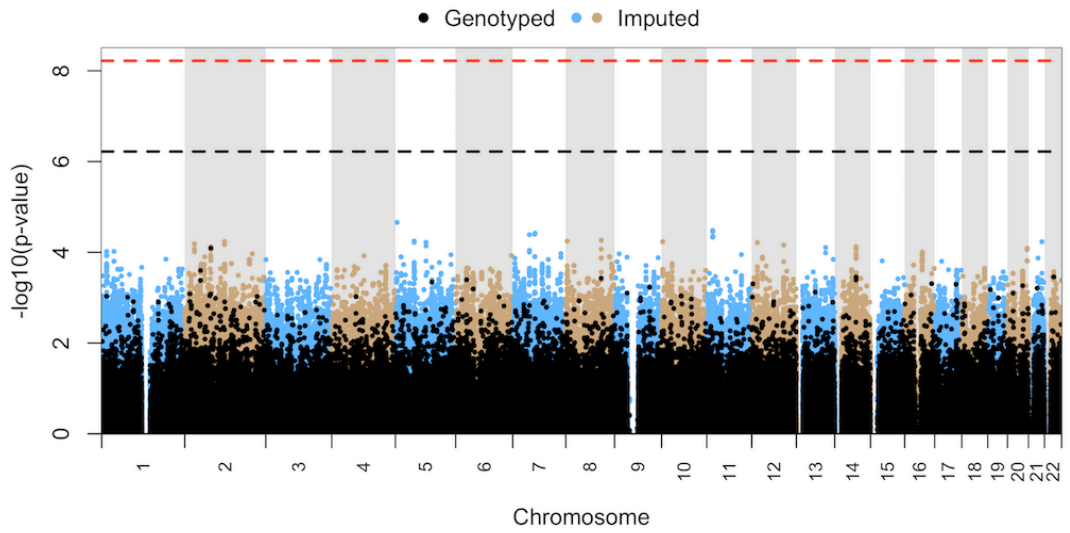

Figure 2.5 – Manhattan plot for common variant analysis of HS subgroup with fever, rash and eosinophilia (FEV-RASH-EOS) vs. all TOL patients. No SNPs were suggestive of ($p \leq 6.02 \times 10^{-7}$) or reached genome-wide significance ($p \leq 6.02 \times 10^{-9}$).

No SNP or locus within the candidate gene set was significant for either subgroup in either the common or rare variant analysis.

Discussion

In the past 15 years, many pharmacogenetic studies have used novel, genome-wide techniques to identify previously unknown genetic risk factors for drug HS reactions.[29-32] Many of the strongest associations have been found for the HLA loci, but other metabolic, transporter, and drug-target genes have also been implicated.[29-32,48-55] Such associations are particularly important to the rapidly growing field of personalized medicine; in fact, the FDA now recommends prospective genotyping for some of these implicated drugs.[56] Although a genetic basis for sulfonamide HS has long been suspected, little work has been done in immunocompetent patients and none at the genomic level.[9] Therefore, the aim of this study was to identify possible genetic risk factors for sulfonamide HS in the general population using a

GWAS approach. Unfortunately, the present study did not demonstrate any genetic associations for sulfonamide HS. These negative findings could result from insufficient study sensitivity, inadequate clinical phenotyping, or represent a true lack of high impact genetic effects for this drug HS syndrome in this population.

The power of a GWAS lies in its ability to simultaneous assess millions of variants across tens of thousands of genes. This provides superior sensitivity over a traditional candidate gene approach in which only one or few genes are investigated. Candidate studies also require an index of suspicion on the part of the investigators that a gene may be mechanistically important to the trait of interest, whereas a genome-wide approach is free from such investigator bias. However, the ability of any study to detect significant differences between groups relies on its sample size and, because a GWAS involves millions of comparisons, large sample sizes are usually required to maintain adequate study sensitivity. The present study was powered to detect a 3-fold increase in relative risk. Pharmacogenetic studies of drug outcomes are typically powered to detect only variants with large genetic effects, because polymorphisms with small effects are unlikely to be adequately predictive to impact clinical decision making.[57] Although the present study was sufficiently powered to detect a relative risk of ≥ 3.0, genotyping more HS and TOL patients would have increased sensitivity and may have allowed us to detect significant risk of lesser magnitude.

Because it is likely that not all of the SNPs are independent, our significance cutoff value of 6.03×10^{-9} may have been overly conservative. If the significance cutoff value were based only on the genotyped SNPs, the p-value would increase from 6.03×10^{-9} to 2.21×10^{-7}. Since

the smallest p-value found in this study was 4.98×10^{-6}, the study conclusions remain the same whether the genotyped SNPs alone, or the genotyped and the imputed SNPs, are used in calculating the significance cutoff value.

The rare variant analysis used in this study accounted for the genetic effects of low MAF SNPs that would have been missed in a traditional GWAS approach. However, this technique does not detect genetic interactions. Development of methods to detect interactions at the SNP, gene, and pathway level is an ongoing area of bioinformatics research.[58-60] Study sensitivity may also have been improved had we used a different modality for genotyping. This GWAS was performed using SNP arrays, which included hundreds of thousands of known variants. Combined with imputation, several million genetic markers were used in this study. However, next-generation, whole-genome sequencing exponentially increases the number of possible SNPs included and also assesses non-SNP variants (e.g. indels, inversions, transpositions). Whole-genome sequencing would also allow direct identification of the causative variant, rather than relying on linkage disequilibrium to identify an area of the genome, which then must be re-sequenced.

Our negative findings emphasize the difficulties in studying patients with a diagnosis of drug hypersensitivity as manifested by exanthematous rash, which can have other etiologies that could be misdiagnosed as a drug reaction. We attempted to minimize this by direct medical record review and use of a validated adverse drug reaction scale. Despite these measures, errors and misinterpretation of medical records can still occur. For example, upon secondary review of the cases, it was discovered that one TOL subject failed to complete the minimum 10-day course

of TMP/SMX due to GI upset. Given that our analyses did not reveal any significant findings, this oversight is unlikely to affect our results, but it does highlight the difficulties in reviewing medical record information in retrospective studies. Furthermore, drug rechallenge remains the gold standard for confirming adverse drug reactions,[36,61] and this was not performed in most patients in our HS group for ethical reasons. In addition, only banked DNA samples were available for most patients, so we were unable to include other potential measures of causality, such as the lymphocyte transformation test or the *in vitro* cytotoxicity assay.[16,62,63] These additional biomarkers may have refined our HS population and possibly provided a more clinically uniform phenotype to study.

Because of concerns about phenotype, we also analyzed a subgroup of patients with fever and rash (FEV-RASH) and a subgroup of patients with characteristics of the DRESS (drug reaction with eosinophilia and systemic signs) syndrome (FEV-RASH-EOS).[64] A single gene, *COL12A1*, was significant for the FEV-RASH group in the rare variant analysis. This gene encodes for type XII collagen, a regulator protein mediating the interaction between type I collagen and the extracellular matrix.[65] Although *COL12A1* has been associated with certain familial myopathies,[66] it is not known to be an immunoreactive protein, and there does not seem to be a logical role for a collagen protein in our current understanding of sulfonamide HS pathogenesis. Thus, this modest association was likely due to a Type I error, particularly since the subgroup analyses were underpowered and at risk for sample size bias.

The lack of identifiable genetic associations for sulfonamide HS could be due to the methodical concerns discussed above, but could also represent a true lack of genetic effect for

this syndrome. Although a few studies do support a familial inheritance pattern,[17,18] most of the evidence is anecdotal. Recent methods developments have allowed for an estimate of the overall phenotypic variance from GWAS data, but the current study was underpowered to get a reliable estimate.[67] Such estimates are often a challenge in pharmacogenomics studies.[68] With a potentially heritable trait, a genome-wide linkage study among members of an affected family appears an attractive option to minimize enrollment and maximize results. However, it is unusual for all family members to have been treated with the same drug. Furthermore, ethical considerations preclude prospective phenotyping by drug challenge in persons who may have a hereditary predisposition to sulfonamide HS.

Epigenetic and environmental factors may also play a role in the development of sulfonamide HS reactions. For example, patients infected with HIV have an incidence of sulfonamide HS of 20-57% compared to 3% in the general population.[69-72] This significant overrepresentation, along with the fact that risk for a reaction increases with declining immune status in AIDS patients,[70,73-76] implies that HIV is an acquired risk factor for sulfonamide HS. Several cases of TMP-SMX-induced HS reactions have also been reported during recrudescence of human herpesvirus-6 (HHV6) infection.[77-79] Although a cause and effect relationship has not been established, it has been suggested that HHV6 reactivation induces pro-inflammatory cytokines, which might predispose to a cell-mediated immune response, constituting another possible acquired risk factor for sulfonamide HS even in the immunocompetent population.[78,79]

In conclusion, we did not identify any convincing genetic associations for sulfonamide HS, manifested primarily as delayed onset of exanthematous drug eruption with or without fever

and eosinophilia, in immunocompetent patients in our Caucasian-American population. These negative findings highlight the need for careful population phenotyping. Clinical use of assays for drug specific T cells or *in vitro* cytotoxicity may be useful, but new biomarkers for confirming HS reactions are also needed. Large, prospective, multicenter studies would allow for real-time evaluation of phenotype rather than relying on medical record adjudication. Additional studies are currently underway in our laboratory to further investigate the immunopathogenesis of sulfonamide HS.

Acknowledgments

The authors would like to thank Terrie Kitchner, Rob Strenn, and the Marshfield Clinic Research Foundation for data abstraction, as well as the University of Wisconsin Biotechnology Center Gene Expression Center for providing the Illumina iScan genotyping services. The authors would also like to thank Skylar Marvel from the North Carolina State University Bioinformatics Research Center for his assistance with data analyses. This study was funded by the by grant R01 GM100784 from the National Institutes of Health. Dr. Reinhart was supported by grant T32 OD010423 from the National Institutes of Health.

References

1. Avgeri SG, Matthaiou DK, Dimopoulos G, Grammatikos AP, Falagas ME. Therapeutic options for Burkholderia cepacia infections beyond co-trimoxazole: a systematic review of the clinical evidence. Int J Antimicrob Ag. 2009;33(5):394–404.
2. Drekonja DM, Johnson JR. Urinary tract infections. Prim Care. 2008;35(2):345–367.
3. Korbila IP, Manta KG, Siempos II, Dimopoulos G, Falagas ME. Penicillins vs trimethoprim-based regimens for acute bacterial exacerbations of chronic bronchitis: meta-analysis of randomized controlled trials. Can Fam Physician. 2009;55(1):60–67.
4. Drekonja DM, Traynor LM, DeCarolis DD, Crossley KB, Johnson JR. Treatment of non-

life-threatening methicillin-resistant *Staphylococcus aureus* infections with alternative antimicrobial agents: a 2-year retrospective review. Diagn Microbiol Infect Dis. 2009;63(2):201–207.

5. Gupta D, Zachariah A, Roppelt H, Patel AM, Gruber BL. Prophylactic antibiotic usage for *Pneumocystis jirovecii* pneumonia in patients with systemic lupus erythematosus on cyclophosphamide: a survey of US rheumatologists and the review of literature. J Clin Rheumatol. 2008;14(5):267–272.

6. Rodriguez M, Sifri CD, Fishman JA. Failure of low-dose atovaquone prophylaxis against *Pneumocystis jiroveci* infection in transplant recipients. Clin Infect Dis. 2004;38(8):e76–78.

7. Seger RA. Modern management of chronic granulomatous disease. Br J Haematol. 2008;140(3):255–266.

8. Thomas M, Rupali P, Woodhouse A, Ellis-Pegler R. Good outcome with trimethoprim 10 mg/kg/day-sulfamethoxazole 50 mg/kg/day for *Pneumocystis jirovecii* pneumonia in HIV infected patients. Scand J Infect Dis. 2009;41(11-12):862–868.

9. Cribb AE, Lee BL, Trepanier LA, Spielberg SP. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. Adverse Drug React Toxicol Rev. 1996;15(1):9–50.

10. Morelli JG, Tay YK, Rogers M, Halbert A, Krafchik B, Weston WL. Fixed drug eruptions in children. J Pediatr. 1999;134(3):365–367.

11. Patel RM, Marfatia YS. Clinical study of cutaneous drug eruptions in 200 patients. Indian J Dermatol Venereol Leprol. 2008;74(4):430.

12. Ozkaya-Bayazit E, Akar U. Fixed drug eruption induced by trimethoprim-sulfamethoxazole: evidence for a link to HLA-A30 B13 Cw6 haplotype. J Am Acad Dermatol. 2001;45(5):712–717.

13. Ortiz JE, Horn MS, Peterson HD. Toxic epidermal necrolysis-case report and review of the literature. Ann Plast Surg. 1982;9(3):249–253.

14. Saka B, Kombaté K, Mouhari-Toure A, Akakpo S, Tchangaï-Walla K, Pitché P. [Stevens-Johnson syndrome and toxic epidermal necrolysis in a teaching hospital in Lomé, Togo: retrospective study of 89 cases]. Med Trop (Mars). 2010;70(3):255–258.

15. Wolkenstein P, Charue D, Laurent P, Revuz J, Roujeau JC, Bagot M. Metabolic predisposition to cutaneous adverse drug reactions. Role in toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. Arch Dermatol. 1995;131(5):544–551.

16. Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. Clin Biochem. 2000;33(7):517–524.

17. Shear NH, Spielberg SP, Grant DM, Tang BK, Kalow W. Differences in Metabolism of Sulfonamides Predisposing to Idiosyncratic Toxicity. Ann Intern Med. 1986;105(2):179–

184.

18. Mohanasundaram J, Rajaram S, Mohanasundaram S. Familial tendency in hypersensitivity reactions to co-trimoxazole. J Indian Med Assoc. 1998;96(1):21–22.

19. Deloménie C, Grant DM, Mathelier-Fusade P, Jacomet C, Leynadier F, Jacqz-Aigrain E, et al. N-acetylation genotype and risk of severe reactions to sulphonamides in AIDS patients. Br J Clin Pharmacol. 1994;38(6):581–582.

20. Pirmohamed M, Alfirevic A, Vilar J, Stalford A, Wilkins EG, Sim E, et al. Association analysis of drug metabolizing enzyme gene polymorphisms in HIV-positive patients with co-trimoxazole hypersensitivity. Pharmacogenetics. 2000;10(8):705–713.

21. Wolkenstein P, Loriot M-A, Flahault A, Cadilhac M, Caumes E, Eliaszewicz M, et al. Association analysis of drug metabolizing enzyme gene polymorphisms in AIDS patients with cutaneous reactions to sulfonamides. J Invest Dermatol. 2005;125(5):1080–1082.

22. Alfirevic A, Vilar FJ, Alsbou M, Jawaid A, Thomson W, Ollier WE, et al. TNF, LTA, HSPA1L and HLA-DR gene polymorphisms in HIV-positive patients with hypersensitivity to cotrimoxazole. Pharmacogenomics. 2009;10(4):531–540.

23. Wang D, Curtis A, Papp AC, Koletar SL, Para MF. Polymorphism in glutamate cysteine ligase catalytic subunit (GCLC) is associated with sulfamethoxazole-induced hypersensitivity in HIV/AIDS patients. BMC Med Genomics. 2012;5(1):32.

24. Zielińska E, Niewiarowski W, Bodalski J. The arylamine N-acetyltransferase (*NAT2*) polymorphism and the risk of adverse reactions to co-trimoxazole in children. E J Clin Pharmacol. 1998;54(9-10):779–785.

25. Wolkenstein P, Carrière V, Charue D, Bastuji-Garin S, Revuz J, Roujeau JC, et al. A slow acetylator genotype is a risk factor for sulphonamide-induced toxic epidermal necrolysis and Stevens-Johnson syndrome. Pharmacogenetics. 1995;5(4):255–258.

26. Sacco JC, Abouraya M, Motsinger-Reif A, Yale SH, McCarty CA, Trepanier LA. Evaluation of polymorphisms in the sulfonamide detoxification genes *NAT2*, *CYB5A*, and *CYB5R3* in patients with sulfonamide hypersensitivity. Pharmacogenet Genomics. 2012;22(10):733–740.

27. Gill HJ, Tjia JF, Kitteringham NR, Pirmohamed M, Back DJ, Park BK. The effect of genetic polymorphisms in *CYP2C9* on sulphamethoxazole N-hydroxylation. Pharmacogenetics. 1999;9(1):43–53.

28. Ateş NA, Tursen U, Tamer L, Kanik A, Derici E. Glutathione S-transferase polymorphisms in patients with drug eruption. Arch Dermatol Res. 2004;295:429-433.

29. Daly AK, Donaldson PT, Bhatnagar P, Shen Y, Pe'er I, Floratos A, et al. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nat Genet. 2009;41(7):816–819.

30. Del-Aguila JL, Beitelshees AL, Cooper-Dehoff RM, Chapman AB, Gums JG, Bailey K, et

al. Genome-wide association analyses suggest *NELL1* influences adverse metabolic response to HCTZ in African Americans. Pharmacogenomics J. 2014;14(1):35–40.

31. Lucena MI, Molokhia M, Shen Y, Urban TJ, Aithal GP, Andrade RJ, et al. Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. Gastroenterology. 2011;141(1):338–347.

32. SEARCH Collaborative Group, Link E, Parish S, Armitage J, Bowman L, Heath S, et al. *SLCO1B1* variants and statin-induced myopathy--a genomewide study. N Engl J Med. 2008;359(8):789–799.

33. McCarty GA. Electronic medical record embedded functions facilitate attending-trainee care oversight and education. AMIA Annu Symp Proc. 2005:1049.

34. Harle DG, Baldo BA, Wells JV. Drugs as allergens: detection and combining site specificities of IgE antibodies to sulfamethoxazole. Mol Immunol. 1988;25(12):1347–1354.

35. Gruchalla RS, Sullivan TJ. Detection of human IgE to sulfamethoxazole by skin testing with sulfamethoxazoyl-poly-L-tyrosine. J Allergy Clin Immun. 1991;88(5):784–792.

36. Naranjo CA, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts EA, et al. A method for estimating the probability of adverse drug reactions. Clin Pharmacol Ther. 1981;30(2):239–245.

37. Laurie CC, Doheny KF, Mirel DB, Pugh EW, Bierut LJ, Bhangale T, et al. Quality control and quality assurance in genotypic data for genome-wide association studies. Genet Epidemiol. 2010;34(6):591–602.

38. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007;81(3):559–575.

39. Putnam PP, Zhang G, Wilsey PA. A comparison study of succinct data structures for use in GWAS. BMC Bioinformatics. 2013;14(1):369.

40. Azzopardi D, Dallosso AR, Eliason K, Hendrickson BC, Jones N, Rawstorne E, et al. Multiple rare nonsynonymous variants in the adenomatous polyposis coli gene predispose to colorectal adenomas. Cancer Res. 2008;68(2):358–363.

41. Cohen JC, Kiss RS, Pertsemlidis A, Marcel YL, McPherson R, Hobbs HH. Multiple rare alleles contribute to low plasma levels of HDL cholesterol. Science. 2004;305(5685):869–872.

42. Walsh T, McClellan JM, McCarthy SE, Addington AM, Pierce SB, Cooper GM, et al. Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. Science. 2008;320(5875):539–543.

43. Li B, Leal SM. Methods for detecting associations with rare variants for common diseases: application to analysis of sequence data. Am J Hum Genet. 2008;83(3):311–321.

44. Lee S, Abecasis GR, Boehnke M, Lin X. Rare-variant association analysis: study designs and statistical tests. Am J Hum Genet. 2014;95(1):5–23.

45. Wu MC, Lee S, Cai T, Li Y, Boehnke M, Lin X. Rare-variant association testing for sequencing data with the sequence kernel association test. Am J Hum Genet. 2011;89(1):82–93.

46. Lee S, Wu MC, Lin X. Optimal tests for rare variant effects in sequencing association studies. Biostatistics. 2012;13(4): 762–775.

47. Lancaster HO. The combination of probabilities: an application of orthonormal functions. Aust J Stat. 1961;3(1):20–33.

48. Pavlos R, Mallal S, Phillips E. HLA and pharmacogenetics of drug hypersensitivity. Pharmacogenomics. 2012;13(11):1285–1306.

49. Quiralte J, Sánchez-García F, Torres MJ, Blanco C, Castillo R, Ortega N, et al. Association of HLA-DR11 with the anaphylactoid reaction caused by nonsteroidal anti-inflammatory drugs. J Allergy Clin Immunol. 1999;103(4):685–689.

50. Hung SI, Chung WH, Liou LB, Chu CC, Lin M, Huang HP, et al. HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. Proc Natl Acad Sci. 2005;102(11):4134–4139.

51. Mallal S, Nolan D, Witt C, Masel G, Martin AM, Moore C, et al. Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. Lancet. 2002;359(9308):727–732.

52. Chung WH, Hung SI, Hong HS, Hsih MS, Yang LC, Ho HC, et al. Medical genetics: a marker for Stevens-Johnson syndrome. Nature. 2004;428(6982):486–486.

53. Rizzo R, Spaggiari F, Indelli M, Lelli G, Baricordi OR, Rimessi P, et al. Association of *CYP1B1* with hypersensitivity induced by taxane therapy in breast cancer patients. Breast Cancer Res Treat. 2010;124(2):593–598.

54. Westphal GA, Schnuch A, Schulz TG, Reich K, Aberer W, Brasch J, et al. Homozygous gene deletions of the glutathione S-transferases M1 and T1 are associated with thimerosal sensitization. Int Arch Occup Environ Health. 2000;73(6):384–388.

55. Takeuchi F, McGinnis R, Bourgeois S, Barnes C, Eriksson N, Soranzo N, et al. A genome-wide association study confirms *VKORC1*, *CYP2C9*, and *CYP4F2* as principal genetic determinants of warfarin dose. PLoS Genet. 2009;5(3). doi:10.1371/journal.pgen.1000433

56. Ziagen [package insert]. Research Triangle Park, NC: GlaxoSmithKline; 2008.

57. Nelson MR, Bacanu S-A, Mosteller M, Li L, Bowman CE, Roses AD, et al. Genome-wide approaches to identify pharmacogenetic contributions to adverse drug reactions. Pharmacogenomics J. 2009;9(1):23–33.

58. Li P, Guo M, Wang C, Liu X, Zou Q. An overview of SNP interactions in genome-wide association studies. Brief Funct Genomics. 2015;14(2):143–155.

59. Xu J, Yuan Z, Ji J, Zhang X, Li H, Wu X, et al. A powerful score-based test statistic for detecting gene-gene co-association. BMC Genet. 2016;17:31.

60. Mooney MA, Nigg JT, McWeeney SK, Wilmot B. Functional and genomic context in pathway analysis of GWAS data. Trends Genet. 2014;30(9):390–400.

61. Bousquet PJ, Gaeta F, Bousquet-Rouanet L, Lefrant JY, Demoly P, Romano A. Provocation tests in diagnosing drug hypersensitivity. Curr Pharm Des. 2008;14:2792–2802.

62. Rieder MJ, Uetrecht J, Shear NH, Cannon M, Miller M, Spielberg SP. Diagnosis of sulfonamide hypersensitivity reactions by *in vitro* rechallenge with hydroxylamine metabolites. Ann Intern Med. 1989;110(4):286–289.

63. Reilly TP, Bellevue FH, Woster PM, Svensson CK. Comparison of the *in vitro* cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. Biochem Pharmacol. 1998;55(6):803–810.

64. Kardaun SH, Sekula P, Valeyrie-Allanore L, Liss Y, Chu CY, Creamer D, et al. Drug reaction with eosinophilia and systemic symptoms (DRESS): an original multisystem adverse drug reaction. Results from the prospective RegiSCAR study. Br J Dermatol. 2013;169(5):1071–1080.

65. Koch M, Bohrmann B, Matthison M. Large and small splice variants of collagen XII: differential expression and ligand binding. J Cell Biol. 1995;130(4):1005–1014.

66. Hicks D, Farsani GT, Laval S, Collins J, Sarkozy A, Martoni E, et al. Mutations in the collagen XII gene define a new form of extracellular matrix-related myopathy. Human Molecular Genetics. 2014;23(9):2353–2363.

67. Yang J, Less SH, Goddard ME, Visscher PM. GCTA: a tool for Genome-wide complex trait analysis. Am J Hum Genet. 2011;88(1):76–82.

68. Motsinger-Reif AA, Jorgenson E, Relling MV, Kroetz DL, Weinshilboum R, Cox NJ, et al. Genome-wide association studies in pharmacogenomics: successes and lessons. Pharmacogenet Genomics. 2013;23(8):383–94.

69. Gordin FM, Simon GL, Wofsy CB, Mills J. Adverse reactions to trimethoprim-sulfamethoxazole in patients with the acquired immunodeficiency syndrome. Ann Intern Med. 1984;100(4):495–499.

70. Hennessy S, Strom BL, Berlin JA, Brennan PJ. Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary *Pneumocystis carinii* pneumonia prophylaxis. J Gen Intern Med. 1995;10(7):380–386.

71. Walmsley SL, Khorasheh S, Singer J, Djurdjev O. A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in

Pneumocystis carinii pneumonia prophylaxis (CTN 057). J Acquir Immune Defic Syndr Hum Retrovirol. 1998;19(5):498–505.

72. Medina I, Mills J, Leoung G, Hopewell PC, Lee B, Modin G, et al. Oral therapy for *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome. A controlled trial of trimethoprim-sulfamethoxazole versus trimethoprim-dapsone. N Engl J Med. 1990;323(12):776–782.

73. Carr A, Swanson C, Penny R, Cooper DA. Clinical and laboratory markers of hypersensitivity to trimethoprim-sulfamethoxazole in patients with *Pneumocystis carinii* pneumonia and AIDS. J Infect Dis. 1993;167(1):180–185.

74. Eliaszewicz M, Flahault A, Roujeau JC, Fillet AM. Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. J Am Acad Dermatol. 2002;47:40-46.

75. Rabaud C, Charreau I, Izard S, Raffi F, Meiffredy V, Leport C, et al. Adverse reactions to cotrimoxazole in HIV-infected patients: predictive factors and subsequent HIV disease progression. Scand J Infect Dis. 2001;33(10):759–764.

76. Ryan C, Madalon M, Wortham DW, Graziano FM. Sulfa hypersensitivity in patients with HIV infection: onset, treatment, critical review of the literature. Wisc Med J. 1998;97(5):23–27.

77. Hubiche T, Milpied B, Cazeau C, Taïeb A, Léauté-Labrèze C. Association of immunologically confirmed delayed drug reaction and human herpesvirus 6 viremia in a pediatric case of drug-induced hypersensitivity syndrome. Dermatology. 2011;222(2):140–141.

78. Morimoto T, Sato T, Matsuoka A. Trimethoprim-sulfamethoxazole-induced hypersensitivity syndrome associated with reactivation of human herpesvirus-6. Int Med. 2006;45(2)101–105.

79. Neuman MG, McKinney KK, Nanau RM, Kong V, Malkiewicz I, Mazulli T, et al. Drug-induced severe adverse reaction enhanced by human herpes virus-6 reactivation. Transl Res. 2013;161(5):430–440.

Chapter 3: RNA expression profiling in sulfamethoxazole-treated patients with a range of lymphocyte cytotoxicity phenotypes

Jennifer M. Reinhart, Warren Rose, Daniel Panyard, Michael Newton, Tyler Liebenstein, Jeremiah Yee, Lauren A. Trepanier

Manuscript in preparation.

Abstract

Background: The lymphocyte toxicity assay (LTA) is a proposed surrogate marker of sulfonamide antibiotic hypersensitivity. In the LTA, peripheral blood mononuclear cells (PBMCs) die more readily in hypersensitive versus tolerant patients when exposed to drug-hydroxylamine metabolites *in vitro*. The underlying mechanism of enhanced cytotoxicity is unknown.

Objective: To identify key gene transcripts associated with increased cytotoxicity from sulfamethoxazole-hydroxylamine in the LTA.

Methodology: The LTA was performed on PBMCs of 10 patients hypersensitive to trimethoprim-sulfamethoxazole (HS) and 10 drug-tolerant controls (TOL), using two cytotoxicity assays: YO-PRO (n = 20) and MTT (n = 12). mRNA expression profiles of PBMCs, enriched for $CD8^+$ T cells, were compared between HS and TOL patients. Transcript expression was interrogated for correlation with % cytotoxicity from YO-PRO and MTT assays. Correlated transcripts of interest were validated by qPCR.

Results: LTA results were not significantly different between HS and TOL patients, and no transcripts were differentially expressed between the two groups. 96 transcripts were correlated with cytotoxicity by YO-PRO. Transcripts were selected for validation based on mechanistic

plausibility, and three were significantly differentially expressed by qPCR in high cytotoxicity patients: mitoferrin-1 (*SLC25A37*, r = -0.63, p = 0.037), multi-specific organic anion transporter C (*ABCC5*, r = -0.49, p = 0.030), and Porimin (*TMEM123*, r = 0.53, p = 0.017).
Conclusions: These data identify novel transcripts that could contribute to sulfonamide-hydroxylamine induced cytotoxicity. These include *SLC25A37*, encoding a mitochondrial iron transporter, *ABCC5*, encoding an arylamine drug transporter, and *TMEM123*, encoding a transmembrane protein that mediates cell death.

Introduction

Sulfonamide antibiotics, such as sulfamethoxazole (SMX) in combination with trimethoprim (TMP), are indicated for the treatment of urinary and respiratory tract infections and resistant infections such as methicillin-resistant *Staph. aureus* (MRSA).[1-4] TMP-SMX is also the drug of choice for the prevention of opportunistic protozoal infections in immunocompromised patients, including those with AIDS.[5-8] Unfortunately, potentiated sulfonamide antibiotics can cause idiosyncratic drug toxicity, also called sulfonamide hypersensitivity (HS) or "sulfa allergy,"[9] which is characterized by delayed onset of fever and pruritic skin rash after 5-14 days of treatment. Less commonly, HS can progress to multi-organ involvement and/or severe bullous skin eruptions such as Stevens-Johnson syndrome and toxic epidermal necrolysis, with up to 30% mortality.[10,11]

Sulfonamide HS occurs in both immunocompromised and immunocompetent populations with incidence rates of 20-57% and ~3%, respectively.[12-16] In the former, risk for drug reaction is primarily an acquired risk and associated with advancing disease status.[13,17-19] In contrast, there

is some evidence that sulfonamide HS in immunocompetent patients has a familial component, suggesting that genetic risk factors may be present.[20-22]

A few polymorphic candidate genes have been investigated for associations with sulfonamide HS, focusing predominantly on SMX biotransformation and detoxification. Genotype of N-acetyltransferase (*NAT2*), the primary SMX detoxification enzyme, has previously been associated with sulfonamide HS,[23,24] but a larger, more recent study in our laboratory, no associations with were present despite resequencing of the *NAT2* coding region.[25] In that same study, polymorphism frequencies in another detoxification system, *CYB5A* and *CYB5R3*, were not significantly different between sulfonamide HS and tolerant patients.[25] An increased risk for cutaneous drug eruptions overall was reported for patients with *GSTM1* and *GSTT1* null genotypes, but only 4/36 patients were hypersensitive to sulfonamide antibiotics.[26] Also, an association between sulfonamide-induced skin eruptions and an HLA-A haplotype was found in a Turkish population.[27] Given these sparse results, our group recently performed a genome-wide association study in immunocompetent individuals, but did not identify any convincing genetic associations with sulfonamide HS,[28] emphasizing the complex nature of this trait. Therefore, alternate approaches are needed to understand the mechanisms of risk for sulfonamide HS in the general population.

In both immunocompetent and HIV-infected patients, sulfonamide HS has been associated with a surrogate marker, the lymphocyte toxicity assay (LTA). Results of this *in vitro* cytotoxicity assay show that peripheral blood mononuclear cells (PBMCs) from HS patients are more susceptible to apoptosis in the presence of sulfonamide metabolites, particularly SMX-HA,

when compared to cells from sulfonamide-tolerant patients.[29-32] Furthermore, $CD8^+$ T cells appear to be most susceptible to the LTA response.[33] Cytotoxicity in the LTA has been measured by multiple methods, including trypan dye exclusion,[21,34] MTT conversion,[29-31,35-37] and YO-PRO fluorescence.[38]

These findings have been interpreted as a "detoxification defect" in HS but not tolerant patients.[32,39,40] Furthermore, this apparent defect can be demonstrated in family members of HS patients who have never been administered potentiated sulfonamides,[20,21,39] which suggests a heritable component. However, the mechanism for this possible defect is not understood. Therefore, the purpose of this study was to identify key gene transcripts and pathways that are associated with increased cytotoxicity in the LTA in patients treated with potentiated sulfonamides.

Methods

Subject Recruitment

Patients with sulfonamide HS and controls tolerant of a course of potentiated sulfonamide antibiotics were identified and recruited through the University of Wisconsin-Madison Hospital and Clinics. Additional patients and controls were recruited among the UW School of Veterinary Medicine's faculty, staff, and students. Medical records were searched electronically for a history of TMP-SMX administration or for a diagnosis of sulfonamide HS, and then were reviewed using a structured abstraction form. Each case was adjudicated to ensure consistency and accuracy. The abstraction form included the following eligibility criteria: (1) administration of TMP-SMX for at least 5 days prior to the adverse event;[9] (2) documentation of one or more

new clinical signs after starting TMP-SMX, including fever with or without eosinophilia, skin rash, increases in liver enzyme activities, hyperbilirubinemia, blood dyscrasias (anemia, leukopenia or thrombocytopenia), pneumonitis, myocarditis, aseptic meningitis, polyarthritis, acute interstitial nephritis, toxic epidermal necrolysis, or Stevens-Johnson syndrome;[9] (3) lack of other clinical explanation for the adverse event; and (4) resolution of clinical signs with discontinuation of TMP-SMX alone. Patients with only gastrointestinal symptoms such as nausea, vomiting or diarrhea,[9] or with acute anaphylactoid reactions,[41,42] were excluded. Because some forms of immunosuppression, in particular AIDS, lead to a high acquired risk of SMX hypersensitivity, apparently independent of genotype,[43] immunocompromised patients, including those with HIV infection or undergoing immunosuppressive chemotherapy, were not eligible. These criteria together were designed to yield a score of 6 or more, or "probable" adverse reaction, using the Naranjo Adverse Drug Reaction scale.[44] Control patients ("tolerant;" TOL) must have been prescribed a course of TMP-SMX at a standard therapeutic daily dosage for at least 10 days, with adequate follow-up to indicate that the drug was taken and tolerated without adverse event. Clinical and demographic variables, including dosage, duration of treatment, and reason for TMP-SMX prescription were also abstracted. This protocol was approved by the UW Health Sciences Institutional Review Board (Protocol # 2011-0512).

Sample Collection and Processing

For each subject, 40 ml of heparinized blood were collected and PBMCs isolated using standard density centrifugation. $CD8^+$ T cells were isolated from an aliquot of PBMCs (10.0 $x10^6$) using an antibody-based, negative selection magnetic bead system (MACS $CD8^+$ T cell Isolation Kit; Miltenyi Biotec Inc., Auburn, CA, USA). However, based on flow cytometry, the

resultant cell population was not purely $CD8^+$ T cells; therefore, these cells were considered $CD8^+$ T cell-enriched PBMCs. These enriched PBMCs were placed in RNAlater and stored at -80°C until RNA isolation. The remainder of the PBMCs was immediately used for the lymphocyte toxicity assay (LTA). Unless otherwise noted, chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Lymphocyte Toxicity Assay

Assays were performed in quadruplicate in 96-well microtiter plates. PBMCs were suspended in cell media (Hank's balanced salt solution, 5% fetal bovine serum, 1% penicillin-streptomycin solution, 15 mM HEPES) and incubated with 25–800 μM SMX-HA (Dalton Chemicals; Toronto, CA), and 1 mM SMX or vehicle as negative controls, at 37°C for 2 hours. To remove free drug, cells were washed twice in phosphate buffered saline (PBS), resuspended in 100 μL cell media, and incubated at 37°C for another 19 hours. Cytotoxicity was determined using the YO-PRO method: YO-PRO (12 μM) was added to each well and fluorescence measured using a 96-well plate reader (ex. 485, em. 530; Synergy 2; BioTek Instruments, Inc., Winooski, VT). To determine the total amount of cells in each well, cells were lysed with the addition 15 μL of 2% Triton X and incubated for at room temperature for 1 hour; fluorescence was then re-measured. For each condition, % cytotoxicity was defined as pre-Triton treatment fluorescence divided by the post-Triton treatment value; values were normalized by subtracting the % cytotoxicity of the vehicle from that of each drug-treated condition.

For a subset of samples, % cytotoxicity was also determined using the MTT detection method: 10 μL of 12 nM MTT was added to each well and incubated at 37°C for 4 hours to

allow generation of formazan. The formazan was solubilized with 100 µL 0.1 N HCl in isopropyl alcohol and absorbance measured at 570 nm. For each condition, cell viability was defined as absorbance divided by the absorbance of the vehicle; % cytotoxicity was then calculated as 100% minus % viability.

RNA Isolation and Expression Microarray of $CD8^+$ T cell-enriched PBMCs

$CD8^+$ T cell-enriched PBMC samples were thawed washed with 1 volume PBS. Samples were centrifuged at 4,000 *x g* at 4°C for 10 minutes and supernatant removed. RNA was isolated using the TRIzol/chloroform method: 1 ml TRIzol (ThermoFisher Scientific; Madison, WI) was added to the cell pellet and homogenized by repeated pipetting. Then 200 µg glycogen (ThermoFisher Scientific; Madison, WI) was added and genomic DNA sheared by passage through a 25 g needle. To each tube, 200 µL chloroform was added, shaken, and centrifuged at 16,100 *xg* at 4°C for 5 minutes. The aqueous phase was transferred to a clean tube and 500 µL ice-cold 100% isopropanol added. RNA was precipitated for 1 hour at -20°C. Tubes were centrifuged at 16,100 *xg* at 4°C for 10 minutes, supernatant removed, and the pellet allowed to dry completely. RNA was then resuspended in nuclease free water. Genomic DNA contaminants were digested using TURBO DNA-free (ThermoFisher Scientific; Madison, WI) and residual extraction reagents removed using RNeasy MinElute Cleanup (Qiagen; Hilden, Germany) according to manufacturers' directions.

Prior to expression profiling, RNA quality was assessed on the Agilent 2100 Bioanalyzer (RNA 6000 Pico Kit; Agilent Technologies; Santa Clara, CA); samples with an RNA Integrity Number ≥ 8 were used for expression arrays.[45] Microarray expression profiling was performed

using the Human Transcriptome Array 2.0 (Affymetrix; Santa Clara, CA) on the Affymetrix GeneChip platform through the University of Wisconsin Biotechnology Center.

Validation of Transcript Expression with Quantitative PCR (qPCR)

Expression levels of genes of interest identified by the microarray associated with % cytotoxicity were verified with qPCR. Reverse transcription with random oligomers (Superscript IV VILO cDNA Synthesis Kit; ThermoFisher Scientific; Madison, WI) was used to generate cDNA. Primers were designed for the target gene transcripts along with two reference genes (*GUSB*, *HPRT1*) and primer efficiencies determined for each using a 10-fold dilution series of pooled cDNA. SYBR Green (LightCycler 480 SYBR Green I Master; Roche Diagnostics; Indianapolis, IN) qPCR with melting curves was performed on the LightCycler 96 Instrument (Roche; Diagnostics; Indianapolis, IN) with parameters adapted from manufacturer's directions (**Table 3.1**).

Target (°C)	**Acquisition Mode**	**Hold (mm:ss)**	**Ramp Rate (°C/s)**
Pre-Incubation			
95	None	05:00	4.4
Amplification			
95	None	00:10	4.4
55	None	00:20	2.2
72	Single	00:20	4.4
Melting Curve			
95	None	00:05	4.4
65	None	01:00	2.2
97	Continuous	-	-
Cooling			
40	None	00:10	1.5

Table 3.1 – LightCycler 96 parameters used for SYBR Green qPCR with 45 amplification cycles and 20 µL reaction volumes.

Statistical Analysis

LTA analysis: Continuous data are presented as mean ± standard deviation. For both YO-PRO and MTT detection methods, mean % cytotoxicity at 800 μM SMX-HA was compared between TOL and HS patients using a Student's t test. Correlation between YO-PRO and MTT methods was assessed by comparing % cytotoxicity (at 800 μM SMX-HA) using the Pearson's correlation coefficient. Analyses were performed with commercial statistical software (Prism 7; GraphPad Software Inc.; La Jolla, CA).

Microarray analysis: Microarray analysis was performed in R (www.r-project.org) and Bioconductor (bioconductor.org). Raw data were retrieved from .CEL files and normalized by the Robust Multi-chip Average (RMA) method. Probe level signals were collapsed to the gene level and gene-specific effects were removed using the allez function to generate gene-level scores. Three separate analyses were performed on these scores. The first assessed for differential expression based on clinical status (TOL vs. HS patients). The second and third assessed for correlations between gene expression and % cytotoxicity as determined by the YO-PRO and MTT methods, respectively. For assessment of correlation, independent filtering was used to remove genes of low variance from the analyses.[46] Of the genes assessed, candidate genes previously hypothesized to be involved in sulfonamide HS pathogenesis (**Table 3.2**) were of particular interest. Transcripts significantly associated with % cytotoxicity by YO-PRO were included in a pathway analysis, which grouped transcripts by gene ontology (GO) terms.

Gene	Protein	Rationale
CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	SMX biotransformation
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8	SMX biotransformation
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	TMP biotransformation
GCLC	Glutamate-cysteine ligase, catalytic subunit	Glutathione pathways for reactive drug metabolites
GCLM	Glutamate-cysteine ligase, modifier subunit	Glutathione pathways
GSS	Glutathione synthetase	Glutathione pathways
GSTM1	Glutathione S-transferase mu 1	Glutathione pathways
GSTP1	Glutathione S-transferase pi 1	Glutathione pathways
GSTT1	Glutathione S-transferase tau 1	Glutathione pathways
HLA-A	Major histocompatibility complex, class I, A	Antigen presentation
HLA-B	Major histocompatibility complex, class I, B	Antigen presentation
HLA-C	Major histocompatibility complex, class I, C	Antigen presentation
HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1	Antigen presentation
MARC1	Mitochondrial amidoxime reducing component 1	SMX biotransformation
MARC2	Mitochondrial amidoxime reducing component 2	SMX biotransformation
MPO	Myeloperoxidase	SMX biotransformation
NAT1	N-acetyltransferase 1	SMX biotransformation
NAT2	N-acetyltransferase 2	SMX biotransformation

Table 3.2. Candidate genes within the microarray hypothesized to be involved in the pathogenesis of sulfonamide HS, and the mechanistic rationale for inclusion of each candidate gene.

Gene	Primers	Product	Efficiency
RPL37	F 5' AGTGCCTTCTCTTCCGGTCT 3' R 5' CAAACGATGACGTTCCCTTC 3'	70 bp	2.03
SLC25A37	F 5' CCACCCACATGACAGCAG 3' R 5' TCAAACTCTGCATTCGTGTCTT 3'	93 bp	2.01
ABCC5	F 5' CCGCCAGTTGAGATCAATTC 3' R 5' ACCCTGCCCTTTCTCTTC 3'	150 bp	1.96
TMEM123	F 5' CACAACTCCAGTGCTAACTC 3' R 5' CGCTGTAGGTTTCATGGTG 3'	147 bp	2.02
TMEM129	F 5' CTCGCCAGACTCGAACTTG 3' F 5' TCCCCGTACTCAGTGGAGTT 3'	108 bp	1.90
GUSB	F 5' CGCCCTGCCTATCTGTATTC 3' R 5' TCCCCACAGGGAGTGTGTAG 3'	91 bp	1.94
HPRT1	F 5' TGACCTTGATTTATTTTGCATACC 3' R 5' CGAGCAAGACGTTCAGTCCT 3'	66 bp	2.03

Table 3.3 – Primers used in qPCR validation of gene transcripts of interest.

qPCR validation analysis: For each target gene (**Table 3.3**), ratios normalized to a pooled cDNA calibrator were generated using the LightCycler 96 software (Roche Diagnostics; Indianapolis, IN). The correlation between normalized expression ratios and % cytotoxicity were assessed by the Pearson's correlation coefficient in Prism (GraphPad Software Inc.; La Jolla, CA).

Results

Patient Population

Ten HS and 10 TOL patients were including in this study. Patient demographics are summarized in **Table 3.4**. The drug reaction was characterized by cutaneous rash in all 10 HS patients, and only one patient had a documented fever. No other clinical symptoms of sulfonamide HS were reported in this population.

	TOL (n=10)	**HS (n=10)**
Age at Administration (yr)	42 (17-73)	38 (21-58)
Body weight (kg)	81 (42-123)	60 (55-68)
Total Daily Dose (mg/kg)	14.2 (6.5-23.2)	16.5 (11.7-28.4)
GENDER		
Female	8	10
Male	2	0
RACE		
Caucasian	10	9
African American	0	1
DIAGNOSIS		
Urinary Tract Infection	3	3
Respiratory Tract Infection	3	5
Other Soft Tissue Infection	3	1
Unknown/Multiple	1	1

Table 3.4 – Demographic information for drug-tolerant (TOL) and sulfonamide hypersensitive (HS) patients. Continuous data are presented as mean (range).

Lymphocyte Toxicity Assays (LTAs)

The LTA with YO-PRO detection was performed for all 20 patients (10 TOL, 10 HS); the LTA with MTT detection was only available for 12 patients (6 TOL, 6 HS) because of limited PBMC availability. Unexpectedly, there was no significant difference in % cytotoxicity between TOL and HS patients for either the YO-PRO (41.5 ± 19.2% vs. 38.1 ± 15.4%, p = 0.67; **Figure 3.1A**) or MTT (50.8 ± 21.3% vs. 39.9 ± 34.2%, p = 0.52; **Figure 3.1B**) assays. Furthermore, % cytotoxicity between the two methods did not correlate across patients (r = 0.11, 95% CI -0.50 – 0.64, p = 0.74). Microarray data are available in the GEO database (www.ncbi.nlm.nih.gov/geo; accession number GSE100443).

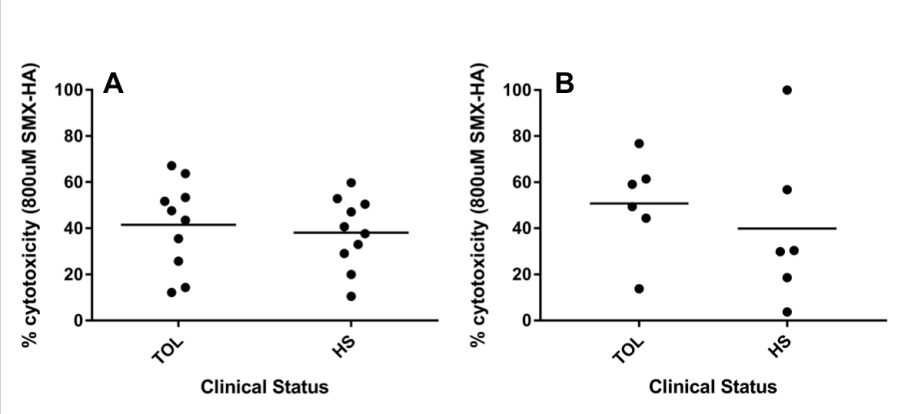

Figure 3.1 – Relationship between clinical status and LTA results detected by the YO-PRO and MTT methods. (A) Scatter plot of % cytotoxicity with YO-PRO detection for TOL vs. HS patients, p = 0.669; the black line represents the mean for each group. (B) Scatter plot of % cytotoxicity with YO-PRO detection for TOL vs. HS patients, p = 0.522; the black line represents the mean for each group.

Gene Expression by Clinical Status and Cytotoxicity

Expression data from all 20 patients (10 HS, 10 TOL) were included in the final comparison. No transcripts (candidate gene or otherwise) were significantly up- or down-regulated in the HS compared to the TOL group.

For cytotoxicity using YO-PRO, 96 gene transcripts were significantly correlated with % cytotoxicity at a FDR of 0.188 (**Figure 3.2; Table 3.5**). No candidate genes were among these transcripts. Seven GO terms were identified in the pathway analysis that contained more than one correlated transcript (**Figure 3.3**). Of interest, a group of 8 ribosomal protein genes (*RPL13*, *RPL21*, *RPL24*, *RPL37*, *RPL38*, *RPL9*, *RPS11*, *RPS29*) was identified in 4 of the 7 GO terms. All 8 of these genes were positively correlated with cytotoxicity.

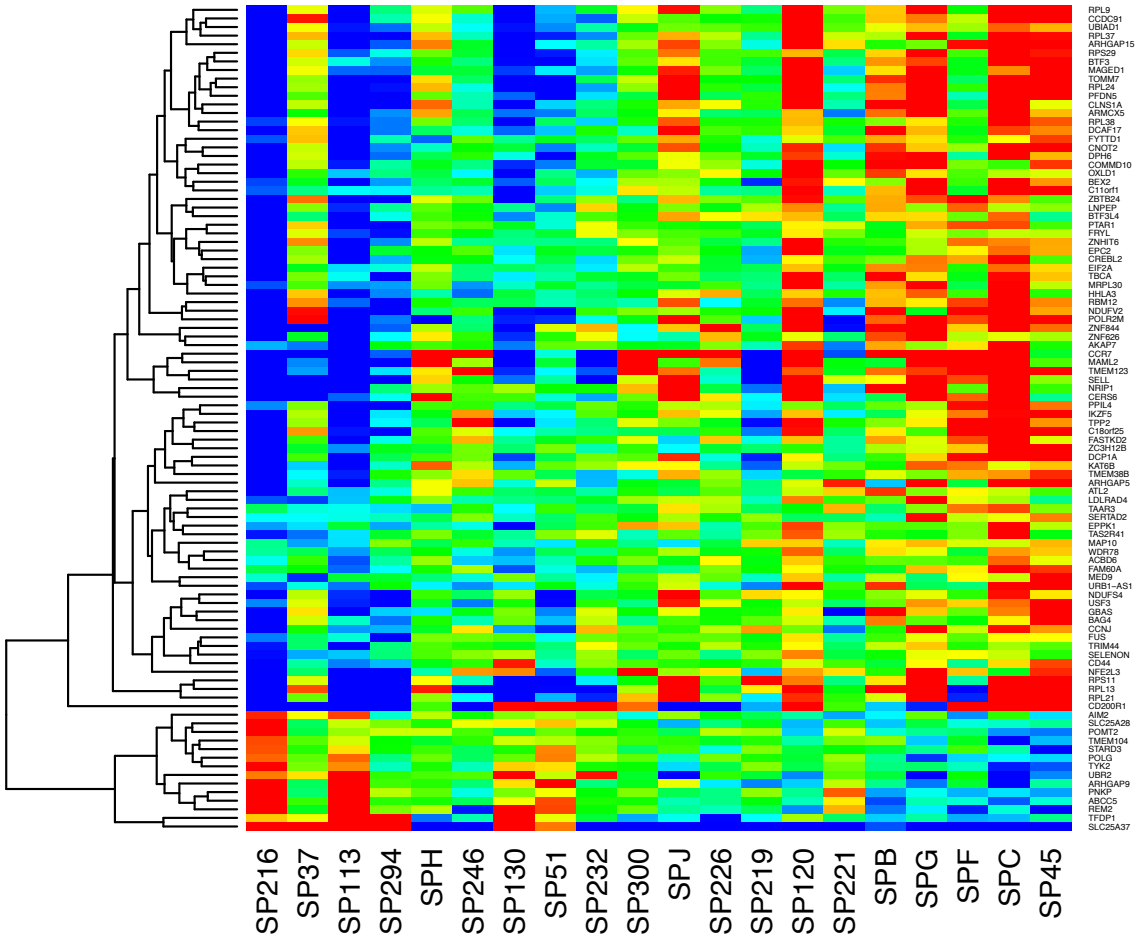

Figure 3.2 – Heat map of 96 transcripts correlated with LTA results by the YO-PRO method. The 20 samples are listed along the x-axis in order of lowest (left) to highest (right) % cytotoxicity.

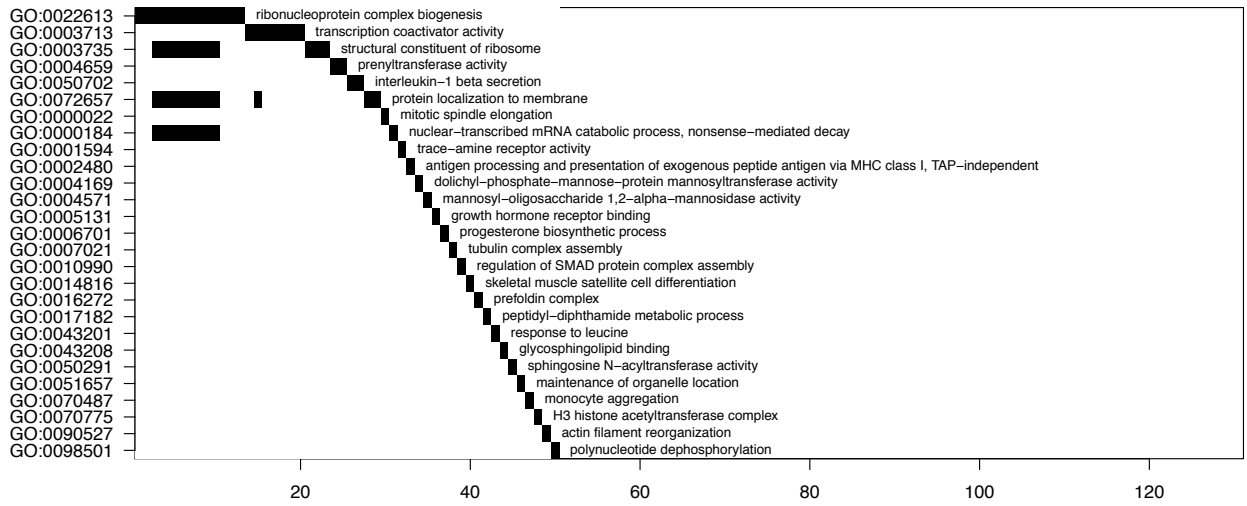

Figure 3.3 – Waterfall plot of pathway analysis. GO terms are listed along the y-axis, number of transcripts identified for each term is indicated on the x-axis.

Gene Symbol	Name	r	Gene Symbol	Name	r
ABCC5	ATP Binding Cassette Subfamily C Member 5	-0.7	NDUFV2	NADH:Ubiquinone Oxidoreductase Core Subunit V2	0.64
ACBD6	Acyl-CoA Binding Domain Containing 6	0.68	NFE2L3	Nuclear Factor, Erythroid 2 Like 3	0.64
AIM2	Absent In Melanoma 2	-0.69	NRIP1	Nuclear Receptor Interacting Protein 1	0.66
AKAP7	A-Kinase Anchoring Protein 7	0.64	OXLD1	Oxidoreductase Like Domain Containing 1	0.64
ARHGAP15	Rho GTPase Activating Protein 15	0.69	PFDN5	Prefoldin Subunit 5	0.69
ARHGAP5	Rho GTPase Activating Protein 5	0.67	PNKP	Polynucleotide Kinase 3'-Phosphatase	-0.64
ARHGAP9	Rho GTPase Activating Protein 9	-0.64	POLG	DNA Polymerase Gamma, Catalytic Subunit	-0.66
ARMCX5	Armadillo Repeat Containing, X-Linked 5	0.65	POLR2M	RNA Polymerase II Subunit M	0.66
ATL2	Atlastin GTPase 2	0.7	POMT2	Protein O-Mannosyltransferase 2	-0.68
BAG4	BCL2 Associated Athanogene 4	0.64	PPIL4	Peptidylprolyl Isomerase Like 4	0.7
BEX2	Brain Expressed X-Linked 2	0.72	PTAR1	Protein Prenyltransferase Alpha Subunit Repeat Containing 1	0.64
BTF3	Basic Transcription Factor 3	0.65	RBM12	RNA Binding Motif Protein 12	0.64
BTF3L4	Basic Transcription Factor 3 Like 4	0.68	REM2	RRAD And GEM Like GTPase 2	-0.67
C11orf1	Chromosome 11 Open Reading Frame 1	0.66	RPL13	Ribosomal Protein L13	0.65
C18orf25	Chromosome 18 Open Reading Frame 25	0.66	RPL21	Ribosomal Protein L21	0.65
CCDC91	Coiled-Coil Domain Containing 91	0.65	RPL24	Ribosomal Protein L24	0.66
CCNJ	Cyclin J	0.64	RPL37	Ribosomal Protein L37	0.65
CCR7	C-C Motif Chemokine Receptor 7	0.64	RPL38	Ribosomal Protein L38	0.64
CD200R1	CD200 Receptor 1	0.71	RPL9	Ribosomal Protein L9	0.63
CD44	CD44 Molecule (Indian Blood Group)	0.67	RPS11	Ribosomal Protein S11	0.66
CERS6	Ceramide Synthase 6	0.64	RPS29	Ribosomal Protein S29	0.65
CLNS1A	Chloride Nucleotide-Sensitive Channel 1A	0.65	SELENON		0.68
CNOT2	CCR4-NOT Transcription Complex Subunit 2	0.73	SELL	Selectin L	0.74
COMMD10	COMM Domain Containing 10	0.64	SERTAD2	SERTA Domain Containing 2	0.71
CREBL2	CAMP Responsive Element Binding Protein Like 2	0.67	SLC25A28	Solute Carrier Family 25 Member 28	-0.66

DCAF17	DDB1 And CUL4 Associated Factor 17	0.65	SLC25A37	Solute Carrier Family 25 Member 37	-0.71
DCP1A	Decapping MRNA 1A	0.65	STARD3	StAR Related Lipid Transfer Domain Containing 3	-0.64
DPH6	Diphthamine Biosynthesis 6	0.64	TAAR3	Trace Amine Associated Receptor 3 (Gene/Pseudogene)	0.73
EIF2A	Eukaryotic Translation Initiation Factor 2A	0.71	TAS2R41	Taste 2 Receptor Member 41	0.67
EPC2	Enhancer Of Polycomb Homolog 2	0.64	TBCA	Tubulin Folding Cofactor A	0.65
EPPK1	Epiplakin 1	0.7	TFDP1	Transcription Factor Dp-1	-0.63
FAM60A	Family With Sequence Similarity 60 Member A	0.66	TMEM104	Transmembrane Protein 104	-0.67
FASTKD2	FAST Kinase Domains 2	0.68	TMEM123	Transmembrane Protein 123	0.72
FRYL	FRY Like Transcription Coactivator	0.65	TMEM38B	Transmembrane Protein 38B	0.67
FUS	FUS RNA Binding Protein	0.67	TOMM7	Translocase Of Outer Mitochondrial Membrane 7	0.69
FYTTD1	Forty-Two-Three Domain Containing 1	0.64	TPP2	Tripeptidyl Peptidase 2	0.63
GBAS	Glioblastoma Amplified Sequence	0.64	TRIM44	Tripartite Motif Containing 44	0.68
HHLA3	HERV-H LTR-Associating 3	0.67	TYK2	Tyrosine Kinase 2	-0.67
IKZF5	IKAROS Family Zinc Finger 5	0.66	UBIAD1	UbiA Prenyltransferase Domain Containing 1	0.67
KAT6B	Lysine Acetyltransferase 6B	0.64	UBR2	Ubiquitin Protein Ligase E3 Component N-Recognin 2	-0.65
LDLRAD4	Low Density Lipoprotein Receptor Class A Domain Containing 4	0.64	URB1-AS1	URB1 Antisense RNA 1 (Head To Head)	0.68
LNPEP	Leucyl And Cystinyl Aminopeptidase	0.64	USF3	Upstream Transcription Factor Family Member 3	0.69
MAGED1	MAGE Family Member D1	0.63	WDR78	WD Repeat Domain 78	0.69
MAML2	Mastermind Like Transcriptional Coactivator 2	0.64	ZBTB24	Zinc Finger And BTB Domain Containing 24	0.64
MAP10	Microtubule Associated Protein 10	0.65	ZC3H12B	Zinc Finger CCCH-Type Containing 12B	0.72
MED9	Mediator Complex Subunit 9	0.67	ZNF626	Zinc Finger Protein 626	0.63
MRPL30	Mitochondrial Ribosomal Protein L30	0.64	ZNF844	Zinc Finger Protein 844	0.75
NDUFS4	NADH:Ubiquinone Oxidoreductase Subunit S4	0.64	ZNHIT6	Zinc Finger HIT-Type Containing 6	0.64

Table 3.5 – Gene transcripts of which expression was significantly correlated with % cytotoxicity by YO-PRO detection.

Transcripts were selected for validation by qPCR based on strength of correlation and mechanistic plausibility with SMX-HA-induced cytotoxicity (**Table 3.1**). *RPL37*, which encodes ribosomal protein, large subunit 37, was selected to represent the group of ribosomal proteins identified in the pathway analysis. *SLC25A37*, which encodes a mitochondrial iron transporter, was selected because of the relationship between mitochondrial function and cell survival. *ABCC5*, which encodes an organic anion pump, is known to transport arylamine-containing drugs including methotrexate and leucovorin.[47] Finally, *TMEM123*, which encodes Porimin, was

selected because of its role as a cell membrane protein associated with cell death. In contrast to the correlation identified by the expression array for *RPL37* (r = 0.65), only a weak correlation was found between % cytotoxicity and *RPL37* expression by qPCR that did not reach statistical significance (r = 0.32, p = 0.17; **Figure 3.4**). A negative correlation between *SLC25A37* expression by qPCR and % cytotoxicity approached statistical significance (r = -0.42, p = 0.067; **Figure 3.5**); when an outlier was removed (> 3 SD above the mean), the correlation (r = -0.63, p = 0.0037) was comparable to that found in the array (r = -0.71). The inverse correlation between *ABCC5* expression and cytotoxicity in the array (r = -0.70) was confirmed by qPCR (r = -0.49, p = 0.02; **Figure 3.6**), as was the positive correlation between *TMEM123* expression and cytotoxicity (r = 0.71 in the array and r = 0.53, p = 0.017 by qPCR; **Figure 3.7**).

In addition to YO-PRO cytotoxicity, a subset of patients (6 HS, 6 TOL) was also evaluated in the LTA using the MTT method. Using independent filtering, no transcripts were significantly correlated with % cytotoxicity. However, when all genes were included, a correlation was found for a single transcript, *TMEM129* (r = 0.92), with FDR < 0.05. *TMEM129* expression was also assessed by qPCR, but an association with % cytotoxicity could not be confirmed (r = 0.40, p = 0.19), possibly due to low power in just 12 patients.

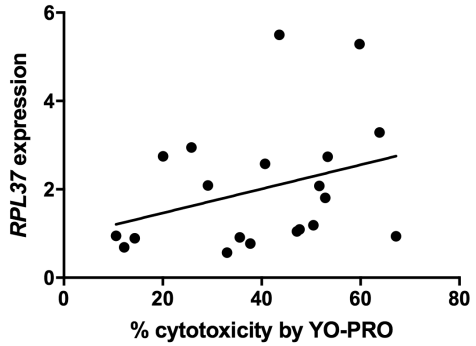

Figure 3.4 – Correlation between % cytotoxicity by YO-PRO detection and expression of *RPL37* (r = 0.322, p = 0.166) by qPCR.

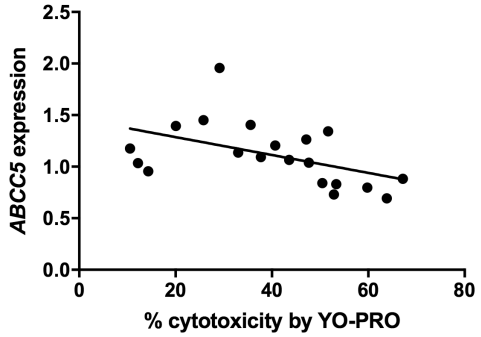

Figure 3.6 – Correlation between % cytotoxicity by YO-PRO detection and expression of *ABCC5* (r = -0.486, p = 0.030) by qPCR.

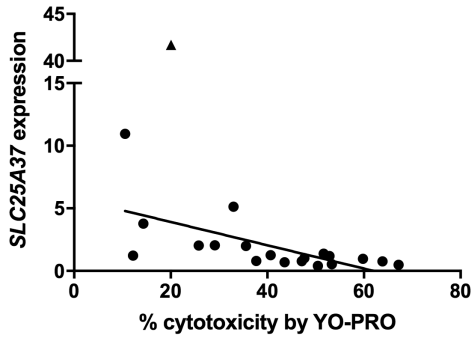

Figure 3.5 – Correlation between % cytotoxicity by YO-PRO detection and expression of *SLC25A37* by qPCR without (r = -0.632, p = 0.037) inclusion of the outlier. Circles represent included expression levels; triangle represents the outlier data point.

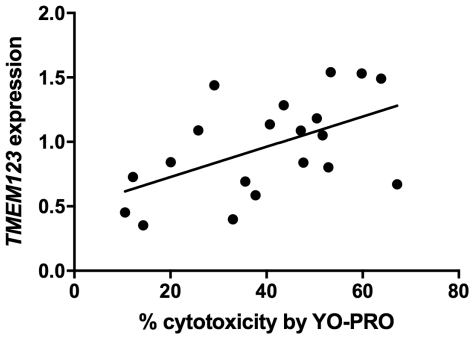

Figure 3.7 – Correlation between % cytotoxicity by YO-PRO detection and expression of *TMEM123* (r = 0.526, p = 0.017) by qPCR

Discussion

The lymphocyte toxicity assay (LTA) has been proposed as an *in vitro* marker of sulfonamide HS with possible clinical applications.[32,39,40] However, the mechanism underlying enhanced cytotoxicity of PBMCs to reactive SMX metabolites has not been established. Therefore, we set out to define differences in gene expression in patients with high versus low susceptibility to SMX-HA cytotoxicity *in vitro,* using two methods for evaluation of cytotoxicity. We chose YO-PRO-1 as our primary method, which is a DNA-intercalating fluorophore that measures increased cell permeability and has been used as a marker of cellular apoptosis and death. [48] We also used the classical MTT assay, which measures mitochondrial viability, in a subset of patients with adequate PBMC for both assays.[49]

In our hands, there was poor correlation between cytotoxicity from SMX-HA using the YO-PRO assay versus the MTT assay across patients. Part of this may have been due to small sample size, since we had inadequate PBMC numbers to perform the MTT assay on all patients. However, the lack of correlation could also be due to different assay outcomes (cell permeability versus mitochondrial function) and both assays have diagnostic weaknesses. As a vital dye, YO-PRO is considered a conclusive test for cell death. [50] However, false negatives can occur with cell death that does not involve increased membrane permeability[50] and false positives are possible with high expression of transporters for large cations.[51] In the MTT assay, impaired mitochondrial reductase function in otherwise healthy cells will yield falsely low viability results. [50] These factors may lead to discordant results between YO-PRO and MTT assays.[52]

We used cytotoxicity of SMX-HA at 800 μM for the purposes of statistical comparisons of LTA results, since this metabolite concentration appeared to highlight most variability among individuals. However, we did not detect increased cytotoxicity in HS patients compared to TOL patients at this concentration for either method, which was a concern given the results of previous studies.[21,31,53] The reported specificity of the LTA is quite high (78-99%), but sensitivity varies greatly (41-100%).[37,39,53,54] In the relatively small numbers of patients recruited for expression arrays in our study, the lack of sensitivity reported by some groups could have led to poor discrimination between HA and TOL patients. Additionally, the performance of the LTA in drug HS appears to vary with the severity of clinical signs. In patients with only an exanthemous rash, sensitivity is quite low (41%), but is much higher in patients with more serious systemic organ involvement (92%).[53] These differences in apparent assay sensitivity may reflect problems not with the assay but with accurate phenotyping of patients with simple rash, which can have causes other than drug HS.

Although we did not find differences in cytotoxicity outcomes by clinical phenotype in our population, we were still interested in underling gene expression patterns associated with the wide range of SMX-HA cytotoxicity that we did observe. After independent filtering, which removes transcripts with low variance between samples, expression levels for 96 transcripts were significantly correlated with SMX-HA cytotoxicity using the YO-PRO method. These genes represent a wide range of cellular localization and functions, but none were candidate genes known or hypothesized to

metabolize sulfonamide antibiotics (**Table 3.2**). Since we used a relatively low threshold for false discovery (18.8%) in this exploratory study, we selected key transcripts with very high cytotoxicity correlations (*RPL37*, *TMEM129*) or possible mechanistic significance (*SLC25A37*, *ABCC5*, *TMEM123*) for qPCR validation.

RPL37 was one of a group of 8 ribosomal proteins that were significantly and positively correlated with SMX-HA cytotoxicity by YO-PRO (r = 0.63 to 0.68). While it is difficult to resolve a mechanistic relationship between upregulation of ribosomal proteins (which was assessed in an aliquot of PBMC that were not exposed to drug metabolite *in vitro*) and increased drug cytotoxicity, *RPL37* overexpression has been shown to lead to increased cell death *in vitro*.[55] However, the correlation between cytotoxicity and *RPL37* expression by qPCR in our study was poor and not statistically significant (r = 0.32, p = 0.17).

SLC25A37 encodes mitoferrin 1, a solute carrier in the inner mitochondrial membrane that is known to transport iron. *SLC25A37* expression was inversely correlated with SMX-HA induced cytotoxicity in the microarray, and was confirmed with qPCR (r = -0.63). *SLC25A37* has been most studied relative to heme biosynthesis in erythroid cells, but its role in non-erythroid cells is not well characterized.[56] Down-regulation of mitoferrin 1 in lymphoid cells could plausibly lead to iron-induced redox stress, and possible increased susceptibility to reactive xenobiotics such as SMX-HA. Of note, *SLC25A28*, which encodes for mitoferrin 2, another mitochondrial iron transporter that is highly homologous to mitoferrin 1,[57] was also inversely correlated with % cytotoxicity in

the microarray (r = -0.66).

ABCC5 encodes a multi-specific organic anion transporter, and its expression was negatively correlated with SMX-HA cytotoxicity in the microarray and by qPCR (r = -0.49). This transcript is of interest because it mediates cellular efflux of the drugs methotrexate and leucovorin (folinic acid),[47] which, like sulfonamide antibiotics, are arylamine compounds with therapeutic targets in folate metabolism. The *ABCC5* gene is polymorphic[58] and variants have been associated with altered risk for toxicity to a variety of drugs including methotrexate,[59] irinotecan,[60] and azathioprine.[61] These variants are thought to either modulate transporter affinity for the drug or drug metabolite or alter expression of the transporter itself. Therefore, down-regulation of *ABCC5* observed in the present study could possibly lead to increased intracellular accumulation of SMX and its metabolites, contributing to increased cytotoxicity *in vitro* and possibly increased immunogenic drug adduct formation *in vivo*. This hypothesis merits further investigation.

Finally, *TMEM123*, which encodes Porimin, was positively correlated with % cytotoxicity by YO-PRO detection in the microarray and confirmed by qPCR (r = 0.53). Porimin is of interest because it has been shown to increase membrane permeability and cell death in Jurkat (lymphoid) cells.[62] This has direct relevance for the cytotoxicity observed in the *in vitro* LTA, but could also influence susceptibility to sulfonamide HS *in vivo* through increased cell death from relatively lower concentrations of reactive SMX metabolites, and enhanced "danger signals" to trigger a systemic immune response to cellular drug adducts. Like *ABCC5*, *TMEM123* deserves further study relative to the risk

of sulfonamide HS.

The major limitation of this study was its small sample size, which was limited in part by the expense of expression array analyses ($650 per sample) and by the logistics of prospective recruitment and adjudication of patients for fresh sample collection from a single hospital. However, our sample size was based on an *a priori* power calculation that 10 patients per group would provide 80% power to detect 2.5-fold or greater differences in transcript expression.[63] We also used a lenient false positive discovery rate to detect novel transcripts that might lead to novel mechanistic insights. Another potential limitation of the study was the use of $CD8^+$ enriched, but not pure, lymphocytes. While the LTA is traditionally performed on mixed PBMC, a previous study had demonstrated that $CD8^+$ T lymphocytes were the predominant cell type affected by SMX-HA cytotoxicity.[33] We chose to focus on the cell type with the highest toxicity phenotype for expression arrays by using negative selection for enrichment of $CD8^+$ T cells. This provided adequate RNA for robust array analyses, but since cell yields were not pure, our results may have been clouded by expression patterns in other mononuclear leukocytes in the samples.

In summary, although high SMX-HA cytotoxicity towards patient PBMC has been previously attributed to a possible innate defect in drug detoxification,[32,39,40] we found no evidence for an association between increased cytotoxicity and altered expression of genes known or hypothesized to be involved in sulfamethoxazole biotransformation. Instead, expression array analyses suggested other novel pathways

that could contribute mechanistically to SMX-HA cytotoxicity, and by extension, to sulfonamide HS in some populations. Novel genes of interest include *ABCC5*, which encodes an arylamine drug transporter, and *TMEM123,* which encodes a transmembrane protein that mediates cell death. *ABCC5* in particular is polymorphic,[58] and additional work is needed to determine whether this transporter mediates SMX or SMX-HA efflux from cells, and whether this is affected by known polymorphisms of the transporter.

Acknowledgements

The authors would like to thank the University of Wisconsin Gene Expression Center for providing Agilent Bioanalyzer and Affymetrix GeneChip services. This study was funded by the by grant R01 GM100784 from the National Institutes of Health. Dr. Reinhart was supported by grant T32 OD010423 from the National Institutes of Health.

References

1. Avgeri SG, Matthaiou DK, Dimopoulos G, Grammatikos AP, Falagas ME. Therapeutic options for Burkholderia cepacia infections beyond co-trimoxazole: a systematic review of the clinical evidence. International Journal of Antimicrobial Agents. 2009;33(5):394–404.

2. Drekonja DM, Traynor LM, DeCarolis DD, Crossley KB, Johnson JR. Treatment of non-life-threatening methicillin-resistant Staphylococcus aureus infections with alternative antimicrobial agents: a 2-year retrospective review. Diagn Microbiol Infect Dis. 2009;63(2):201–207.

3. Drekonja DM, Johnson JR. Urinary tract infections. Prim Care. 2008;35(2):345–367.

4. Korbila IP, Manta KG, Siempos II, Dimopoulos G, Falagas ME. Penicillins vs trimethoprim-based regimens for acute bacterial exacerbations of chronic bronchitis: meta-analysis of randomized controlled trials. Can Fam Physician. 2009;55(1):60–67.

5. Gupta D, Zachariah A, Roppelt H, Patel AM, Gruber BL. Prophylactic antibiotic

usage for Pneumocystis jirovecii pneumonia in patients with systemic lupus erythematosus on cyclophosphamide: a survey of US rheumatologists and the review of literature. J Clin Rheumatol. 2008;14(5):267–272.

6. Rodriguez M, Sifri CD, Fishman JA. Failure of low-dose atovaquone prophylaxis against Pneumocystis jiroveci infection in transplant recipients. Clin Infect Dis. Oxford University Press; 2004;38(8):e76–78.

7. Seger RA. Modern management of chronic granulomatous disease. Br J Haematol. 2008;140(3):255–266.

8. Thomas M, Rupali P, Woodhouse A, Ellis-Pegler R. Good outcome with trimethoprim 10 mg/kg/day-sulfamethoxazole 50 mg/kg/day for Pneumocystis jirovecii pneumonia in HIV infected patients. Scand J Infect Dis. 2009;41:862–868.

9. Cribb AE, Lee BL, Trepanier LA, Spielberg SP. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. Adverse Drug React Toxicol Rev. 1996;15(1):9–50.

10. Ortiz JE, Horn MS, Peterson HD. Toxic epidermal necrolysis-case report and review of the literature. Ann Plast Surg. 1982;9(3):249–253.

11. Saka B, Kombaté K, Mouhari-Toure A, Akakpo S, Tchangaï-Walla K, Pitché P. Stevens-Johnson syndrome and toxic epidermal necrolysis in a teaching hospital in Lomé, Togo: retrospective study of 89 cases. Med Trop (Mars). 2010;70(3):255–258.

12. Gordin FM, Simon GL, Wofsy CB, Mills J. Adverse Reactions to Trimethoprim-Sulfamethoxazole in Patients with the Acquired Immunodeficiency Syndrome. Ann Intern Med. American College of Physicians; 1984;100(4):495–499.

13. Hennessy S, Strom BL, Berlin JA, Brennan PJ. Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary Pneumocystis carinii pneumonia prophylaxis. J Gen Intern Med. 1995;10(7):380–386.

14. Medina I, Mills J, Leoung G, Hopewell PC, Lee B, Modin G, et al. Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. A controlled trial of trimethoprim-sulfamethoxazole versus trimethoprim-dapsone. N Engl J Med. 1990;323(12):776–782.

15. Walmsley SL, Khorasheh S, Singer J, Djurdjev O. A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in Pneumocystis carinii pneumonia prophylaxis (CTN 057). J Acquir Immune Defic Syndr Hum Retrovirol. 1998;19(5):498–505.

16. Bigby M. Drug-induced cutaneous reactions. A report from the Boston

Collaborative Drug Surveillance Program on 15,438 consecutive inpatients, 1975 to 1982. J Am Med Assoc. 1986;256(24):3358–3363.

17. Carr A, Swanson C, Penny R, Cooper DA. Clinical and laboratory markers of hypersensitivity to trimethoprim-sulfamethoxazole in patients with Pneumocystis carinii pneumonia and AIDS. J Infect Dis. 1993;167(1):180–185.

18. Eliaszewicz M, Flahault A, Roujeau JC, Fillet AM. Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. J Am Acad Dermatol. 2002;47:40–46.

19. Rabaud C, Charreau I, Izard S, Raffi F, Meiffredy V, Leport C, et al. Adverse reactions to cotrimoxazole in HIV-infected patients: predictive factors and subsequent HIV disease progression. Scand J Infect Dis. 2001;33(10):759–764.

20. Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. Clin Biochem. 2000;33(7):517–524.

21. Shear NH, Spielberg SP, Grant DM, Tang BK, Kalow W. Differences in Metabolism of Sulfonamides Predisposing to Idiosyncratic Toxicity. Ann Intern Med. 1986;105(2):179–184.

22. Mohanasundaram J, Rajaram S, Mohanasundaram S. Familial tendency in hypersensitivity reactions to co-trimoxazole. J Indian Med Assoc. 1998;96(1):21–22.

23. Wolkenstein P, Carrière V, Charue D, Bastuji-Garin S, Revuz J, Roujeau JC, et al. A slow acetylator genotype is a risk factor for sulphonamide-induced toxic epidermal necrolysis and Stevens-Johnson syndrome. Pharmacogenetics. 1995;5(4):255–258.

24. Zielińska E, Niewiarowski W, Bodalski J. The arylamine N-acetyltransferase (NAT2) polymorphism and the risk of adverse reactions to co-trimoxazole in children. E J Clin Pharmacol. 1998;54(9-10):779–785.

25. Sacco JC, Abouraya M, Motsinger-Reif A, Yale SH, McCarty CA, Trepanier LA. Evaluation of polymorphisms in the sulfonamide detoxification genes NAT2, CYB5A, and CYB5R3 in patients with sulfonamide hypersensitivity. Pharmacogenet Genomics. 2012;22(10):733–740.

26. Ateş NA, Tursen U, Tamer L, Kanik A, Derici E, Ercan B, et al. Glutathione S-transferase polymorphisms in patients with drug eruption. Arch Dermatol Res. 2004;295(10):429–433.

27. Ozkaya-Bayazit E, Bayazit H, Ozarmagan G. Drug related clinical pattern in fixed drug eruption. Eur J Dermatol. 2000;10(4):288–291.

28. Reinhart JM, Motsinger-Reif A, Dickey A, Yale S, Trepanier LA. Genome-Wide

Association Study in Immunocompetent Patients with Delayed Hypersensitivity to Sulfonamide Antimicrobials. PLoS ONE. 2016;11(6):e0156000.

29. Carr A, Penny R, Cooper DA. Efficacy and safety of rechallenge with low-dose trimethoprim-sulphamethoxazole in previously hypersensitive HIV-infected patients. AIDS. 1993;7(1):65–71.

30. Rieder MJ, Uetrecht J, Shear NH, Spielberg SP. Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. J Pharmacol Exp Ther. 1988;244(2):724–728.

31. Rieder MJ, Uetrecht J, Shear NH, Cannon M, Miller M, Spielberg SP. Diagnosis of sulfonamide hypersensitivity reactions by in-vitro rechallenge with hydroxylamine metabolites. Ann Intern Med. 1989;110(4):286–289.

32. Rieder MJ, Krause R, Bird IA. Time-course of toxicity of reactive sulfonamide metabolites. Toxicology. 1995;95(1-3):141–146.

33. Hess DA, Sisson ME, Suria H, Wijsman J, Puvanesasingham R, Madrenas J, et al. Cytotoxicity of sulfonamide reactive metabolites: apoptosis and selective toxicity of CD8(+) cells by the hydroxylamine of sulfamethoxazole. FASEB J. 1999;13(13):1688–1698.

34. Shear NH, Spielberg SP. In vitro evaluation of a toxic metabolite of sulfadiazine. Can J Physiol Pharmacol. 1985;63(11):1370–1372.

35. Leeder JS, Cannon M, Nakhooda A, Spielberg SP. Drug metabolite toxicity assessed in human lymphocytes with a purified, reconstituted cytochrome P-450 system. J Pharmacol Exp Ther. 1988;245(3):956–962.

36. Reilly TP, Bellevue FH, Woster PM, Svensson CK. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. Biochem Pharmacol. 1998;55(6):803–810.

37. Elzagallaai AA, Jahedmotlagh Z, Del Pozzo-Magaña BR, Knowles SR, Prasad AN, Shear NH, et al. Predictive value of the lymphocyte toxicity assay in the diagnosis of drug hypersensitivity syndrome. Mol Diagn Ther. 2010;14(5):317–322.

38. Reilly TP, MacArthur RD, Farrough MJ, Crane LR, Woster PM, Svensson CK. Is hydroxylamine-induced cytotoxicity a valid marker for hypersensitivity reactions to sulfamethoxazole in human immunodeficiency virus-infected individuals? J Pharmacol Exp Ther. 1999;291(3):1356–1364.

39. Wolkenstein P, Charue D, Laurent P, Revuz J, Roujeau JC, Bagot M. Metabolic predisposition to cutaneous adverse drug reactions. Role in toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. Arch Dermatol. 1995;131(5):544–551.

40. Westphal GA, Reich K, Schulz TG, Neumann C, Hallier E, Schnuch A. N-acetyltransferase 1 and 2 polymorphisms in para-substituted arylamine-induced contact allergy. Br J Dermatol. 2000;142(6):1121–1127.

41. Harle DG, Baldo BA, Wells JV. Drugs as allergens: detection and combining site specificities of IgE antibodies to sulfamethoxazole. Mol Immunol. 1988;25(12):1347–1354.

42. Gruchalla RS, Sullivan TJ. Detection of human IgE to sulfamethoxazole by skin testing with sulfamethoxazoyl-poly-L-tyrosine. J Allergy Clin Immun. 1991;88(5):784–792.

43. Pirmohamed M, Alfirevic A, Vilar J, Stalford A, Wilkins EG, Sim E, et al. Association analysis of drug metabolizing enzyme gene polymorphisms in HIV-positive patients with co-trimoxazole hypersensitivity. Pharmacogenetics. 2000;10(8):705–713.

44. Naranjo CA, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts EA, et al. A method for estimating the probability of adverse drug reactions. Clin Pharmacol Ther. 1981;30(2):239–245.

45. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Molecular Aspects of Medicine. 2006;27(2-3):126–139.

46. Bourgon R, Gentleman R. Independent filtering increases detection power for high-throughput experiments. PNAS 2010;107(21):9546–9551.

47. Assaraf YG. The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis. Drug Resist Update. 2006;9(4-5):227–246.

48. Plantin-Carrenard E, Bringuier A, Derappe C, Pichon J, Guillot R, Bernard M, et al. A Fluorescence Microplate Assay Using Yopro-1 to measure apoptosis: Application to HL60 Cells Subjected to Oxidative stress. Cell Biol Toxicol. 2003;19(2):121–133.

49. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal Immunol Method. 1983;65(1-2):55–63.

50. Méry B, Guy J-B, Vallard A, Espenel S, Ardail D, Rodriguez-Lafrasse C, et al. In Vitro Cell Death Determination for Drug Discovery: A Landscape Review of Real Issues. J Cell Death. 2017;10:1179670717691251.

51. Munns CH, Chung M-K, Sanchez YE, Amzel LM, Caterina MJ. Role of the outer pore domain in transient receptor potential vanilloid 1 dynamic permeability to large cations. J Biol Chem. 2015;290(9):5707–5724.

52. Gruhlke MCH, Nicco C, Batteux F, Slusarenko AJ. The effects of allicin, a

reactive sulfur species from garlic, on a selection of mammalian cell lines. Antioxidants 2016;6(1):doi.org:10.3390/antiox6010001.

53. Neuman MG, Shear NH, Malkiewicz IM, Taeri M, Shapiro LE, Krivoy N, et al. Immunopathogenesis of hypersensitivity syndrome reactions to sulfonamides. Transl Res. 2007;149(5):243–253.

54. Riley RJ, Cribb AE, Spielberg SP. Glutathione transferase mu deficiency is not a marker for predisposition to sulphonamide toxicity. Biochem Pharmacol. 1991;42(3):696–698.

55. Daftuar L, Zhu Y, Jacq X, Prives C. Ribosomal proteins RPL37, RPS15 and RPS20 regulate the Mdm2-p53-MdmX network. PLoS ONE. 2013;8(7):e68667.

56. Mühlenhoff U, Hoffmann B, Richter N, Rietzschel N, Spantgar F, Stehling O, et al. Compartmentalization of iron between mitochondria and the cytosol and its regulation. Eur J Cell Biol. 2015;94(7-9):292–308.

57. Haitina T, Lindblom J, Renström T, Fredriksson R. Fourteen novel human members of mitochondrial solute carrier family 25 (SLC25) widely expressed in the central nervous system. Genomics. 2006;88(6):779–790.

58. Saito S, Iida A, Sekine A, Miura Y, Ogawa C, Kawauchi S, et al. Identification of 779 genetic variations in eight genes encoding members of the ATP-binding cassette, subfamily C (ABCC/MRP/CFTR). J Hum Genet. 2002;47(4):147–171.

59. Zaruma-Torres F, Lares-Asseff I, Reyes-Espinoza A, Loera-Castañeda V, Chairez-Hernández I, Sosa-Macías M, et al. Association of ABCB1, ABCC5 and xanthine oxidase genetic polymorphisms with methotrexate adverse reactions in Mexican pediatric patients with ALL. Drug Metab Pers Ther. 2015;30(3):195–201.

60. Chen S, Villeneuve L, Jonker D, Couture FL, Laverdi re I, Cecchin E, et al. ABCC5 and ABCG1 polymorphisms predict irinotecan-induced severe toxicity in metastatic colorectal cancer patients. Pharmacogenet Genomics. 2015;25(12):573–583.

61. Lee M-N, Kang B, Choi SY, Kim MJ, Woo SY, Kim J-W, et al. Impact of Genetic Polymorphisms on 6-Thioguanine Nucleotide Levels and Toxicity in Pediatric Patients with IBD Treated with Azathioprine. Inflammatory Bowel Diseases. 2015;21(12):2897–2908.

62. Ma F, Zhang C, Prasad KV, Freeman GJ, Schlossman SF. Molecular cloning of Porimin, a novel cell surface receptor mediating oncotic cell death. Proc Natl Acad Sci USA. 2001;98(17):9778–9783.

63. Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A. False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics. 2005;21(13):3017–3024.

Chapter 4: A single nucleotide polymorphism in the canine cytochrome b_5 reductase (*CYB5R3*) gene is associated with sulfonamide hypersensitivity and is overrepresented in Doberman Pinschers

Jennifer M. Reinhart, Joanne Ekena, Amelia C. Cioffi, Lauren A. Trepanier

In review with the Journal of Veterinary Pharmacology and Therapeutics

Abstract

Canine sulfonamide hypersensitivity (HS) has been associated with a variant in the cytochrome b_5 reductase gene (*CYB5R3* 729A>G), which encodes a drug-detoxifying enzyme. Study objectives were to confirm this association; determine variant allele frequencies in a predisposed breed, Doberman Pinschers (DOBE); and characterize the effects of *CYB5R3* 729G on gene expression and function. *CYB5R3* 729A>G allele frequencies were compared between HS (n = 24) vs. drug-tolerant (TOL; n = 20) dogs; and DOBE (n = 24) vs. non-Doberman (nonDOBE; n = 60) dogs. *CYB5R3* mRNA expression, protein expression, and enzyme function were compared between banked canine liver samples of AA vs. GG genotype. The 729G allele was overrepresented in HS (0.792) vs. TOL dogs (0.550, $p = 0.022$) and in DOBE (1.00) vs. nonDOBE dogs (0.567, $p < 0.0001$). GG livers expressed less protein than AA (1.35 ± 0.461 vs. 2.80 ± 2.52, $p = 0.037$), but mRNA expression and enzyme activities were similar. All Dobermans in this study were homozygous for *CYB5R3* 729G, which could contribute to this breed's predisposition to sulfonamide HS. Lower enzyme expression in GG livers may suggest a causal link between the variant and sulfonamide HS, but was not associated with decreased SMX-hydroxylamine reduction activity.

Introduction

Hypersensitivity (HS) to sulfonamide antibiotics is an uncommon, but potentially severe, idiosyncratic drug reaction that occurs in both dogs and humans.[1] In dogs, sulfonamide HS occurs a minimum of 5 days and an average of 12 days after initiation of antimicrobial therapy.[2] The most commonly reported clinical signs include fever, blood dyscrasias, hepatopathy, and polyarthropathy, but uveitis, cutaneous and mucocutaneous lesions, proteinuria, and neurologic signs may also occur.[2-5] Treatment is largely symptomatic and requires withdrawal of the sulfonamide drug; our previous retrospective study found a mortality rate of 21%, with hepatopathy and thrombocytopenia carrying a poorer prognosis.[2]

As an idiosyncratic drug reaction, the occurrence of sulfonamide HS is inherently difficult to predict. However, in humans, the condition appears to be heritable[6-8] and, in dogs, breeds such as the Doberman Pinscher appear to be overrepresented.[4,9-13] These findings suggest that a genetic predisposition to sulfonamide HS may be present for both species.

Our laboratory has previously identified a genetic risk factor for sulfonamide HS in dogs: a single nucleotide polymorphism (SNP) in the *CYB5R3* gene (729A>G).[14] In this initial study, the GG genotype was significantly overrepresented in dogs with sulfonamide HS compared to dogs tolerant to a course of sulfonamide antibiotics. *CYB5R3* encodes for cytochrome b_5 reductase, a hepatic microsomal enzyme that detoxifies the sulfonamide-hydroxylamine drug metabolite, which is thought to be

involved in the pathogenesis of the HS reaction.[10] The *CYB5R3* 729A>G variant is a synonymous coding SNP, but is predicted to alter the conformation of the mRNA transcript to a more stable form.[14] We therefore hypothesized that *CYB5R3* 729G creates a more difficult to translate mRNA structure, which leads to decreased enzyme synthesis and cumulative function.

The goal of this study was to better understand the role of *CYB5R3* 729A>G as a genetic risk factor for sulfonamide HS in dogs. The specific objectives of this study were to confirm the association of *CYB5R3* 729G with sulfonamide HS in a somewhat larger group of dogs, to determine whether this risk allele was over-represented in Doberman Pinschers, and to evaluate the effects of the *CYB5R3* 729G allele on gene expression and enzyme activity.

Methods

Genotyping studies

Dogs were recruited for two separate genotyping studies. First, in order to confirm findings from the previous study[14] in a larger population, allele and genotype frequencies for *CYB5R3* 729A>G were compared between dogs with sulfonamide HS and dogs tolerant to a course of sulfonamide antibiotics (TOL). Then, to determine the prevalence of this variant in an overrepresented breed, allele and genotype frequencies were compared between Doberman Pinschers (DOBE) and dogs of other breeds (nonDOBE).

Study population and sample collection. Whole blood (EDTA or heparin) or buccal mucosal swabs were collected from pet dogs between 1995 and 2017. Some of the dogs in the TOL and HS groups were recruited as part of the original study and their genotypes have been previously reported.[14] Additional dogs in the TOL and HS groups were recruited through the University of Wisconsin, Veterinary Medical Teaching Hospital and through referral consultation with one of the authors (LT). All dogs in these two groups had received a therapeutic course of potentiated sulfonamide antibiotics. Dogs were included in the HS group if hepatopathy, blood dyscrasia, polyarthropathy, fever, or skin eruption developed five days or longer after initiation of antibiotic therapy and no other plausible explanation for these clinical signs was present. Dogs were included in the TOL group if they completed a therapeutic course of potentiated sulfonamide antibiotics without developing adverse reactions. Dogs in the DOBE and nonDOBE groups were recruited from the University of Wisconsin Veterinary Medical Teaching Hospital patient population, from dogs owned by School of Veterinary Medicine students, faculty, and staff, at outreach events at local dog training facilities, and through Doberman Pinscher interest group message boards and listservs. These dogs were reported by their owners to be naïve to sulfonamide antibiotics. The study protocol was approved by the University of Wisconsin's Institutional Animal Care and Use Committee.

Genotype determination. Genomic DNA was extracted from blood or buccal swab with a commercial kit (DNEasy Blood and Tissue Kit; Qiagen, Hilden, Germany) and quantified and checked for quality via spectrometry (Take3; BioTek Instruments Inc.,

Winooski, VT). The region around the *CYB5R3* 729 position was amplified via PCR in 20 µl reactions: 10 µl DNA polymerase master mix (AmpliTaq Gold or DreamTaq; Thermo Fisher Scientific, Madison, WI), 1 µl genomic DNA template, 0.5 µM forward primer (5' TCCTCAGGTGAGATGGGGAA 3'), 0.5 µM reverse primer (5' CAGAAGGCCAGCTCTCTGTC 3'). Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, then 30 cycles of 94 °C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minutes, with a final extension of 72°C for 7 minutes. Amplicons were checked for size (564 bp) with gel electrophoresis and sequenced through the University of Wisconsin Biotechnology Center. For each dog, genotype was determined by inspection of electrophoretograms.

Functional studies

The hypothesis that *CYB5R3* 729G leads to a more stable and difficult to translate mRNA transcript, thus decreasing protein expression and total enzyme function, was initially investigated in genotyped canine liver samples. The functional implications of this variant were also assessed in an *in vitro* cell transfection and expression system.

Canine hepatic *CYB5R3* expression and function. Genomic DNA was extracted from the livers of 46 dogs of varying breeds (no Dobermans included) and genotyped for *CYB5R3* 729A>G. Livers with homozygous AA and GG genotypes were used to compare *CYB5R3* mRNA expression, microsomal cytochrome b_5 reductase (b5R) protein expression, and microsomal sulfamethoxazole-hydroxylamine reduction. mRNA expression was determined using qRT-PCR: total RNA was extracted from canine livers

with a commercial kit (RNEasy Mini Plus; Qiagen, Hilden, Germany) and digested to remove genomic DNA (TURBO DNA-free; Thermo Fisher Scientific, Madison, WI). Following RNA quantification (Take3; BioTek Instruments Inc., Winooski, VT) and quality assessment (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA), cDNA was generated from 500 ng of RNA via reverse transcription with random oligomers (Superscript IV VILO cDNA Synthesis Kit; Thermo Fisher Scientific; Madison, WI). Primers were designed for the canine *CYB5R3* transcript along with two canine reference genes (*B2M*, *HPRT1*, **Table 4.1**) and primer efficiencies determined for each using a 10-fold dilution series of pooled cDNA. SYBR Green (LightCycler 480 SYBR Green I Master; Roche Diagnostics; Indianapolis, IN) qPCR with melting curves was performed on the LightCycler 96 Instrument (Roche; Diagnostics; Indianapolis, IN) with parameters adapted from manufacturer's directions. mRNA expression data were reported as normalized ratios.

Gene	Sample	Primers	Product	Efficiency
CYB5R3 canine	canine liver	F 5' TGCCACCTACTATTTGCCAAC 3' R 5' GCTTGAAGCGAGCAGAATGT 3'	94 bp	1.91
B2M canine	canine liver	F 5' GGTTTCCTGGCCTTGCTC 3' R 5' TGGGTGTCGTGAGTACACTTG 3'	93 bp	1.97
HPRT canine	canine liver	F 5' AGCTTGCTGGTGAAAAGGAC 3' R 5' TTATAGTCAAGGGCATATCC 3'	114 bp	1.99
CYB5R3 canine	HepG2 cells	F 5' TGGACCTGGTCATCAAGGTT 3' R 5' TCCAATCTTCATGCTTTCCA 3'	95 bp	2.03
B2M human	HepG2 cells	F 5' CTCCGTGGCCTTAGCTGTG 3' R 5' TTTGGAGTACGCTGGATAGCCT 3'	69 bp	2.02
HMBS human	HepG2 cells	F 5' TGCAACGGCGGAAGAAAA 3' R 5' ACGAAGCTTTCAATGTTGCC 3'	113 bp	2.00

Table 4.1 – Primers used in qPCR measurement of canine *CYB5R3* mRNA expression in canine liver and in HepG2 cells.

For quantification of cytochrome b_5 reductase (b5R) protein expression and enzyme function, canine liver microsomes were prepared using ultracentrifugation. Protein expression was determined by immunoblotting using β-actin as a loading control. For each sample, 2.5 μg of microsomal protein was electrophoresed in polyacrylamide and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% bovine serum albumin (Sigma-Aldrich; St. Louis, MO) for 1 hour, washed 3 times in 0.1% PBS-Tween, and then incubated with anti-mouse β-actin monoclonal antibody conjugated with horseradish peroxidase (HRP, 1:5,000; Abcam, Cambridge, United Kingdom) for 1 hour at room temperature. β-actin protein bands were detected by chemiluminescence (ECL Prime Western Blotting Detection Reagent; GE Healthcare, Buckinghamshire, United Kingdom) and then the membrane was stripped (Restore PLUS Western Blot Stripping Buffer; ThermoScientific, Rockford, IL). The membrane was incubated with polyclonal rabbit anti-human b5R antibody (1:50,000 in 1% milk) for 1 hour at room temperature, followed by HRP-conjugated rabbit anti-IgG (Promega Corp., Fitchburg, WI) (1:5,000 in 1% milk) for 1 hour at room temperature and detection of b5R protein bands. Band densitometry was assessed using ImageJ (National Institutes of Health, Bethesda, MD) and protein expression was reported as b5R:β-actin density ratio.

Microsomal b5R enzyme activity was assessed by measuring reduction of sulfamethoxazole-hydroxylamine (SMX-hydroxylamine; Dalton Chemical Co., Toronto, Ontario, Canada) to sulfamethoxazole (SMX; Sigma-Aldrich; St. Louis, MO) by HPLC as previously described,[15] using 125 μg microsomal protein and 200 μM SMX-hydroxylamine. Enzyme activities were expressed as nmoles SMX generated /mg

microsomal protein/min.

In vitro canine *CYB5R3* expression. The full length *CYB5R3* cDNA sequence was amplified from the liver cDNA of a dog homozygous for the reference 729A allele and cloned into a mammalian expression vector (pcDNA3.1(+); Invitrogen Corp., Carlsbad, CA). A 729G variant-containing plasmid was generated via site-directed mutagenesis (QuickChange Multi Site-Directed Mutagenesis Kit; Agilent Technologies, Santa Clara, CA) with the following primers: forward 5' GTGGACAAAGCCCCGGAAGCCTGGGACTA 3', reverse 5' TAGTCCCAGGCTTCCGGGGCTTTGTCCAC 3'. Both the A- and G-containing plasmids were transfected into HepG2 cells (ATCC, Manassas, VA), using a cationic transfection reagent (FuGENE HD; Promega Corp., Fitchburg, WI). Cells were incubated for 48 hours at 37°C, 5% CO_2 prior to downstream applications.

In order to ensure that transfection efficiency was similar between 729A- and G-plasmid transfected cells, HepG2 cells were co-transfected with a plasmid expressing green fluorescent protein (pmaxGFP; Lonza Group, Basel, Switzerland). In separate experiments, transfection efficiencies were determined by counting GFP-expressing cells normalized to the total number of cells in each culture as determined by SYTOX Orange (Thermo Fisher Scientific; Madison, WI) counterstaining. Cell counts were assessed in ImageJ (National Institutes of Health, Bethesda, MD). There was no significant difference in transfection efficiencies between A- and G-plasmid transfected cells (10.6 ± 6.5% vs. 12.5 ± 6.6%, $p = 0.33$) using a Student's t-test.

Following cell transfection and incubation, RNA was extracted for cDNA generation and protein was extracted for quantification. mRNA expression was assessed by qPCR using primers for canine *CYB5R3* with human *B2M* and *HMBS* as reference genes (**Table 4.1**). Protein was extracted from separate cell culture preparations using passive lysis buffer (Promega Corp., Fitchburg, WI) and quantified via immunoblotting, as described above, with the following antibody concentrations: rabbit polyclonal anti-b5R 1:10,000, anti-rabbit HRP conjugate 1:2,500, and monoclonal mouse anti-β-actin HRP conjugate 1:5,000.

Statistical analyses

Normality was assessed for all continuous data using the D'Agostino and Pearson normality test. Parametric data were expressed as mean ± SD; non-parametric data were expressed as median [range]. Differences in age and sex between groups were compared with Mann-Whitney U and Chi-square tests, respectively. Allele and genotype frequencies were compared between groups using Fisher's exact and Chi-square tests, respectively. Expression levels and activities were compared between AA vs. GG genotype livers and A- vs. G-plasmid transfected cells using the Student's t test or Mann-Whitney U test, depending on distribution. All analyses were performed using commercial software (Prism; GraphPad Software Inc., San Diego, CA). Statistical significance was set at $p < 0.05$.

Results

Genotyping by clinical status

Forty-four dogs were included in the comparison of *CYB5R3* 729A>G allele and genotype frequencies between dogs hypersensitive and tolerant to sulfonamide antibiotics (24 HS, 20 TOL). Of these, 34 were previously reported[14] and 10 were newly recruited dogs. Demographic data are reported in **Table 4.2**. There was no significant difference in age ($p = 0.39$) or sex ($p = 0.69$) between groups. The G allele was significantly over-represented in HS dogs (allele frequency 0.792) compared to TOL dogs (0.550, $p = 0.022$, **Figure 4.1A**) in this expanded population. The GG genotype frequency appeared higher in the HS dogs (0.625) vs. the TOL dogs (0.300), but this difference did not reach statistical significance ($p = 0.06$).

Genotyping by breed

Eighty-four dogs were included in the breed comparison of allele frequencies (24 DOBE, 60 nonDOBE). Demographic data are reported in **Table 4.2**. There was no significant difference in age ($p = 0.08$) between DOBE and nonDOBE dogs. However, intact animals were overrepresented in the DOBE group compared to the nonDOBE group ($p = 0.0005$). The G allele was significantly over represented in DOBE dogs (1.00) compared to nonDOBE dogs (0.567, $p < 0.0001$, **Figure 4.1B**). Similarly, the GG genotype was overrepresented in DOBE dogs (1.00) compared to nonDOBE dogs (0.417, $p < 0.0001$).

	HS **n = 24**	**TOL** **n = 20**	**DOBE** **n = 24**	**nonDOBE** **n = 60**
Age (years)	5.5 [0.5 – 14]	3.0 [0.3 – 12]	7.3 [0.3 – 12]	5.0 [0.8 – 14]
Sex	MC 5 FS 16 MI 2 FI 1	MC 6 FS 10 MI 2 FI 2	MC 9 FS 6 MI 5 FI 4	MC 33 FS 25 MI 1 FI 1
Breed (# of dogs)	Labrador retriever (6), Golden retriever (5), Great Dane (3), Mixed breed (3), Samoyed (2); 1 each: German Shepherd, Doberman Pinscher, Malamute, Springer Spaniel, Dalmatian	Mixed breed (6), Boxer (2), Cocker Spaniel (2); 1 each: Australian Shepherd, Poodle, Shar Pei, Labrador retriever, German Shepherd, Brittany Spaniel, Shih Tzu, Doberman Pinscher, Bernese Mountain Dog, Golden retriever	Doberman Pinscher (24)	Mixed breed (25), Golden retriever (5), German Shepherd (5), Australian Shepherd (4), Labrador retriever (4), Australian Cattle Dog (3), Chihuahua (2), Cavalier King Charles Spaniel (2), Border collie (2); 1 each: Boxer, English Bulldog, Brittany Spaniel, Cocker Spaniel, Plott Hound, Chow Chow, Bernese Mountain Dog, Mallinois
***CYB5R3* 729A>G Allele Frequency**				
A	0.208	0.450	0.00	0.433
G	0.792*	0.550	1.00**	0.567
***CYB5R3* 729A>G Genotype Frequency**				
AA	0.042	0.200	0.00	0.283
AG	0.333	0.500	0.00	0.300
GG	0.625	0.300	1.00	0.417

Table 4.2 – Demographic data of dogs included in *CYB5R3* genotyping. MC = male castrated, FS = female spayed, MI = male intact, FI = female intact. * = significantly different from TOL, $p = 0.022$; ** = significantly different from nonDOBE, $p < 0.0001$.

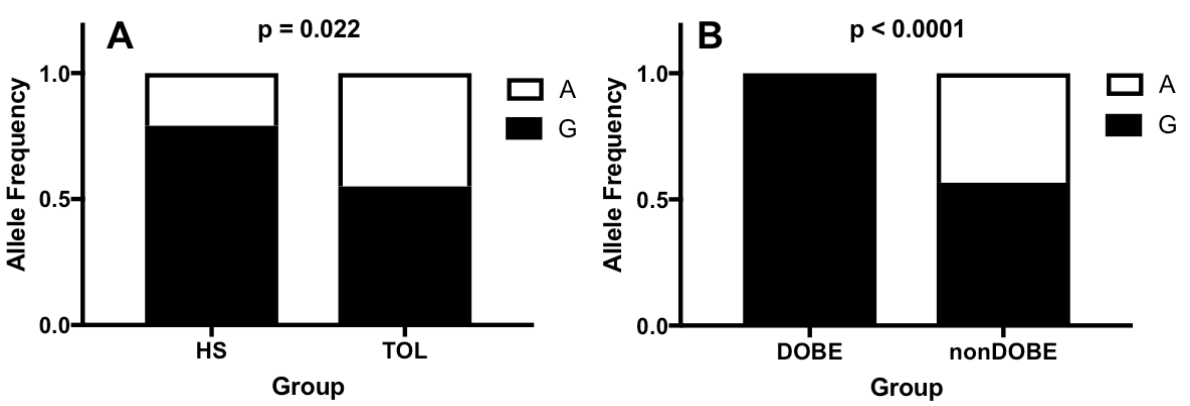

Figure 4.1. Allele frequencies for *CYB5R3* 729A>G in HS vs. TOL dogs (A) and in DOBE vs. nonDOBE dogs (B). The G allele was significantly over represented in HS dogs (0.792) compared to TOL dogs (0.550, $p = 0.022$). The G allele was also significantly over represented in DOBE dogs (1.00) compared to nonDOBE dogs (0.567, $p < 0.0001$).

Functional effects of CYB5R3 729G: canine liver expression and activity

Forty-six banked dog livers of adequate RNA quality were available for analysis. Of these, 12 were 729AA and 15 were 729GG; 19 were heterozygotes that were not further phenotyped. Expression of *CYB5R3* mRNA was not significantly different between AA and GG livers (normalized ratio, 1.16 [0.17 – 1.62] vs. 0.97 [0.19 – 1.72], $p = 0.17$, **Figure 4.2A**). Expression of b5R protein was significantly lower in liver microsomes of GG livers (1.35 ± 0.461) vs. those of AA livers (2.80 ± 2.52, $p = 0.037$, **Figures 4.2B**). However, there was no significant difference in microsomal SMX-hydroxylamine reduction activities between AA and GG livers (1.08 ± 0.301 vs. 0.977 ± 0.310 nmol/min/mg, $p = 0.379$, **Figure 4.2C**).

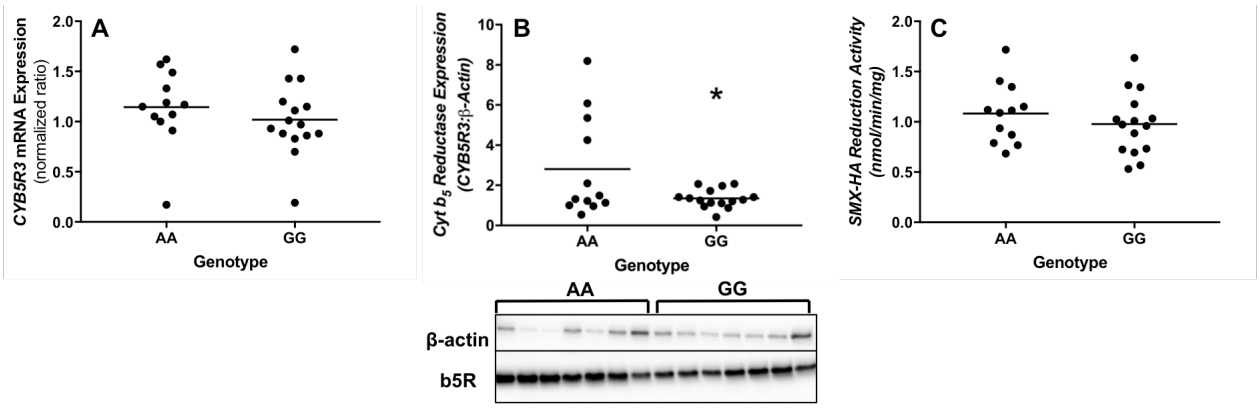

Figure 4.2. *CYB5R3* mRNA expression (A), b5R protein expression (B), and SMX-hydroxylamine reduction (C) in canine livers homozygous for *CYB5R3* 729A (n = 12) and *CYB5R3* 729G (n = 15). * p = 0.037. *Panel B insert*: immunoblotting of canine b5R expression in canine microsomes of dogs homozygous for the A (n = 12) and G (n = 15) allele at the *CYB5R3* 729A>G SNP. β-actin was used as a loading control.

Functional effects of CYB5R3 729G: in vitro expression

In transfection experiments, there was no significant difference in expression of canine *CYB5R3* mRNA between HepG2 cells transfected with the A vs. G allele (normalized ratio, 4391 [2499 – 7936] vs. 4867 [4127 – 9484], p = 0.31, **Figure 4.3A**). Similarly, there was no significant difference in b5R protein expression in cells transfected with the A vs. G allele (5.06 [4.43 – 7.38] vs. 5.93 [2.88 – 11.0], p = 1.00, **Figures 4.3B**).

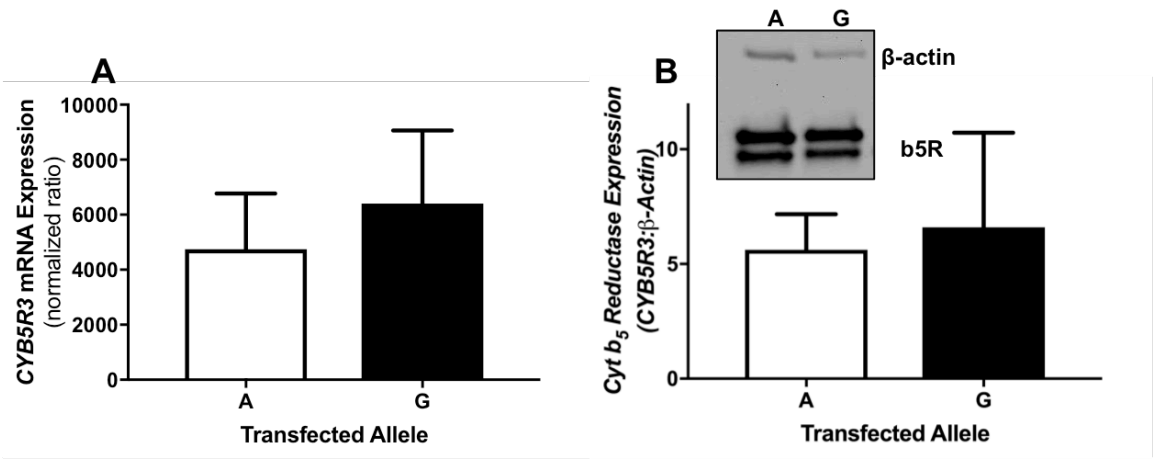

Figure 4.3. *CYB5R3* mRNA (A) and b5R protein expression (B) in HepG2 cells transfected with the *CYB5R3* gene containing either the 729A or G allele. Five separate preparations of A- and G-plasmid transfected cells were used for mRNA quantification and three preparations were used for protein quantification. There were no significant differences between groups for mRNA or protein expression. *Panel B insert*: immunoblotting of canine b5R expression in HepG2 cells transfected with the *CYB5R3* gene containing either the 729A or G allele. The upper b5R band co-localized with canine b5R and thus was used for densitometry readings. The lower b5R band co-localized with human b5R representing endogenous expression by the HepG2 cells (data not shown). β-actin was used as a loading control.

Discussion

Cytochrome b_5 reductase, along with cytochrome b_5, catalyzes the reduction of the reactive sulfonamide metabolite, SMX-hydroxylamine, back to the stable parent drug.[15,16] Since the hydroxylamine metabolite contributes to the immunogenicity of sulfonamide antibiotics such as sulfamethoxazole and sulfadiazine,[10,17,18] hydroxylamine reduction should be protective against sulfonamide HS. Dogs may rely more heavily on the cytochrome b_5/b_5 reductase system for sulfonamide detoxification, because dogs lack the other major detoxifying pathway employed by humans, the *N*-acetyltransferases.[19]

We previously examined the role of variants in the cytochrome b_5 reductase (*CYB5R3*) and cytochrome b_5 (*CYB5A*) genes in the risk of sulfonamide HS in dogs. We found a synonymous SNP in the *CYB5R3* gene, 729A>G, that was significantly

associated with sulfonamide HS in a group of 18 affected and 16 control dogs.[14] The purpose of this study was to confirm this association in a somewhat larger group of dogs, to determine whether the risk allele was over-represented in Doberman Pinschers, and to evaluate the effects of the *CYB5R3* 729G allele on gene expression and enzyme activity.

Similar to previous findings,[14] the frequency of the *CYB5R3* 729G allele was significantly higher in dogs with sulfonamide HS (0.792) than dogs tolerant to sulfonamide antibiotics (0.550) and the difference in GG genotype approached significance (HS 0.625 vs. TOL 0.300, $p = 0.062$). Thus, the G allele may predispose to sulfonamide HS. However, it is important to note that 80% (AG + GG genotype frequencies) of sulfonamide-tolerant dogs do carry the G allele and 30% are homozygous, but the incidence of canine sulfonamide HS is much lower (~0.25% by one estimate).[20] Therefore, it follows that carriage of the *CYB5R3* 729G allele is not sufficient as a single risk factor for sulfonamide HS.

When Doberman Pinschers were evaluated as a breed, all 24 Dobermans were homozygous for the 729G allele. If this variant is truly associated with sulfonamide HS, then this may contribute to breed risk in the Doberman Pinscher. However, not all Dobermans exposed to sulfonamide antibiotics develop a HS reaction; in fact, one of our drug-tolerant controls was a Doberman Pinscher. Therefore, other factors clearly influence the risk of sulfonamide HS. Further evaluation and genotyping of Dobermans tolerant and hypersensitive to sulfonamide antibiotics is warranted to identify other possible genetic risk factors that might interact with *CYB5R3* 729G.

We next investigated the effects of *CYB5R3* 729G on gene expression and activity in both canine liver and an *in vitro* mammalian expression system. The *CYB5R3* 729A>G variant is a synonymous coding SNP, so it should not affect enzyme structure or function. However, based on *in silico* predictions, the G allele may confer a more stable and difficult to translate structure to the mRNA transcript.[14] Therefore, we hypothesized that 729G would lead to increased mRNA levels but decreased protein synthesis and cumulative enzyme function.

We found that dog livers with the 729GG genotype expressed significantly less microsomal cytochrome b_5 reductase (b5R) protein than those with the AA genotype. However, endogenous *CYB5R3* mRNA expression did not differ and SMX-hydroxylamine reduction was not measurably decreased, even at lower substrate concentrations (10 μM SMX-hydroxylamine, data not shown). In addition, a direct effect of the 729G allele on protein expression was not confirmed by *in vitro* transfection in HepG2 cells. Of note, SMX-hydroxylamine reduction was not measurable in transfected HepG2 cells over background activities observed in non-transfected cells (data not shown), which may reflect a requirement for parallel co-transfection of *CYB5A* at an optimized stoichiometry,[16] which was not pursued. Overall, these data provide some evidence for decreased endogenous b5R protein expression with the 729G allele, but it is difficult to relate this mechanistically to sulfonamide HS without evidence of impaired SMX-hydroxylamine reduction.

This follow-up study was still limited by a small sample size for genotyping experiments. As an idiosyncratic reaction, sulfonamide HS is intrinsically an uncommon occurrence;[20] furthermore, sulfonamides are less commonly used today because other antibiotics with lower risks of adverse effects are available. However, as antimicrobial resistance to "safer" antibiotics (e.g. beta-lactams, fluoroquinolones) increases, we may see increased sulfonamide use and a resurgence of HS reactions. Therefore, it is important to understand this idiosyncratic reaction and its risk factors as best as possible.

In conclusion, the *CYB5R3* 729A>G SNP was significantly overrepresented in dogs with sulfonamide HS in a somewhat larger sample size, and all Doberman Pinschers genotyped were homozygous for the G allele. However, this variant was not convincingly associated with impaired enzyme function. It is possible that *CYB5R*3 729G is an indirect marker for another gene locus that does influence clinical phenotype; alternatively our *ex vivo* liver experiments may not have adequately reflect *in vivo* metabolite clearance. Further work is underway to identify additional genetic risk factors for sulfonamide HS in dogs.

Acknowledgements

The authors would like to thank Ms. Gerianne Holzman and Badger Kennel Club (DeForest, WI) for assistance with collection of genetic samples as well as Ms. Giselle Huerta for assistance with genotyping. The authors would also like to thank Dr. Michael Court for providing additional canine liver samples. This study was partially funded by the American Kennel Club Canine Health Foundation's 2017 Clinician Scientist

Fellowship awarded to Dr. Reinhart. Dr. Reinhart is also supported by grant T32 OD010423 from the National Institutes of Health. The authors have no conflicts of interest to report.

References

1. Trepanier, LA. Idiosyncratic toxicity associated with potentiated sulfonamides in the dog. J Vet Pharmacol and Ther, 2004;27:129–138.

2. Trepanier LA, Danhof R, Toll J, Watrous D. Clinical findings in 40 dogs with hypersensitivity associated with administration of potentiated sulfonamides. J Vet Intern Med. 2003;17:647–652.

3. Fox LE, Ford S, Alleman AR, Homer BL, Harvey JW. Aplastic anemia associated with prolonged high-dose trimethoprim-sulfadiazine administration in two dogs. Vet Clin Pathol. 1993;22:89–92.

4. Giger U, Werner LL, Millichamp NJ, Gorman NT. Sulfadiazine-induced allergy in six Doberman pinschers. J Am Vet Med Assoc.1985;186:479–484.

5. Harvey RG. Possible sulphadiazine-trimethoprim induced polyarthritis. Vet Rec. 1987;120(22):537–538.

6. Mohanasundaram J, Rajaram S, Mohanasundaram S. Familial tendency in hypersensitivity reactions to co-trimoxazole. J Ind Med Assoc. 1998;96:21–22.

7. Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. Clin Biochem. 2000;33:517–524.

8. Shear NH, Spielberg SP, Grant DM, Tang BK, Kalow W. Differences in Metabolism of Sulfonamides Predisposing to Idiosyncratic Toxicity. Ann Intern Med. 1986;105;179–184.

9. Cribb AE. Idiosyncratic reactions to sulfonamides in dogs. J Am Vet Med Assoc. 1989;195:1612–1614.

10. Cribb AE, Spielberg SP. An in vitro investigation of predisposition to sulphonamide idiosyncratic toxicity in dogs. Vet Res Comm. 1990;14:241–252.

11. Gray A. Trimethoprim-sulphonamide hypersensitivity in dogs. Vet Rec.1990;127:579–580.

12. Werner LL, Bright JM. Drug-induced immune hypersensitivity disorders in two dogs treated with trimethoprim sulfadiazine: case reports and drug challenge studies. J Am Anim Hosp Assoc. 1983;19:783–790.

13. Whur P. (Possible trimethoprim-sulphonamide induced polyarthritis. Vet Rec. 1987;121:91–92.

14. Funk-Keenan J, Sacco J, Wong YY, Rasmussen S, Motsinger-Reif A, Trepanier LA. Evaluation of polymorphisms in the sulfonamide detoxification genes CYB5A and CYB5R3 in dogs with sulfonamide hypersensitivity. J Vet Intern Med. 2012;26:1126–1133.

15. Trepanier LA, Miller JL. NADH-dependent reduction of sulphamethoxazole hydroxylamine in dog and human liver microsomes. Xenobiotica. 2000;30:1111–1121.

16. Kurian JR, Bajad SU, Miller JL, Chin NA, Trepanier LA. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. J Pharmacol Exp Ther. 2004;311;1171–1178.

17. Farrell J, Naisbitt DJ, Drummond NS, Depta JPH, Vilar FJ, Pirmohamed M, Park BK. Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. J Pharmacol Exp Ther. 2003;306:229–237.

18. Shear NH, Spielberg SP. In vitro evaluation of a toxic metabolite of sulfadiazine. Can J Physiol Pharmacol, 1985;63:1370–1372.

19. Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. (1997). Cytosolic arylamine N-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. Biochem Pharmacol. 1997;54:73–80.

20. Noli C, Koeman JP, Willemse,T. A retrospective evaluation of adverse reactions to trimethoprim-sulphonamide combinations in dogs and cats. Vet Quart. 1995;17:123–128.

Chapter 5: Reflections and future directions

Jennifer M. Reinhart

As an idiosyncratic drug reaction, sulfonamide HS cannot be predicted by drug dose. Thus, identifying risk factors for this potentially severe adverse drug reaction is of great importance. The studies presented in this thesis represent several different approaches to understanding genetic risk for sulfonamide HS in humans and dogs. These results highlight the successes and difficulties that can be encountered when studying a trait that is genetically, clinically, and mechanistically complex. These findings also suggest next steps and new avenues of research to pursue, with the eventual goal of improving treatment and prevention strategies for sulfonamide HS.

In the genome-wide association study (**Chapter 2**) including 91 patients with sulfonamide HS and 184 tolerant patients, we found no SNP associations with sulfonamide HS. This is in contrast to previous candidate gene studies, some of which have suggested genetic risk factors for this condition.[1-4] It is possible that the true strength of association of the variants identified in these previous studies was not great enough to be detectable in a moderately-sized genome-wide study in which several million comparisons are made and must be accounted for statistically. It is also important to note that the study population characteristics can greatly impact study results. In our GWAS, the majority of HS patients exhibited only a maculopapular rash – a fairly mild form of the reaction. It is possible that some these rashes were incorrectly attributed to sulfonamide HS, "diluting" the power of the study of our study to detect a difference

between HS and TOL patients. This highlights a major issue with idiosyncratic drug reactions at both a clinical and research level, in that there is no definitive way to confirm a drug HS reaction without drug rechallenge, and this has inherent risk. Hence, future research efforts should include development of better biochemical or immunologic tests for confirming the diagnosis of sulfonamide HS.

In our GWAS study, when more stringent diagnostic criteria for sulfonamide HS were used (rash plus fever), a genetic association was identified for *COL12A1* by the rare variant analysis. These patients with more severe manifestations were more likely to truly be HS, thus finding an association only in this subgroup may be indirect support for misclassification in patients with more mild symptoms. Alternately, it is possible that genetic risk factors influence the severity rather than the occurrence of the reaction. *COL12A1* encodes for an extracellular matrix adaptor protein and has not been associated with any other immune-mediated conditions. Thus, a mechanistic rationale for this association is unclear, but further investigation of this locus may be warranted including full resequencing of the gene in HS and TOL patients. Given that many other idiosyncratic drug reactions have been associated with HLA haplotypes, it may also be interesting to reevaluate the MHC region in our patients. Finally, it is possible that heritable risk factors for sulfonamide HS are epigenetic rather than genetic. Future studies may include evaluation of the epigenome in HS and tolerant patients.

In the expression array study (**Chapter 3**), we found several transcripts whose expression levels correlated (directly or inversely) with susceptibility to

sulfamethoxazole-hydroxylamine induced cell death in the lymphocyte toxicity assay (LTA). Next steps should include investigations of the interaction between products of these transcripts and sulfamethoxazole (SMX) metabolites. For example, it is unknown whether SMX is a substrate for the ABCC5 transporter; in fact, SMX transport has not been well studied despite some transporter variants being identified as genetic risk factors for other adverse drug reactions.[5] It would also be interesting to know whether the association of these transcripts with cytotoxicity is specific to SMX metabolites or if these pathways are important in the cellular response to multiple xenobiotics. Hypersensitivity to other aromatic and arylamine drugs has been associated with increased cytotoxicity in the LTA, so these compounds, which include dapsone and some anticonvulsant medications, provide a logical starting place for such investigations.[6,7]

In this study, LTA results did not differentiate between HS and TOL patients. Although this is not the first report of a lack of association between LTA and clinical status,[8] most other studies do describe a moderate discriminatory ability. Similar to the GWAS results, this again highlights the problem with accurately phenotyping drug HS reactions without definitive drug rechallenge. All but one of the HS patients included in the expression arrays had mild symptoms (rash only) so it is possible that some of these individuals were misdiagnosed. Elzagallaai et al.[9] demonstrated that the diagnostic performance of the LTA is much lower in patients with only a rash compared to those with more severe manifestations. Thus, this concern with accurate clinical phenotyping does not appear to be limited to our work. However, it is also possible different pathogenic mechanisms may exist across the spectrum of clinical signs, some of which

may be more closely linked to the *in vitro* LTA. Another area of future research may be evaluating what factors determine the severity of a drug HS reaction and which organ systems are involved. Tracer studies of drug adduct formation in animal models would be worthwhile as would examination of local metabolic enzyme expression and activity of in various target tissues. In the clinical setting, genetic and environmental risk factors could be compared among patients with differing clinical manifestations. Such investigations may be relevant to idiosyncratic reactions to other immunogenic drugs as well.

Our investigations in dogs (**Chapter 4**) reconfirmed the association between the *CYB5R3* 729G allele and canine sulfonamide HS. We also found that all Doberman Pinschers genotyped in this study were homozygous for the 729G allele, which may contribute to the apparent breed predisposition to this adverse drug reaction. Based on *in silico* predictions,[10] we hypothesized that this synonymous SNP encoded for a more conformationally stable and difficult to translate mRNA transcript, leading to less lower production of cytochrome b_5 reductase, an SMX-detoxifying enzyme. However, we found little evidence to support this hypothesis in hepatic and *in vitro* expression studies. It is possible that our techniques were not sensitive enough to detect a difference between alleles, but if this locus truly impacts total detoxification function and clinical outcome, then a measurable effect on enzyme activity would be expected. It is also possible that the *CYB5R3* 729A>G SNP is not functionally related to canine sulfonamide HS; rather it may be in linkage disequilibrium with another variant that is. Full sequencing of the *CYB5R3* gene as well as areas up- and downstream of this locus would be a logical next step in pursuing this genetic association.

Although the evidence for overrepresentation of the *CYB5R3* 729G allele in HS dogs is fairly convincing, this allele is also fairly common in tolerant dogs and is, in fact, the major allele in the general, naïve population (MAF 0.570). Given the much lower low population prevalence of sulfonamide HS in dogs, this variant cannot be the major determinant of whether a dog develops a reaction. Thus, other candidate genes should be evaluated for an association. Of particular interest are the dog leukocyte antigen (DLA) genes. In humans, many drug HS reactions have been correlated with certain HLA alleles,[11] and several canine immune-mediated diseases have been associated with DLA variants.[12-15] In a way, these genes represent the antigenic repertoire of an individual, determining what molecules can elicit an adaptive immune response. Therefore, investigating variation in these genes for drug HS in dogs is a logical step. Genome-wide approaches are another potential tool for identifying genetic risk factors in dogs and have been successfully used for many adverse drug reactions in humans[5,16-21] as well as for certain diseases in dogs.[22] Finally, acquired risk factors for sulfonamide HS have not been as well studied in dogs. Evaluating antioxidant concentrations, immune status, and comorbidities in drug-tolerant and HS dogs could yield valuable and clinically relevant information. In all of these potential investigations, the lessons learned in human studies regarding the importance of careful clinical phenotyping should be heeded for work in dogs.

Sulfonamide HS remains an incompletely understood medical phenomenon – not surprising for a syndrome that is classically termed "idiosyncratic." The research

presented here highlights the complex nature of genetic risk for non-monogenic traits. Future work should continue to focus on identifying risk factors, but be expanded to include other genetic loci in dogs, as well as investigations of epigenetic and environmental risk in both species. Given the numerous potential clinical manifestations of sulfonamide HS, understanding the elements that determine presentation and severity of the reaction is also important. While sulfonamide HS is typically treated as a single clinical entity, it is possible that different subsets of patients have different pathogeneses and risk factors, and so must be considered separately. Finally, diagnosis of sulfonamide HS is clinically inexact and rarely definitive, so great care must be taken when selecting patient populations of any species when studying this adverse drug reaction.

References

1. Ozkaya-Bayazit E, Akar U. Fixed drug eruption induced by trimethoprim-sulfamethoxazole: evidence for a link to HLA-A30 B13 Cw6 haplotype. J Am Acad Dermatol. 2001;45(5):712–717.

2. Zielińska E, Niewiarowski W, Bodalski J, Rebowski G, Skretkowicz J, Mianowska K, et al. Genotyping of the arylamine N-acetyltransferase polymorphism in the prediction of idiosyncratic reactions to trimethoprim-sulfamethoxazole in infants. Pharm World Sci. 1998;20(3):123–130.

3. Zielińska E, Niewiarowski W, Bodalski J. The arylamine N-acetyltransferase (NAT2) polymorphism and the risk of adverse reactions to co-trimoxazole in children. E J Clin Pharmacol. Springer-Verlag; 1998;54(9-10):779–785.

4. Wolkenstein P, Carrière V, Charue D, Bastuji-Garin S, Revuz J, Roujeau JC, et al. A slow acetylator genotype is a risk factor for sulphonamide-induced toxic epidermal necrolysis and Stevens-Johnson syndrome. Pharmacogenetics. 1995;5(4):255–258.

5. Link E, Parish S, Armitage J, Bowman L, Heath S, et al. SLCO1B1 variants and statin-induced myopathy--a genomewide study. N Engl J Med. 2008;359(8):789–799.

6. Reilly TP, Bellevue FH, Woster PM, Svensson CK. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone.

Biochem Pharmacol. 1998;55(6):803–810.

7. Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. Clin Biochem. 2000;33(7):517–524.

8. Reilly TP, MacArthur RD, Farrough MJ, Crane LR, Woster PM, Svensson CK. Is hydroxylamine-induced cytotoxicity a valid marker for hypersensitivity reactions to sulfamethoxazole in human immunodeficiency virus-infected individuals? J Pharmacol Exp Ther. 1999;291(3):1356–1364.

9. Elzagallaai AA, Jahedmotlagh Z, Del Pozzo-Magaña BR, Knowles SR, Prasad AN, Shear NH, et al. Predictive value of the lymphocyte toxicity assay in the diagnosis of drug hypersensitivity syndrome. Mol Diagn Ther. 2010;14(5):317–322.

10. Funk-Keenan J, Sacco J, Wong YYA, Rasmussen S, Motsinger-Reif A, Trepanier LA. Evaluation of polymorphisms in the sulfonamide detoxification genes CYB5A and CYB5R3 in dogs with sulfonamide hypersensitivity. J Vet Intern Med. 2012;26(5):1126–1133.

11. Pavlos R, Mallal S, Phillips E. HLA and pharmacogenetics of drug hypersensitivity. Pharmacogenomics. 2012;13(11):1285–1306.

12. Threlfall AJ, Boag AM, Soutter F, Glanemann B, Syme HM, Catchpole B. Analysis of DLA-DQB1 and polymorphisms in CTLA4 in Cocker spaniels affected with immune-mediated haemolytic anaemia. Canine Genetics and Epidemiology; 2015;2:8–17.

13. Kennedy LJ, Davison LJ, Barnes A, Short AD, Fretwell N, Jones CA, et al. Identification of susceptibility and protective major histocompatibility complex haplotypes in canine diabetes mellitus. Tissue Antigens. 2006;68(6):467–476.

14. Peiravan A, Allenspach K, Boag AM, Soutter F, Holder A, Catchpole B, et al. Single nucleotide polymorphisms in major histocompatibility class II haplotypes are associated with potential resistance to inflammatory bowel disease in German shepherd dogs. Vet Immunol Immunopathol. 2016;182:101–105.

15. Friedenberg SG, Buhrman G, Chdid L, Olby NJ, Olivry T, Guillaumin J, et al. Evaluation of a DLA-79 allele associated with multiple immune-mediated diseases in dogs. Immunogenetics. 2015;68(3):205–217.

16. Takeuchi F, McGinnis R, Bourgeois S, Barnes C, Eriksson N, Soranzo N, et al. A genome-wide association study confirms VKORC1, CYP2C9, and CYP4F2 as principal genetic determinants of warfarin dose. PLoS Genet. 2009;5(3):e1000433.

17. Ochi H, Maekawa T, Abe H, Hayashida Y, Nakano R, Kubo M, et al. ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy--a genome-wide study of Japanese HCV virus patients. Gastroenterology. 2010;139(4):1190–1197.

18. Lucena MI, Molokhia M, Shen Y, Urban TJ, Aithal GP, Andrade RJ, et al. Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. Gastroenterology. 2011;141(1):338–347.

19. Fernandez CA, Smith C, Yang W, Date M, Bashford D. HLA-DRB1* 07: 01 is associated with a higher risk of asparaginase allergies. Blood. 2014;124(8):1266–76.

20. Del-Aguila JL, Beitelshees AL, Cooper-Dehoff RM, Chapman AB, Gums JG, Bailey K, et al. Genome-wide association analyses suggest NELL1 influences adverse metabolic response to HCTZ in African Americans. Pharmacogenomics J. 2014;14(1):35–40.

21. Daly AK, Donaldson PT, Bhatnagar P, Shen Y, Pe'er I, Floratos A, et al. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nat Genet. 2009;41(7):816–819.

22. Tonomura N, Elvers I, Thomas R, Megquier K, Turner-Maier J, Howald C, et al. Genome-wide association study identifies shared risk loci common to two malignancies in golden retrievers. PLoS Genet. 2015;11(2):e1004922.