DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS: AN INVESTIGATION OF DRUG TRANSPORTER P-GLYCOPROTEIN AND AN EVALUATION OF IMPROVED DIAGNOSIS OF DRUG-RESISTANT TUBERCULOSIS IN CAMEROONIAN PULMONARY TUBERCULOSIS PATIENTS

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DEDICATION

This PhD dissertation is dedicated to my loving Lord and Savior, Jesus Christ. The experience has been a challenging journey but his love and grace guided me through. He is my refuge and stronghold and I am blessed because he knows me and loves me. Also, to my loving family; you fostered my love for the biomedical sciences and made sure I always aimed high. Your love, support and words of encouragement always kept me going. Lastly to the International Baptist Fellowship (IBF) family especially; the Newmans, the Katayamas, the Dezzanis, the Lawrences, the Wens, the Youngs, the Wilsons, the Wangs, Naomi, Gayle, Mike and Clyde. You made Hawaii home for me and all through the years you supported, advised, prayed and always had words to encourage me. So thank you very much, and God bless you in all do especially for the international student.

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MANUSCRIPTS

Manuscripts contributing directly to this dissertation

1) **Abanda NN,** Riches Z, Collier AC. 2017. Lobular Distribution and Variability in Hepatic ATP Binding Cassette Protein B1 (ABCB1, P-gp): Ontogenetic Differences and Potential for Toxicity. Pharmaceutics. 9(1). pii: E8.

2) **Abanda NN.** Djieugoué JY. Lim E. Pefura-Yone EW. Mbacham FW. Vernet G. Penlap VM. Eyangoh SI. Taylor DW. Leke RGF. Diagnostic Accuracy and Usefulness of the Genotype MTBDRplus Assay in Diagnosing Multidrug-resistant Tuberculosis in Cameroon? A Cross-Sectional Study. (*This manuscript has been submitted, reviewed, and subsequently revised. Waiting for final decision by the BMC Infectious Diseases*)

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Manuscripts arising from the candidate's research not directly included in this dissertation.

1) Riches Z, **Abanda N**, Collier AC. 2015. BCRP protein levels do not differ regionally in adult human livers, but decline in the elderly. Chemical Biological Interaction.242:203-10.

ABSTRACT

Tuberculosis (TB) is a major health problem, especially in Africa and Southeast Asia. The disease, caused by *Mycobacterium tuberculosis*, (*Mtb*) is curable with a multidrug regimen of Rifampicin (RIF), Isoniazid (INH), Ethambutol and Pyrazinamide. However, treatment is not always successful, as ~ 2 million patients fail treatment each year, in part, because the bacterium has become resistant to these drugs. Thus, efficient diagnosis of drug-resistant *Mtb* is extremely important. This study sought to determine if a new molecular assay, the Genotype MTBDR*plus*, could accurately detect drug-resistant *Mtb* in Cameroon. When compared to the conventional drug susceptibility testing assay, the molecular assay identified 98% (48/49) RIF-resistant isolates, 92% (55/60) INH-resistant isolates, and 94% (46/49) of *Mtb* resistant to both drugs in Cameroonian TB patients. Further evaluation of the molecular assay with an additional 50 *Mtb isolates,* identified 6% (16/275) of isolates with questionable RIF-resistant results. To determine if these 16 isolates were truly RIF-resistant, the Rifampicin Resistance-determining Region of the *rpoB* gene were sequenced. Mutations were found known as 'disputed' RIF mutations, i.e., mutations that are not always associated with resistance to RIF. Thus, sequencing results confirm that when DNA from *Mtb* isolates do not hybridize with the wild-type *rpoB* probe, it is wise to assume the *Mtb* isolate is RIF-resistant and treat accordingly. The Genotype MTBDR*plus* assay can be adopted by the government of Cameroon to diagnose drug-resistant TB. In addition to mycobacterial mutations, host factors that cause sub-therapeutic plasma drug concentration could lead to treatment failure. One of such host factors could be variation in the liver efflux pump, P-glycoprotein (p-gp). Therefore, the relative amount of p-gp

protein in 87 human liver samples was measured. P-gp was found to be present from birth reaching 90 % of adult levels by age 5. Since low variability (2.9 $\pm$ 0.32 fold) in p-gp occurred among individuals, it is unlikely variability in p-gp protein accounts for wide variation in RIF plasma levels. The data are important because expression p-gp in children provides a plausible explanation as to why, when given the same dose/kg of RIF as adults, children clear the drug faster.

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LIST OF ABBREVIATIONS

TB	Tuberculosis
WHO	World Health Organization
Mtb	*Mycobacterium tuberculosis*
TST	Tuberculin Skin Test
RIF	Rifampicin
INH	Isoniazid
PZA	Pyrazinamide
EMB	Ethambutol
DOTS	Directly Observed Treatment and Short-course drug therapy
MDR-TB	Multidrug-resistant TB
XDR-TB	Extensively Drug-resistant TB
XXDR-TB	Extremely or Totally drug-resistant TB
DST	Drug susceptibility testing
MIC	Minimum Inhibitory Concentration
PK	Pharmacokinetics
PD	Pharmacodynamics
MGIT	Mycobacterium Growth Indicator Tube
LJ	Lowenstein-Jensen
LPA	Line Probe Assay
OATP1B1	Organic Anion Transporter Polyprotein 1B1
P-gp	P-glycoprotein
PLHIV	People Living with HIV

AFB	Acid-fast Bacilli
EXPAND-TB	Expand Access to New Diagnostics for TB
NTCP	National Tuberculosis Control Program
PTB	Pulmonary Tuberculosis
EXPANDx-TB	Expand Access to New Diagnostics for TB
DST	Drug Susceptibility Testing
BSL	Biosafety Level
WHO	World Health Organization
IUATLJ	International Union Against Tuberculosis and Lung Disease
NALC-NaOH	N-acetyl-L-cysteine-sodium hydroxide
MGIT	Mycobacterial Growth Indicator Tube
SIRE	Streptomycin, Isoniazid, Rifampicin, Ethambutol
CI	Confidence Interval
WT	Wild type
MUT	Mutation
ETH	Ethionamide
NIH	National Institute of Health, USA
NPGHF	Northern Pacific Global Health Fellowship

CHAPTER 1

INTRODUCTION

1.1 Global Burden of Tuberculosis

Tuberculosis (**TB**) is currently the world's most common cause of death from an infectious disease, including HIV and malaria. According to the World Health Organization (**WHO**), in 2015, 10.4 million people developed active TB disease, of whom, 1.8 million people died (1). TB is caused by the bacterium *Mycobacterium tuberculosis (**Mtb**)*. *Mtb* predominantly affects the lungs causing active pulmonary TB disease but could also affect any part of the body causing extrapulmonary TB disease (2).

Exposure to *Mtb* may cause either active disease or latent TB. People become exposed when they inhale aerosolized droplets generated by a patient with pulmonary TB, e.g., when they cough, sing, or shout. As shown in Fig 1, exposure to *Mtb* does not always lead to infection, because only 30% of those exposed become infected. Among those infected, 5 - 10% will develop active TB disease within two years of exposure, while the remaining 90-95% of infected individuals will develop latent TB. Latent TB is defined as having evidence of *Mtb* infection by a positive reaction to *Mtb* antigens in a

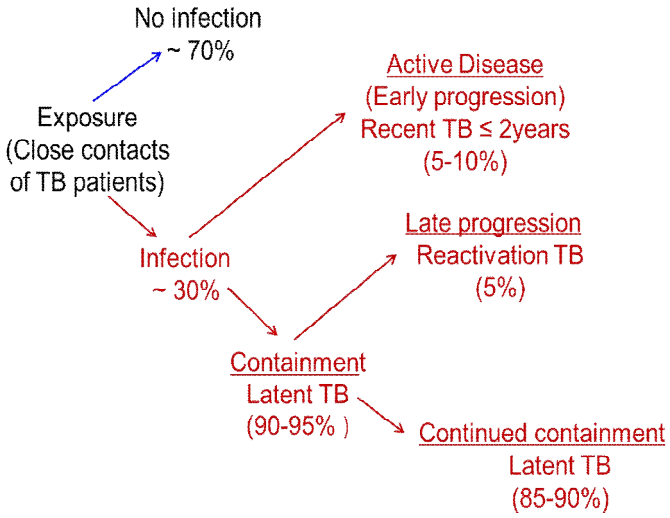


Fig.1. Spectrum of exposure to TB: from exposure to active disease or latent infection

tuberculin skin test (**TST**), but no clinical signs of disease and a negative chest radiograph. About 2 billion people in the world are infected with latent TB and 5-10% of these latently infected individuals will develop active TB disease (reactivation TB) during their lifetime (1–3).

1.2 Treatment of Tuberculosis

TB is a treatable disease. Patients with active TB disease are treated with a 6-month regimen of combined therapy composed of the four drugs: Rifampicin (**RIF**), Isoniazid (**INH**), Pyrazinamide (**PZA**), and Ethambutol (**EMB**). All four drugs are administered during the first 2-months but for the remaining 4 months, only RIF and INH are administered (4). To ensure that patients comply with the lengthy duration of treatment, pharmacotherapy is usually administered through the Directly Observed Treatment and Short-course drug therapy (**DOTS**) program. In the DOTS program, the health worker or community volunteer provides the drugs to the patient and observes as the patient takes the drugs (5). A TB patient is classified “treatment success” if sputum from the patient is smear- or culture-negative during the last month of treatment and the person ingested the drugs for the entire 6-month program without any evidence of treatment being interrupted (6).

1.3 Factors that increase the risk of treatment failure in TB patients

Treatment of TB patients with the standard 6-month drug regimen of RIF, INH, PZA and EMB is expected to lead to treatment success in all patients who adhere to the regimen (7). Unfortunately, about 12 - 20% of TB patients fail treatment and another 3 – 10% relapse within 2 years of completing their medication regimen, despite having drug-susceptible *Mtb* (1,8,9). Treatment failure is due to two major factors: (i) microbial

and (ii) host factors. As shown in Fig 2, patients harboring persistent or drug-resistant *Mtb* are likely to fail treatment (10–14). Likewise, patients with an impaired or compromised immune response or with inadequate plasma anti-TB drug levels are also likely to fail treatment (15,16).

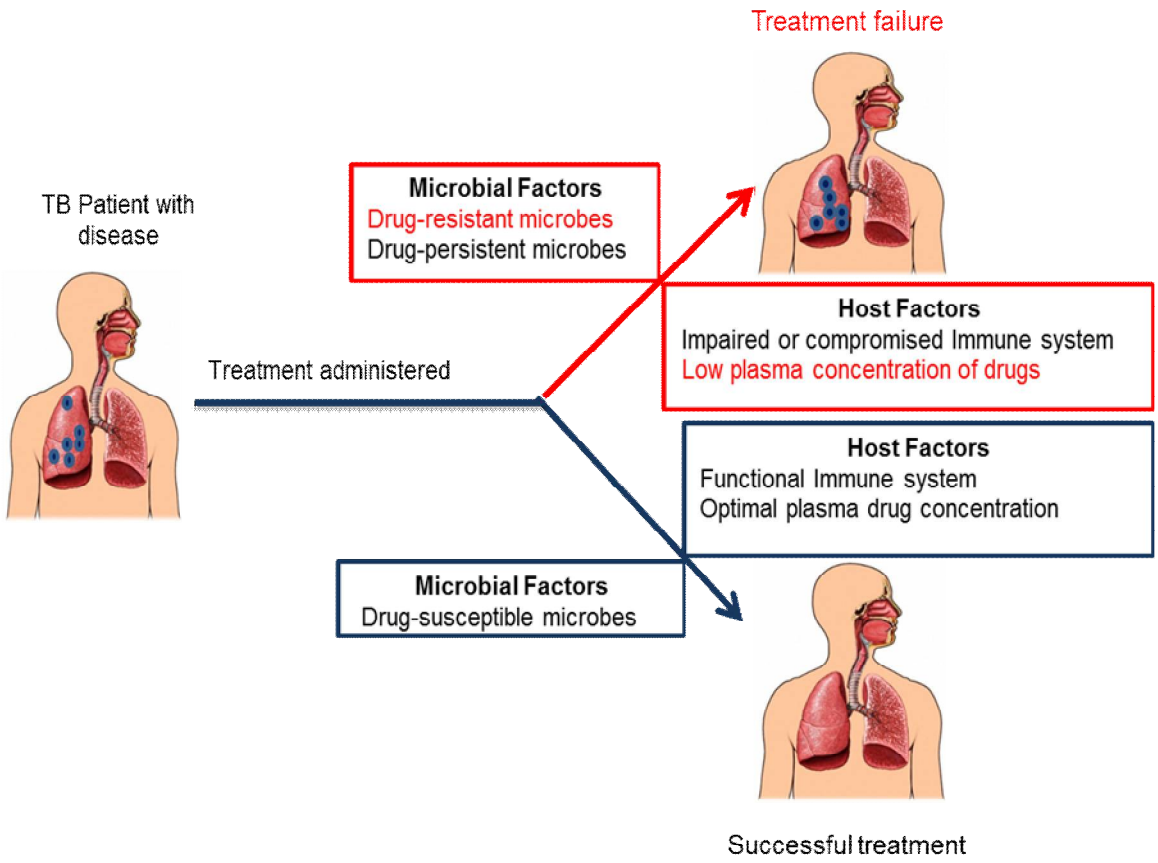


Fig 2: Microbial and Host factors that increase risk of TB treatment failure

1.4 Microbial factors that increase risk of treatment failure in TB patients

1.4.1 Drug-persistent *Mycobacterium tuberculosis*

Microbial drug-persistence is a phenomenon whereby some bacterial cells within a population survive the effects of antimicrobial treatment without acquiring resistance-conferring genetic changes. These surviving bacterial cells are called drug-persistent microbes. Drug-persistent microbes are genetically identical to drug-susceptible microbes and appear to be either slow-growing or non-replicating in the presence of the drug but resume growth once the drug dissipates. Unlike drug-resistant microbes, drug-persistent microbes have nonheritable phenotypic resistance to antimicrobial drugs and their progenies remain fully drug-susceptible to antimicrobials upon regrowth (11,17–19). To date, all bacterial species including *Mtb* have been reported to produce drug-persistent microbes (20).

When exposed to *Mtb* bacilli, most anti-TB drugs demonstrate a biphasic killing activity. Effectively, the fast growing bacilli (that are one *Mtb* subpopulation), are rapidly killed while another subpopulation, the slowly or non-replicating bacilli, respond more slowly to the anti-TB drugs. This biphasic killing indicates that there are at least two *Mtb* subpopulations and these subpopulations differ in their intrinsic drug susceptibility (10,20–26). The slowly or non-replicating subpopulation are drug-tolerant and referred to as drug-persistent *Mtb bacilli* (22).

Mtb bacilli can become drug-persistent through several mechanisms. These mechanisms include: expression of toxin-antitoxin modules that suppress metabolic activities (27); asymmetric growth of bacilli during cell division producing physiologically distinct daughter bacilli with differential susceptibility to antimicrobials (28); stochastic

expression of drug activating genes, such as no or reduced expression of catalase-peroxidase *KatG* enzyme that activates INH into its active form (29); and up-regulation of efflux pumps, such as the efflux pump *Rv1258* to inhibit cellular accumulation of RIF (12). The formation of drug-persistent bacilli following exposure to the anti-TB drug could be viewed as an adaptation of a subpopulation of *Mtb* bacilli to resist the adverse effect of the drugs. These drug-persistent *Mtb* bacilli can impair treatment, resulting in treatment failure or relapse but more disturbingly, they could eventually evolve to drug-resistant *Mtb* bacilli (30,31).

1.4.2 Drug-resistant *Mycobacterium tuberculosis*

According to MacGowan (2008), drug resistance in pathogenic bacteria can be defined in two ways: first, in terms of the clinical outcome that is a patient receives the drug and is not cured and secondly with reference to the drug-susceptibility of the bacterial population prior to treatment. As clinical outcome cannot be determined prior to treatment, drug-resistance is generally defined based on drug-susceptibility of the bacterial population prior to treatment (32). Drug-resistant TB is also defined based on the drug-susceptibility of the *Mtb* population prior to treatment (33). As a consequence, a patient with drug resistant-TB is a patient who harbors *Mtb* bacilli that are resistant to the drug concerned and the patient will fail to respond to treatment with the drug (33).

Globally, drug-resistant TB has been an area of growing concern and poses a dire threat to the effective control of TB (1,34,35). This threat is exemplified by the outbreak of drug-resistant TB in New York City in the early 1990s (36), with nearly all major hospitals reporting drug resistant TB cases and fatality rates greater than 80% (37). Today, fewer than 20 cases of drug-resistant TB (38) are reported in New York

City each year, but high numbers of cases are still reported in other parts of the world where drug-resistant TB is on the rise (1). Drug-resistant TB can be acquired in two ways: (i) during TB treatment owing to poor dosing or patient non-adherence to treatment or (ii) following contact with a drug-resistant TB patient. The former accounts for more drug-resistant TB cases than the latter (1,34).

There are several types of drug-resistant TB (Table 1), but the most prevalent is multidrug-resistant TB (**MDR-TB**). MDR-TB is defined as *Mtb* with *in vitro* resistance to both RIF and INH. According to the WHO, in 2015, an estimated 480,000 new cases of MDR-TB occurred. About 21% of previously treated TB cases developed MDR-TB, while 3.9% of new TB patients had MDR-TB. Still, in the same year, 250,000 patients with MDR-TB died. More disturbing is the tendency of MDR-TB patients to progress to more severe forms of drug-resistance such as extensively drug-resistance TB (**XDR-TB**). XDR-TB is *Mtb* with in-vitro resistance to at least RIF, INH, a quinolone, and 1 of 3 second-line injectables agents. Globally, about 9.5% of MDR-TB cases have XDR-TB (1,34).

Table 1: Different types of drug-resistant tuberculosis*

	Form of drug-resistant TB	Definition
1	Mono-drug-resistant TB	*Mtb* with in-vitro resistance to one of the drugs used for the treatment of drug-susceptible TB. Drug-susceptible TB is treated with RIF+INH+PZA+EMB
2	Poly-drug-resistant TB	*Mtb* with *in-vitro* resistance to more than one of the drugs used for the treatment of drug-susceptible TB other than both RIF and INH.
3	Multidrug-resistant TB (MDR-TB)	*Mtb* with in-vitro resistance to at least RIF and INH
4	Pre-extensively drug-resistant TB (Pre-XDR TB)	*Mtb* with in-vitro resistance to at least RIF and INH and either a fluoroquinolone or 1 of 3 second-line injectables agents (amikacin, kanamycin or capreomycin) but not both.
5	Extensively drug-resistant TB (XDR-	*Mtb* with in-vitro resistance to at least RIF, INH a quinolone, and 1 of 3 second-line injectables agents

	TB)	
6	Totally drug-resistant TB or extremely drug-resistant TB (XXDR TB)	*Mtb* with in-vitro resistance to all drugs used to treat drug-susceptible TB as well as all drugs used to treat at least RIF, INH a quinolone, and 1 of 3 second-line injectables agents
Definitions obtained and modified from (39)		

As the incidence of drug-resistant TB increases, new types of drug-resistant TB emerge. If nothing is done to combat the increasing number of drug-resistant TB cases, it will lead to the emergence of a group of patients for whom there are no drugs available. Although there is hope that new drugs will be quickly discovered, preventive methods, such as early and rapid detection of patients at risk of developing drug resistance, could be used to reduce the spread of drug-resistant TB. Early and rapid drug susceptibility testing of patients will lead to the immediate initiation of appropriate treatment of patients thereby limiting spread (40).

1.4.3 Diagnosing Drug-resistant Tuberculosis

As previously defined, drug-resistant TB refers to *Mtb* isolates with *in vitro* resistance to the drug being tested. As such, diagnosing drug-resistant TB implies conducting drug susceptibility testing (**DST**) of *Mtb* isolates to the various drugs. Several assays are available for the DST of *Mtb* isolates. These assays could be classified as either phenotypic or genotypic.

1.4.3.1 Phenotypic assays for diagnosing drug-resistant TB

Prior to 1960, diagnostic laboratories used diverse phenotypic or culture-based assays for DST of *Mtb* isolates. Although the underlying principles were the same, the

assays used different methods to categorize *Mtb* isolates as either drug-resistant or drug-sensitive. The three methods used were the absolute concentration method, the resistance-ratio method, and the proportion method. Following a consensus meeting organized by WHO in 1961, the culture-based proportion method was adopted as the standard method for DST of *Mtb* isolates (33,41).

In the culture-based proportion method, a clinical specimen from a patient is cultured in a solid or liquid medium for growth of *Mtb* (33,41). Once growth of Mtb is observed, a suspension of the bacteria is made and adjusted to a turbidity equivalent to that of a 0.5 McFarland standard units and used for DST (33,41). To perform DST, two slants or tubes are used. One slant or tube contains only medium and is referred to as the drug-free slant or tube, while the other slant or tube contains both medium and a single concentration of the drug being tested. This single concentration is referred to as the critical concentration (33,41). A 1:100 dilution of the growth suspension is made and added to the drug-free slant or tube, while the undiluted growth suspension is added to the drug-containing slant or tube. Either slants or tubes are incubated and checked for growth of *Mtb* bacilli. Once growth is observed, the numbers of colonies on the drug-free and drug-containing slants or tubes are counted (33,41). According to the proportion method, the *Mtb* isolate is classified as resistant to the drug if the number of colonies on the drug-containing medium is ≥ 1 % of the colonies on the drug-free slant plate (33,41). The underlying assumption in this method is that drug resistance develops by natural selection even without exposure to the drug. As such every *Mtb* isolate will be heterogeneous consisting of both drug-resistant and drug-susceptible bacilli. Hence, for a susceptible *Mtb* isolate, the proportion of potentially resistant bacilli

based on the mutation rate of 1 in 100 million cells would theoretically be only 0.000001% of the entire population (33,41). Thus, the presence of 1% resistant bacilli represents a substantial increase in resistance. This assumption further implies that the difference between a resistant and susceptible *Mtb* isolate is that the former has a larger proportion of resistant bacilli, comprising at least 1% of the bacillary population than the later. As such, the culture-based proportion method seeks to measure the proportion of resistant bacilli within an *Mtb* population(33,41).

Although the culture-based proportion method is universally regarded as the standard method for determining drug susceptibility of *Mtb* isolates, there are multiple problems with this method (42). Majority of the concerns are with the single drug concentration referred to as the critical concentration used to determine if an *Mtb bacillus* is resistant or susceptible. First, the critical concentrations of anti-TB drugs were set by general consensus without any scientific evidence (41,43,44). Secondly, the critical concentration of RIF and INH are set too high and ought to be lowered to 0.0625mg/L and 0.0312mg/L, respectively (43,45). Thirdly, as shown in Table 2, the critical concentration of some drugs bear little relation to the concentration of the drug in plasma (46). Lastly, the critical concentration of drugs varies according to the growth medium used (Table 2) (47). The critical concentrations were initially established in Lowenstein-Jensen (**LJ**) medium. Following the development of modern solid and liquid media, the critical concentration in LJ media was used as a reference to establish the critical concentrations of other media. Although this may have seemed a straightforward approach, recent studies have identified some irregularities with some media such as with the automated BACTEC Mycobacterium Growth Indicator Tube (MGIT) 960 system

(44). Van Deun and colleagues (2013) showed that drug susceptibility testing using the BACTEC MGIT 960 system misclassified some drug-resistant Mtb isolates as drug-susceptible. These misclassified isolates had a mutation in their drug target genes and were classified drug-resistant on Lowenstein Jensen media (48).

Table 2. Critical concentrations for drugs used to treat drug-susceptible *Mycobacterium tuberculosis**

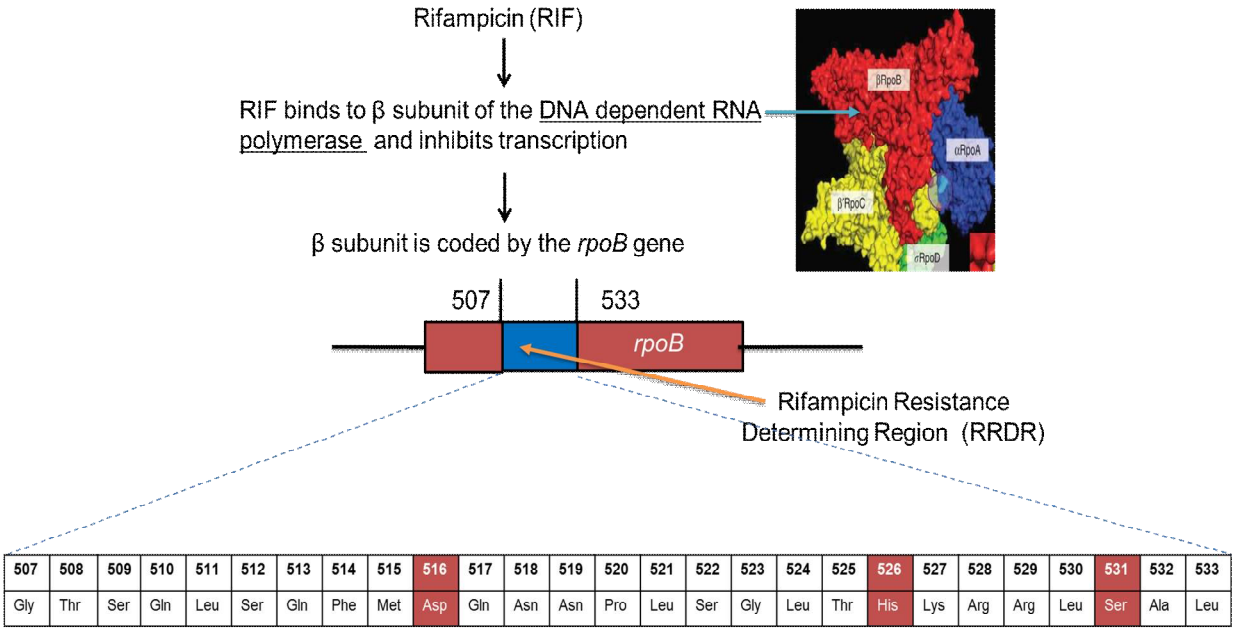
Drug	Concentration in serum (µg /mL)	Critical concentration (µg/mL) (Single drug concentration used in testing).			
		Lowenstein Jensen	Middlebrook 7H10	Middlebrook 7H11	MGIT 960
RIF	10	40	1.0	1.0	1.0
INH	5 -10	0.2	0.2	0.2	0.1
EMB	2 - 5	2.0	5.0	7.5	5.0
PZA	40-50	-	-	-	100.0

*Obtained with permission from (46,47).

Despite the technical limitations of the culture-based proportion method, it is still considered the 'gold standard' for DST of *Mtb* isolates (42). However, the long turnaround time of this method makes it unfit for rapid DST of *Mtb* isolates. One to three months is often required before DST results become available following collection of specimen. Also, culturing large numbers of *Mtb* bacilli imposes the need for specialized and skilled personnel available only at centralized units. As such, the culture-based method is not a rapid test and cannot be used at peripheral sites. The shortcomings of the culture-based proportion method have fostered interest to the more rapid genotypic-assays for DST of *Mtb* (40).

1.4.2.1.2 Genotypic assays for diagnosing drug-resistant tuberculosis

In 2006, the Stop TB Partnership launched its 2006-2015 initiative entitled Global Plan to Stop TB. One of the laudable goals of this plan was to diagnose and treat 56% of MDR-TB patients by 2015 (49). However, by 2008 only 7% (29,423) of the then annually estimated 440,000 MDR-TB cases were being diagnosed (50). The general consensus was that if this goal were to be attained, new drug-resistant diagnostic techniques had to be created. Since then, substantial progress has been made especially with the development of genotypic or molecular-based assays. Some of these molecular-based assays are now being used for the diagnosis of drug-resistant TB. The fast turnaround time and ease of use have led to the adoption of molecular-based assays as the initial test for diagnosing suspected drug-resistant TB cases in most countries (51,52).



507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533
Gly	Thr	Ser	Gln	Leu	Ser	Gln	Phe	Met	Asp	Gln	Asn	Asn	Pro	Leu	Ser	Gly	Leu	Thr	His	Lys	Arg	Arg	Leu	Ser	Ala	Leu

Fig. 3. Adapted with permission from (111,112) . Mutations in the Rifampicin Resistance Determining Region (RRDR) of the *rpoB* gene: target of genotypic-based assays.

The principle of the molecular-based assays is that *Mtb* develops resistance to drugs through genetic mutations in drug-target genes (53). For example (Fig 3), RIF inhibits mycobacterial transcription by binding to the β-subunit of the DNA-dependent RNA polymerase enzyme. The β-subunit of this enzyme is encoded by the *rpoB* gene. Development of resistance to RIF is due to mutations in a well-defined, 81 base pair (bp) region of the *rpoB* gene referred to as the Rifampicin Resistance Determining Region (**RRDR**).

This 81bp region, codes for amino acids 507 through 533 of the β subunit. Ninety-five to Ninety-six % of RIF-resistant *Mtb* isolates harbor mutations in this 81-bp region with codons 531, 526, and 516 being the most frequently reported mutation sites (54). As such, genotypic-based assays diagnose resistance to RIF by identifying mutations in the RRDR of the *rpoB* gene.

Conversely, INH is a pro-drug and must be activated to its active form by the *Mtb* enzyme catalase-peroxidase (**KatG**) (55). The active form of INH inhibits mycolic acid synthesis, a cell wall component of *Mtb,* by binding to the enzyme keto-acyl reductase (inhA). As a consequence, resistance to INH could arise at one or more points in the *KatG* gene where a mutation would result in altered enzyme structure (56). This structural change could result to decrease conversion of INH to its active form. Likewise, mutations in the promoter region of the *inhA* gene may lead to over-expression of the enzyme, thereby diminishing the effectiveness of INH (57). About 80% of INH resistant isolates have mutations in either *KatG gene* or inhA promoter region (58). As such, genotypic assays diagnose resistance to INH by detecting mutations in

the *KatG* gene and in the promoter region of *inhA* gene. Specifically, these assays seek to detect a mutation in codon 315 of the *KatG* gene that leads to a change in amino acid from Ser to Thr, and four nucleic acid mutations in the promoter region of the *inhA* gene (59,60). These four nucleic acid mutations are a mutation at position -15 from C-T, a mutation at position -16 from A to G and a mutation at position -8 from T to either C or A (57,61). Besides RIF and INH, a list of genes and mutations associated with drug-resistance in *Mtb* to other drugs is available in several reviews (53,62,63).

At present, WHO has endorsed two commercially available molecular-based assays for drug susceptibility testing of *Mtb* to RIF and INH; the line probe assay (LPA) (64) and the Xpert *Mtb*/RIF assay (40). The LPA is a 3-step assay. First DNA is extracted from cultured *Mtb* isolates or directly from clinical specimens. Secondly, the gene under investigation is amplified by PCR using biotinylated primers. Lastly, the PCR products are hybridized to specific oligonucleotide sequences immobilized on a strip. Once captured, the biotin-labelled hybridized products are detected by colorimetric reactions that create colored bands on the strip at the sites of hybridization. The LPA detects the presence of *Mtb* and the presence of mutations in the drug target genes for RIF and INH. If a mutation is present in one of the target regions, the amplicon will not hybridize to the wild-type probe on the membrane. If the mutation is one of the frequent mutations, the amplicon will bind with the mutation probe. Two commercial LPAs are currently available: the INNO-LiPA Rif. TB test (Innogenetics NV, Gent, Belgium) and the Genotype MTBDR*plus* test (Hain Lifescience Gmbh, Nehren, Germany) (65). Both LPAs detect *Mtb* and resistance to RIF, but only the Genotype MTBDR*plus* detects resistance to INH. Systematic reviews on the performance of LPAs reported good

accuracy for detecting resistance to RIF and INH (31,66–69). Arentz and colleagues (2013) reported a pooled sensitivity and specificity of 94.1% and 98.8%, respectively for the detection of resistance to RIF for the INNO LiPA RIF test (66). In a recent meta-analysis, Bai and colleagues (2016) reported a pooled sensitivity of 91% for resistance to INH, 96% for resistance to RIF and 91% for MDR-TB. The pooled specificity were 99% for INH; 98% for RIF and 99% for MDR-TB for the Genotype MTBDR*plus* assay (68).

The Xpert Mtb/RIF assay (Cepheid, Sunnyvale, CA, USA) is a semi-nested real-time nucleic acid amplification test. The test simultaneously detects *Mtb* and resistance to RIF within 2 hours. A 2014 Cochrane review concluded that the Xpert Mtb/RIF assay provides accurate results when used for the diagnosis of RIF resistance (70). The review reported a pooled sensitivity and specificity of 95% and 98% for the detection of RIF resistance by the Xpert MTB/RIF assay (70).

To date, only two published studies have directly compared the performance of the LPA and Xpert MTB/RIF for the detection of resistance to RIF. Unfortunately, the studies reported different results. Rufai and colleagues (2014) reported a superiority of the LPA over the Xpert MTB/RIF, whereas Rahman and colleagues (2016) reported the opposite (71,72).

1.5 Host factors that increase the risk of tuberculosis treatment failure

Besides microbial factors, host factors also play a major role towards treatment success or failure in TB patients. Two host factors that may increase the risk of drug

failure are an impaired immune response and low plasma concentration of anti-TB drugs (16,17,68).

1.5.1 Impaired Immune Response

Successful treatment of bacterial infections generally requires both the antibiotic and the host's immune response. The antibiotic is believed to rapidly reduce the growing bacterial population, while the immune response eliminates any antibiotic-evading (resistant or persistent) bacterial cells. However, when designing antibiotic treatment regimens, the contribution of the immune response is not considered (74,75), as the immune response to bacterial infections is complex. Generally, an immune response involves many cell types and cytokines. In addition, the immune response is extremely difficult to identify, making it difficult to establish a model that could be used to evaluate antibiotic treatment (74,75).

Likewise, with TB, the immune response has not generally been considered when designing treatment regimens, even though it contributes towards treatment success or failure. TB treatment outcome studies report a high likelihood of treatment failure in patients with impaired immune systems, such as patients co-infected with HIV or type 2 diabetes (73,76). These patients are unable to mount an effective immune response to augment the drug action. As a result of treatment failure, these patients may suffer adverse outcomes, such as increased risk of relapse and death (16,73,77).

Although a good immune response during TB disease is generally considered to complement drug action, it can antagonize drug action. A typical "good" immune response to TB is characterized by the formation of granulomas that is an aggregation

of immune cells. In human TB, granulomas are composed of an inner core of *Mtb* infected macrophages, stimulated macrophages that have differentiated into multinucleated giant cells, epithelioid cells, and foamy macrophages and neutrophils. The inner core of cells is surrounded by a rim of lymphocytes, that consist largely of CD4+ T cells, CD8+ T cells, B cells and fibroblasts (2). Granulomas help contain or wall off the infecting *Mtb* thereby avoiding dissemination. However, some *Mtb* bacilli can actually survive and replicate within these granulomas. In addition, some anti-TB drugs are either unable to penetrate granulomas or are present in sub-therapeutic concentrations. Thus, the formation of granuloma induced by the immune response creates a protective niche for *Mtb* to flourish in absence of no or low concentration of a drug that could lead to persisting disease and subsequent treatment failures (10,78).

1.5.2 Low plasma concentration of anti-tuberculosis drugs

Another host factor responsible for treatment failure is low plasma concentration (below therapeutic level) of anti-TB drugs. Low plasma concentrations of a single drug in the TB treatment regimen are associated with treatment failures, relapse and acquired drug resistance (79). As shown in Fig.4, a large proportion of patients have low plasma concentrations of all anti-TB drugs used in the treatment regimen including RIF, an indispensable drug in the TB treatment regimen (80–88). RIF affects both replicating and non-replicating Mtb bacilli, but it is thought to have a greater effect on non-replicating drug-persistent Mtb bacilli residing in hypoxic conditions. This drug action of RIF on non-replicating persistent *Mtb* bacilli allowed the shortening of treatment from 12 to 6 months (89,90). Furthermore, in a recent phase 2B trial TB patients with drug-

susceptible TB, high dose of RIF (35mg/kg) resulted in rapid clearance of *Mtb* bacilli (culture conversion) (91). Thus, the consequences of low plasma concentrations of RIF are serious to the patient and influences the design of new drug-treatment regimens.

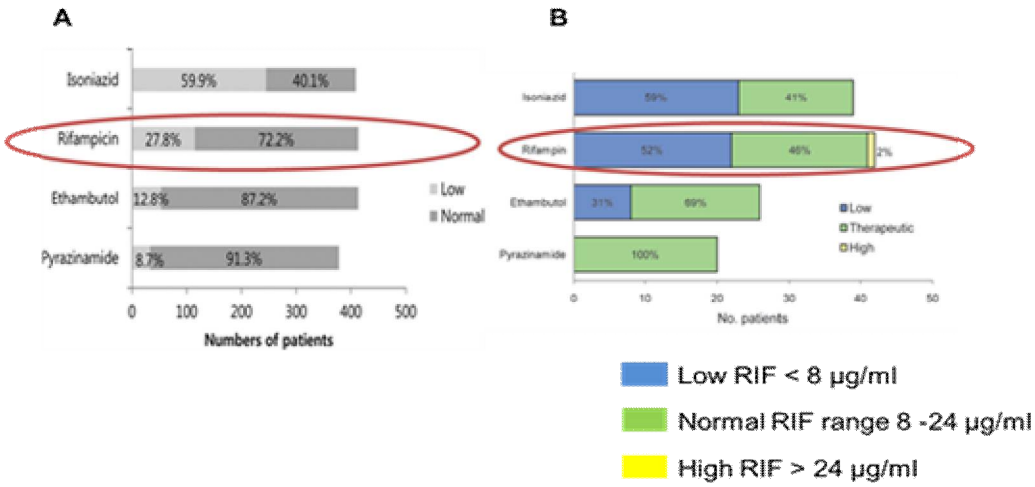


Fig. 4. Proportion of TB patients with low plasma concentration of anti-TB drugs A) Adapted with permission from (88). B) Adapted with permission from (113)

1.5.3 P-glycoprotein and low plasma concentration of Rifampicin

Low plasma concentration of orally administered RIF could be as a result of several factors (i) poor absorption, (ii) pre-systemic metabolism or (iii) rapid systemic clearance. When orally administered under fasting conditions, RIF is usually well absorbed and reaches peak plasma concentration within 2 hours (92). Poor absorption of RIF could occur when taken with food or if the patient is suffering from a predisposing health condition, such as diarrhea, HIV (93), or diabetes (94), that could impair drug absorption (95). Although these conditions may affect the absorption of RIF, not all patients with low plasma concentrations have these prevailing conditions (96). Alternatively, the low plasma concentration of RIF could be due to pre-systemic metabolism. Pre-systemic metabolism refers to the rapid breakdown of a drug by the liver or gut wall enzymes before it reaches systemic circulation (97). Pre-systemic

metabolism mostly affects orally administered drugs. One of the ways to bypass pre-systemic metabolism is to administer the drug intravenously. To determine if pre-systemic metabolism will affect the plasma concentration of a drug if administered orally, the plasma concentration of the drug following intravenous administration is compared to the plasma concentration of the same drug following oral administration (97). Loos and co-workers (1985) compared the plasma concentration of intravenously administered RIF to that of orally administered RIF and reported that the plasma concentration of RIF administered orally was 93% of IV administered dose. This implies that pre-systemic metabolism has little effect on plasma concentration of orally administered RIF (98). However, Loos and colleagues (1985) observed a decrease in plasma concentration of RIF from 93% after the first single oral dose to 68% after three weeks of oral administration. This reduction coincided with an increase in systemic clearance of RIF from 5.69 to 9.03 l/h (98). As such, variation in the systemic clearance of RIF could likely be the cause of low plasma concentration of RIF.

RIF is extensively metabolized in the liver and its metabolic products are predominantly excreted in bile and eliminated in stool (98). As such, variation in systemic clearance of RIF could result from differences in the rate of clearance of RIF into bile. As shown in Fig 5, RIF in blood flowing through the liver is cleared by hepatocytes. Hepatocytes use a series of membrane transporters and metabolizing enzymes to extract substances from blood and excrete them into bile. The uptake transporter, organic anion transporter polypeptide 1b1 (OATP1B1) is expressed on the basolateral membrane of hepatocytes and facilitates the uptake of RIF into hepatocytes (99–101). Once in the hepatocyte, RIF is either metabolized by β-esterases into its

primary metabolite 25 deacetyl rifampicin or excreted un-metabolized (102). Both un-metabolized and metabolized RIF are primarily excreted out of the hepatocyte into the bile canaliculi by the efflux pump p-glycoprotein (**P-gp**) expressed on the canaliculi (apical) membrane. Due to the ability of P-gp to move its substrates into bile even against a 100-1,000-fold concentration gradient (103), it constitutes the primary driving force for the excretion of its substrates into bile. As such, the level of expression and activity of P-gp may have a profound effect on the plasma concentration of RIF and its eventual elimination pattern.

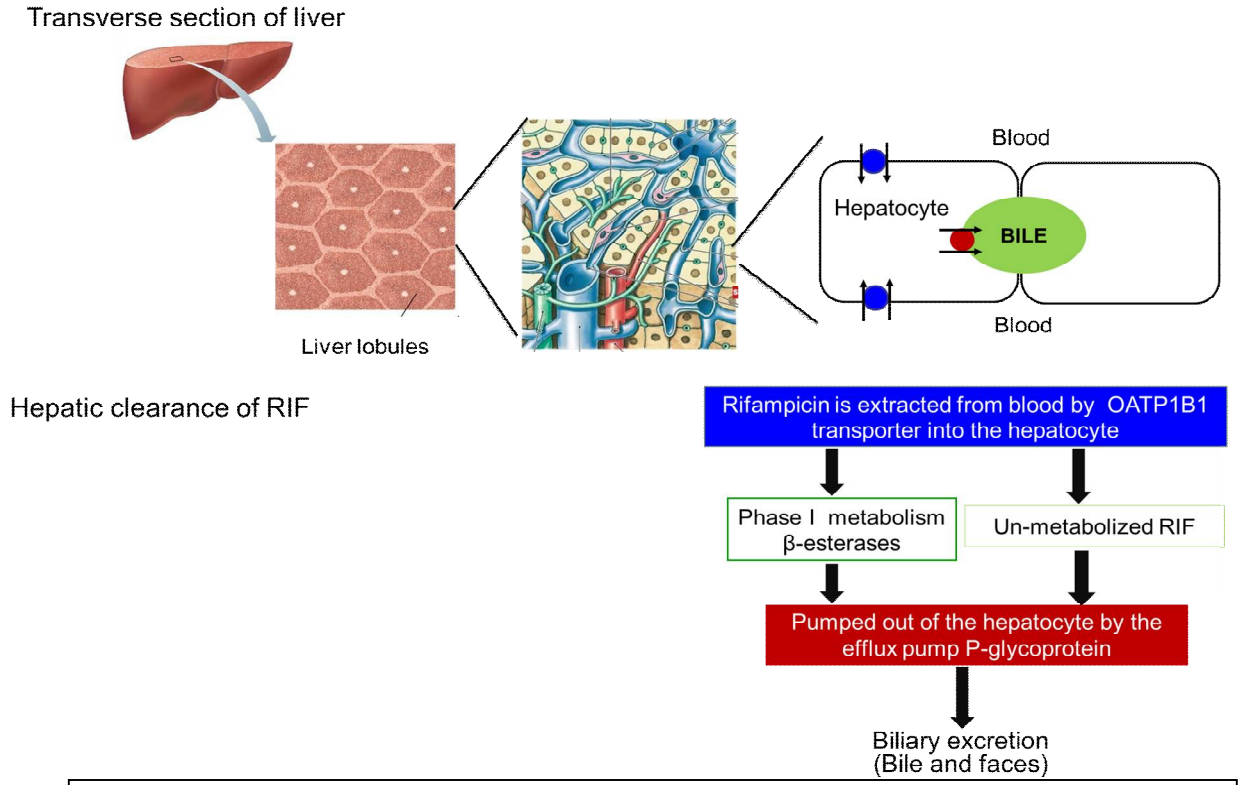


Fig 5. Diagrammatic location of membrane transporters and hepatic clearance of RIF from blood into bile. Pictures of liver obtained from (114)

1.6 Tuberculosis in Cameroon

Cameroon is a central African country with a high TB burden. Cameroon is among the top 20 countries with the highest estimated numbers of incident TB cases among people living with HIV (**PLHIV**) (104). According to WHO, in 2015, an estimated 26,570 people in Cameroon had active TB disease of whom 32% (8,606) were PLHIV (105). Pulmonary TB is the most common form of TB and diagnosis is primarily via detection of *Mtb* bacilli or acid-fact bacilli (**AFB)** in sputum smears of patients by conventional light microscopy. A patient is TB-positive if AFB are detected in at least one of two sputum samples (106). Once a patient is diagnosed with TB, they are immediately placed on the standard 6-month TB regimen and monitored through the DOTs program. Upon completing the 6-month treatment regimen, a smear of the patient's sputum is examined for the presence of AFB. If the patient is AFB- negative, the patient is declared cured. However, if the patient is AFB positive, he or she undergoes to drug susceptibility testing to determine if the patient harbors drug-resistant *Mtb* bacilli. If the AFB-positive patient is drug-susceptible, the patient is classified as 'retreatment' and placed on an 8-month regimen composed of RIF, INH, PZA, EMB, and Streptomycin. Alternatively, if the AFB-positive patient is drug resistant, a new treatment regimen is administered to the patient based on the type of drug resistance (4)

1.6.1 Drug-resistant tuberculosis in Cameroon

The actual burden of drug resistance in Cameroon is not known due to the limited capacity to provide routine DST to all TB patients. Even in high-risk patient groups such previously treated TB patients for whom DST is recommended, not all

patients are tested. Nonetheless, epidemiological studies conducted in different regions of the country report 7 to 23% *Mtb* resistance to a single drug in the 6 months regimen among newly diagnosed TB patients and 16.6 to 41% among previously treated TB patients (107,108). Beside mono-resistance, other severe forms of resistance, such as MDR-TB, have been reported. Recent WHO estimates suggest that each year about 3.2% new pulmonary TB patients in Cameroon have MDR-TB and another 14% of previously treated pulmonary TB patients develop MDR-TB (105).

1.6.2 Diagnosing drug-resistant tuberculosis in Cameroon

Prior to 2010, the only laboratory in Cameroon that performed DST of *Mtb* isolates was The National Tuberculosis Reference Laboratory (109). All clinical specimens or *Mtb* cultures from presumptive drug-resistant patients were sent to this laboratory for DST. This laboratory used culture-based proportion method on Lowenstein-Jensen medium for DST (109). However, in 2010, the government of Cameroon signed an agreement with the Expand Access to New Diagnostics for TB (**EXPAND-TB**) program. The EXPAND-TB program, a UNITAID funded program, built two TB DST laboratories in the cities of Bamenda and Douala and equipped all laboratories with the rapid liquid culture based system, BACTEC MGIT 960 system and the LPA assay, Genotype MTBDR*plus* (110). Subsequently, in 2012, the National Tuberculosis program in Cameroon (**NTCP**) received financial support from several international donor organizations to purchase and introduce the Xpert MTB/RIF assay in the three TB DST laboratories and at some TB treatment centers. At present, the Xpert MTB/RIF assay is used as the initial assay to test all previously treated TB patients for

resistance to RIF. If RIF-resistance positive, the test results must be confirmed with either the Genotype MTBDR*plus* and or BACTEC MGIT 960 system.

1.7 Study Aims

Successful treatment of TB requires consideration of, and medical attention to, a combination of microbial and host factors. Patients infected with drug-resistant Mtb bacilli are likely to fail treatment. Likewise, patients with low plasma drug concentrations are likely to experience treatment failures. Accordingly, to ensure patients have a successful treatment outcome, we need to diagnose drug-resistant *Mtb* and understand why some patients have lower than expected drug plasma concentrations.

Therefore, the overall objectives of this dissertation were to determine if use of the genotypic assay, Genotype MTBDR*plus*, can be used to distinguish resistant from susceptible *Mtb* isolates in Cameroonian TB patients and to investigate if P-gp might be responsible for variation in plasma concentration of RIF. Specifically, we propose to:

Aim # 1: Test the hypothesis that the Genotype MTBDR*plus* assay that detects four mutations for RIF and five mutations for INH will identify drug-resistant TB cases in Cameroon. We hypothesize that the Genotype MTBDR*plus* assay will detect all cases of RIF, but may not detect all INH resistant cases.

Significance: The National Tuberculosis Control Program of Cameroon (NTCP) plans to use the Genotype MTBDR*plus* assay for the rapid diagnosis of patients with drug-resistant TB. Our study will provide the necessary scientific evidence to guide the NTCP on the accuracy of this assay if adopted.

Aim #2: Determine if failure to hybridize to wild type RIF probes and absence of hybridization to known mutation probes in the Genotype MTBDR*plus* assay indicates a

technical problem with the assay or presence of additional mutations. The manufacturer of the Genotype MTBDR*plus* assay recommends that a test sample should be interpreted as resistant if there is absence of hybridization to the wild-type probe and presence or absence of hybridization to a RIF-mutation probe. However, in cases that fail to hybridize to the RIF mutation probe, this outcome does not identify the type of mutation present and whether the mutation is associated with resistance to RIF. Sequencing of the *rpo*B genes of *Mtb* isolates with such Genotype MTBDR*plus* results will help determine if novel, RIF-resistant mutations are present.

Significance: Our study will help determine if one of the recommendations for interpreting the Genotype MTBDR*plus* assay appropriately classifies RIF resistant cases.

Aim #3: Determine the level of expression of P-gp in the liver of children, adults and the elderly. We hypothesize that P-gp is likely expressed from birth based on its critical contribution to hepatic transport functions, but that expression levels take time to reach adult levels and vary between individuals.

Significance: If the expression of P-gp is determined to be the root cause of low plasma concentration of RIF due to developmental dynamics and/or inter-individual variability, then modulating the effect of P-gp on systemic drug levels with specific inhibitors can be conducted to identify inhibitors that prevent low plasma concentration of RIF.

Knowing precisely the drug-susceptibility status of TB patients is important for the adequate treatment of TB patients. Most National Tuberculosis Control Programs, including that of Cameroon, plan to introduce molecular-based assays such as the Genotype MTBDR*plus* assay for drug-susceptibility testing of TB patients. This assay is

able to quickly identify drug-resistant TB; thereby, allowing the administration of adequate and timely treatment regimens. However, it is not known if this assay will adequately detect drug-resistant TB. Our proposed study will provide the necessary scientific evidence to guide National Tuberculosis Program of Cameroon on whether to introduce or not the Genotype MTBDR*plus* system as a means to rapidly diagnose drug resistant TB. Also ensuring TB patients receive optimal concentration of drugs is necessary to ensure good treatment outcome. Our investigation of the p-gp in human livers will provide a plausible explanation for low plasma concentration of RIF in patients. Knowledge of p-gp expression will provide the opportunity to enhance the plasma concentration of RIF by modulating the effect of p-gp.

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CHAPTER 2

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Diagnostic Accuracy and Usefulness of the Genotype MTBDR*plus* Assay in Diagnosing Multidrug-resistant Tuberculosis in Cameroon? A Cross-Sectional Study

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2.1 ABSTRACT

Background

Drug-resistant tuberculosis, especially multidrug-resistant tuberculosis (MDR-TB), is a major public health problem. Effective management of MDR-TB relies on accurate and rapid diagnosis. In this study, we assessed the diagnostic accuracy of the Genotype MTBDR*plus* assay in diagnosing MDR-TB in Cameroon, and then discuss on its utility within the diagnostic algorithm for MDR-TB.

Methods

In this cross-sectional study, 225 isolates of *Mycobacterium tuberculosis* cultured from sputum samples collected from new and previously treated pulmonary tuberculosis patients in Cameroon were used to determine the accuracy of the Genotype MTBDR*plus* assay. We compared the results of the Genotype MTBDR*plus* assay with those from the automated liquid culture BACTEC MGIT 960 SIRE system for sensitivity, specificity, and degree of agreement. The pattern of mutations associated with resistance to RIF and INH were also analyzed

Results

The Genotype MTBDR*plus* assay correctly identified Rifampicin (RIF) resistance in 48/49 isolates (sensitivity, 98% [CI, 89%-100%]), Isoniazid (INH) resistance in 55/60 isolates (sensitivity 92% [CI, 82%-96%]), and MDR-TB in 46/49 (sensitivity, 94% [CI, 83%-98%]). The specificity for the detection of RIF-resistant and MDR-TB cases was 100% (CI, 98%-100%), while that of INH resistance was 99% (CI, 97%-100%). The agreement between the two tests for the detection of MDR-TB was very good (Kappa =

0.96 [CI, 0.92-1.00]). Among the 3 missed MDR-TB cases, the Genotype MTBDR*plus* assay classified two samples as RIF-monoresistant and one as INH monoresistant.

The most frequent mutations detected by the Genotype MTBDR*plus* assay was the *rpoB* S531L MUT3 41/49 (84%) in RIF-resistant isolates, and the *KatG* S315T1 (MUT1) 35/55 (64%) and inhA C15T (MUT1) 20/55 (36%) mutations in INH-resistant isolates.

Conclusion

The Genotype MTBDR*plus* assay had good accuracy and could be used for the diagnosis of MDR-TB in Cameroon. For routine MDR-TB diagnosis, this assay could be used for *Mycobacterium tuberculosis* cultures containing contaminants, to complement culture-based drug susceptibility testing or to determine drug resistant mutations.

Keywords: Multidrug-resistant tuberculosis (MDR-TB), Genotype MTBDR*plus* assay, pulmonary tuberculosis, Cameroon. *InhA* promoter mutation*, KatG* codon 315 mutation*, rpoB* mutations.

2.2 BACKGROUND

The emergence of drug-resistant tuberculosis, especially multidrug-resistant tuberculosis (MDR-TB), threatens TB control efforts in most countries including Cameroon. MDR-TB is caused by strains of *Mycobacterium tuberculosis (Mtb)* with in-vitro resistance to two of the most potent anti-tuberculosis drugs, Rifampicin (RIF) and Isoniazid (INH) (1,2). Recent World Health Organization (WHO) estimates suggest that each year about 460/20882 (2.2%) new pulmonary TB (PTB) patients in Cameroon develop MDR-TB, and another 170/1575 (11%) previously treated PTB patients develop MDR-TB. Unfortunately, only a small fraction (~40%) of these patients have access to drug susceptibility testing (DST) for MDR-TB (3). In response to the need to expand access to diagnostics for patients at risk of MDR-TB, the government of Cameroon signed an agreement with the EXPANDx-TB program (Expand Access to New Diagnostics for TB) project in 2010. The EXPANDx-TB program is a UNITAID funded program with the overall objective to increase access to diagnostics for patients at risk of MDR-TB in 27 low-income and high TB burden countries, including Cameroon. In Cameroon, this program plans to strengthen existing MDR-TB diagnostic laboratories and establish new laboratories with the capacity to culture and conduct culture-based DST of *Mtb*. Also, the program introduced the Genotype MTBDR*plus* assay for the rapid diagnosis of MDR-TB (4).

The Genotype MTBDR*plus* assay (Hain Lifescience GmbH, Nehren, Germany) is a molecular-based test that can rapidly diagnose MDR-TB. This assay detects MDR-TB by identifying mutations in the *rpoB* gene associated with resistance to RIF and in the *KatG* gene and promoter region of the *inhA* gene associated with INH resistance (5,6).

Two independent systematic reviews concluded that the Genotype MTBDR*plus* assay was highly accurate in diagnosing MDR-TB when compared to the culture-based proportion method (7,8). These two reviews reported pooled sensitivities of 88.7% (7) and 91% (8) and specificities of 99.2% (7) and 99% (8), respectively, for detection of MDR-TB. Although there is evidence of the accuracy of the Genotype MTBDR*plus* assay, the prevalence of MDR-TB varies widely worldwide. This variation in prevalence of MDR-TB might have a significant impact on the predictive value of the Genotype MTBDR*plus* assay (9). Thus, it is important to evaluate the assay before its use in routine diagnosis.

In this study, we sought to determine the accuracy of the Genotype MTBDR*plus* assay to diagnose MDR-TB cases in Cameroon. Furthermore, the government of Cameroon has recently adopted, an alternative molecular-based test the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) for the initial screening of TB patients at risk of MDR-TB (10–12). The GeneXpert MTB/RIF assay simultaneously detects TB and resistance to RIF directly from clinical specimen within 2hours. At present, the government has introduced the GeneXpert MTB/RIF assay in 3 of the 10 regions of the country but plans to roll-out the assay to all parts of the Country (12). As such, we discuss on the additional use and role of the Genotype MTBDR*plus* assay in the diagnosis of MDR-TB amidst the availability of the GeneXpert MTB/RIF.

2.3 METHODS

Study design and setting

Participants were enrolled at six health facilities in three towns in Cameroon, between April 2015 and February 2016. The health facilities were the Jamot Hospital in Yaounde

(3.848^0 N, 11.502^0 E), the Bamenda Regional Hospital in Bamenda (5.963^0 N, 10.159^{0}E), and 4 facilities in Douala (4.051^0 N, 9.768^0 E) including Laquintinie Hospital, the St Albert the Great Hospital, the dispensary Catholic Barcelone and the New Bell District Hospital. These health facilities are the major TB diagnosis and treatment centers in their respective locations.

Study participants

Participants aged ≥ 15 years old visiting any of the six health facilities and diagnosed by the laboratory of the health facility to be sputum smear positive for acid-fast bacilli (AFB) or diagnosed by the consulting clinician at the health facility to have clinical symptoms and signs suggestive of PTB were recruited into the study. This recruitment approach, allowed us to enroll patients from whom sputum samples will likely yield positive *Mtb* culture.

Although there are many naïve PTB patients (n =20,882), very few are MDR-TB (2.2%; n=460/20882). To include a high proportion of patients likely to have MDR-TB, , the initial focus was to enrol previously treated PTB patients in whom the proportion of MDR-TB is higher (11%; n= 170/1575) and then expand enrollment to include naïve PTB patients. Written informed consent was obtained from all study participants, and a structured questionnaire was used to collect demographic and medical information, including prior PTB history, age, gender, and HIV status. An expectorated sputum specimen was collected from each participant and transported to the study laboratory for mycobacterial culture, DST and MTBDR*plus* assay.

Specimen processing and microbiology testing

Mycobacterial culture and drug susceptibility testing were performed at the Mycobacteriology unit of Centre Pasteur du Cameroun in Yaounde. Within 48 hours of receipt of sputum samples in the laboratory, smears were made, stained using the auramine staining technique and examined by fluorescence microscopy. The smears were read and graded according to WHO guidelines and that of the International Union against Tuberculosis and Lung Diseases (IUALTD) (13,14). The sputum samples were then processed for culturing by decontaminating in Sodium hydroxide-Sodium citrate-N-acetyl L-cysteine (NaOH-NaC-NALC) solution and cultured in MGIT tubes using the BACTEC MGIT 960 system (Becton Dickinson, Franklin Lakes, NJ). MGIT tubes that gave a positive fluorescent signal with the BACTEC MGIT 960 equipment were checked for acid-fast-bacilli using Ziehl-Neelsen staining and confirmed for *Mtb* complex (MTBC) using the TB Ag MPT64 test (SD Bioline, Standard Diagnostics, Suwon, Korea). Cultures growing MTBC were assessed for contamination with other bacteria or fungi by growth on blood agar medium for 24 hours at 37^0C. If no contaminants (*i.e.,* Bacteria or fungi) were detected on the blood agar, drug susceptibility testing (DST) was performed using the MGIT 960 Streptomycin-Isoniazid-Rifampicin-Ethambutol (SIRE) kit (Becton Dickinson Diagnostic Systems). If contaminants were detected on the blood agar, the MTBC cultures were decontaminated and the culturing process repeated. The critical concentrations of Streptomycin, Rifampicin ,Isoniazid and Ethambutol used in the MGIT 960 SIRE kit were respectively 1.0µg/ml, 1.0µg/ml, 0.1µg/ml and 5.0µg/ml.

Immediately the DST result of a patient's sample was available, an aliquot of the culture was made and stored at $+4^0$C. Once the total number of cultures with DST results reached 226, the stored aliquots were screened using the Genotype MTBDR*plus* assay.

Genotype MTBDR*plus* assay

Samples were screened using the Genotype MTBDR*plus* assay according to manufacturer's instructions (5) and interpreted without knowledge of susceptibility results determined by the MGIT SIRE 960 system. Testing consisted of three steps: DNA extraction using the Genolyse kit (Hain Lifescience, Nehren, Germany), multiplex PCR amplification using biotinylated primers and reverse hybridization. The three steps were carried out in three separated rooms.

Each Genotype MTBDR*plus* strip had 27 reaction zones or probes that hybridize DNA (amplicons). Six of the probes were positive controls, while 21 probes were used to detect resistance to RIF and INH. For the detection of RIF resistance, the strip contained 8 probes that hybridize DNA from codons 506 to 533 of the *rpoB* gene and 4 mutation probes (rpoBMUT1 (D516V), rpoBMUT2A (H526Y), rpoBMUT2B (H526D) and rpoBMUT3 (S531L)). Similarly, for INH resistance, the strip contains probes that hybridized DNA at codon 315 of the *KatG* gene and positions -1 to -22 on the *inhA* promoter region. The mutation probes associated with INH resistance were katGMUT1 (S315T1), katGMUT2 (S315T2), inhAMUT1 (C15T), inhAMUT2 (A16G), inhAMUT3A (T8C), and inhAMUT3B (T8A). When the DNA amplicons hybridized to the probes on the Genotype MTBDR*plus* strip following hybridization, a dark band was produced that was easily interpreted as positive.

The Genotype MTBDR*plus* strips were interpreted in a two-stage process. First, the presence of the 6 control bands was confirmed, demonstrating the assay worked and that MTBC was present. Secondly, susceptibilities to RIF and INH were assessed. A sample was considered to be resistant to the drug if at least one of the wild-type bands was absent or a band indicating a common mutation in the drug resistance-related gene was present. Likewise, a sample was considered sensitive to the drug if all the wild-type bands of the gene were present and no common mutation was detected.

Statistical methods

Participants were classified as new, previously treated PTB patients or patients with unknown PTB history. Participant characteristics were compared between new and previously treated TB patients using two-tailed Fisher's exact test or Chi-square test for categorical variables and non-parametric Mann Whitney test for continuous numerical variables. No adjustment for multiple comparisons was made because only a few planned comparisons were made and the data evaluated are actual observations (15).

To determine the accuracy of the Genotype MTBDRplus assay to diagnose MDR-TB, Genotype MTBDR*plus* results were compared to the gold standard MGIT 960 SIRE system. We calculated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with 95% confidence intervals (CI) of the Genotype MTBDR*plus* assay for the detection of MDR-TB and resistance to RIF and INH. Sensitivity was defined as the proportion of isolates correctly determined as resistant by the Genotype MTBDR*plus* assay compared with MGIT 960 SIRE system. Specificity was defined as the proportion of isolates correctly determined as susceptible by the Genotype MTBDRplus assay compared with MGIT 960 SIRE system. PPV was defined

as the proportion of resistant isolates determined by the MGIT 960 SIRE system among isolates determined as resistant by Genotype MTBDR*plus* assay. NPV was defined as the proportion of susceptible isolates determined by the MGIT 960 SIRE system among isolates determined as susceptible by Genotype MTBDR*plus* assay. The degree of agreement between MGIT 960 SIRE system and the Genotype MTBDR*plus* assay was also assessed using Cohen's kappa (κ) coefficient. κ values of >0.75 defined as showing very good agreement, κ values of <0.4 defined as showing poor agreement and κ values of 0.4-0.75 defined as showing fair to good agreement (16).

All analyses and comparisons were done with GraphPad prism software, version 6 (GraphPad Software, California, USA) and all results having a $p<0.05$ were considered statistically significant.

2.4 RESULTS

Demographic characteristic of the study population

Figure 1 shows a schematic diagram of sample processing. Among the 288 participants recruited from 6 health facilities, 270 participants were eligible to participate in the study. Each eligible participant provided a sputum sample that resulted in smear microscopy and MGIT culture data. Of 270 MGIT cultures, 239 samples were positive for MTBC, 21 (7.8%) were negative for growth, and 10 (3.7%) cultures had growth, but the bacteria were not of the MTBC. Of the 239 MGIT-MTBC positive cultures, DST was successfully performed on 226 (95%); Ziehl-Neelsen staining of the 13 isolates without DST results showed that either non-tuberculous mycobacteria or other contaminants like bacteria or fungi had been cultured. Among the 226 isolates with DST results, 152 (67%) were

susceptible to both RIF and INH, 49 (22%) were resistant to both RIF and INH, 6 (3%) were INH monoresistant, 5 (2%) were poly-drug-resistant (*i.e.,* to INH plus Streptomycin (n=4) or INH plus Ethambutol (n=1)), and 14(6%) were Streptomycin monoresistant.

Table 1 shows characteristics of the 270 eligible participants. The 169 previously treated TB patients were more likely to be recruited from Yaounde ($p < 0.0001$), more likely to be HIV-negative ($p = 0.0008$), more likely to be sputum smear positive ($p = 0.0012$), and more likely to be drug resistant ($p < 0.0001$) than the 79 new TB patients.

Genotype MTBDR*plus* test results

The Genotype MTBDR*plus* assay was performed on 225 cultures with MGIT DST results, as one isolate was not recovered following culture of the stored sediment sample. Valid Genotype MTBDR*plus* results were obtained for all 225 cultured isolates on the first attempt. Table 2 provides results comparing the Genotype MTBDR*plus* and MGIT DST assays for the detection RIF-resistant, INH-resistant, and MDR-TB cases.

Table 2: Performance of the Genotype MTBDR*plus* assay in detecting resistance in clinical isolates of Mycobacterium tuberculosis

Gold standard: BACTEC MGIT DST 960 System(# of *Mtb* isolates)							
		RIF		INH		MDR-TB	
Test assay		R (n=49)	S (n=176)	*R (n=60)	S (n=165)	R (n=49)	S (n=176)
Genotype MTBDR*plus*	R	48	0	55	1	46	0
	S	1	176	5	164	3	176
Sensitivity (95% CI)		98 (89-100)		92 (82-96)		94 (83-98)	
Specificity (95% CI)		100 (98-100)		99 (97-100)		100 (98-100)	
PPV (95% CI)		100 (93-100)		98 (91-100)		100 (92-100)	
NPV (95% CI)		99 (97-100)		97 (93-99)		98 (95-100)	
Cohen's kappa (95% CI)		0.99 (0.96-1.00)		0.93 (0.88-0.99)		0.96 (0.92- 1.00)	

Abbreviations: R: Resistant; S: Susceptible; CI: Confidence interval; RIF: Rifampicin; INH: Isoniazid; MDR-TB: Multidrug resistant Tuberculosis; PPV: Positive Predictive Value; NPV: Negative Predictive Value

** INH Resistant (n=60) refers to all INH resistant isolates that is; INH and RIF drug-resistant (n=49), INH mono drug-resistant (n=6) and INH poly drug-resistant (n=5)*

Overall, the Genotype MTBDR*plus* assay correctly identified RIF resistance in 48/49 (sensitivity, 98% [CI, 89%-100%]), INH resistance in 55/60 (sensitivity 92% [CI, 82%-

96%]), and MDR-TB 46/49 (sensitivity, 94% [CI, 83%-98%]). The specificity for the detection of RIF resistant and MDR-TB cases was 100% (CI, 98%-100%), while that of INH resistant was 99% (CI, 97% -100%). The PPV and NPV of the Genotype MTBDR*plus* assay were high for RIF resistance, INH resistance and MDR-TB, ranging from 97%-100%. The agreement between both tests for the detection of MDR-TB was very good (Kappa = 0.96 [CI: 0.92-1.00]).

All discordant results between the MGIT DST system and the Genotype MTBDR*plus* assay are shown in Table 3. The discordant isolates presented with different Genotype MTBDR*plus* resistant patterns.

Table 3: Discordant results between MGIT DST and Genotype MTBDR*plus*									
Patient code	Patient Treatment history	MGIT DST				*Genotype* MTBDR*plus* *assay*			Results
		RIF	INH	STM	EMB	*rpoB*	*KatG*	*inhA*	
HJ128	Previously treated	S	S	S	S	WT	WT	ΔWT1, MUT1	INH monoresistant
HJ063	Previously treated	S	R	S	R	WT	WT	WT	Susceptible
HL024	Previously treated	S	R	S	S	WT	WT	WT	Susceptible
HJ088	Previously treated	S	R	S	S	WT	WT	WT	Susceptible
HJ107	Previously treated	R	R	S	R	ΔWT8, MUT3	WT	WT	RIF monoresistant
HJ123	Previously treated	R	R	S	S	ΔWT8, MUT3	WT	WT	RIF monoresistant
HJ064	Previously treated	R	R	R	R	WT	ΔWT1, MUT1	WT	INH monoresistant
Abbreviations: S: Susceptible; R: Resistant; WT: Wild Type probe; MUT: Mutation; Δ Absence of hybridization signal with wild-type probe.									

Genotype MTBDR*plus* mutation patterns

The pattern of mutations associated with INH monoresistant, INH poly-resistant and MDR-TB isolates are shown in Table 4. Overall, the most frequent mutation detected in RIF-resistant isolates was the *rpoB* S531L MUT3 41/49 (84%). In INH-resistant isolates the most frequent mutations were *KatG* S315T1 (MUT1) 35/55 (64%) followed by inhA C15T (MUT1) 20/55 (36%). Majority of the *KatG* S315T1 mutation was detected among INH polydrug resistant and MDR-TB isolates. Concurrent *KatG* S315T1 and *inhA* promoter mutations (T8C (MUT3A)) were detected in only one isolate- an MDR-TB isolate.

Table 4: Pattern of gene mutations detected by the Genotype MTBDR*plus* assay in 60 drug-resistant Mycobacterium tuberculosis isolates

Gene	**Band**	**Gene region or mutations**	***INH monoresistant N=6**	***INH poly-resistant N=5**	***MDR-TB (N=49)**
rpoB					
	WT1	506-509	6	5	49
	WT2	510-513	6	5	48
	WT3	513-517	6	5	46
	WT4	516-519	6	5	47
	WT5	518-522	6	5	49
	WT6	521-525	6	5	49

	WT7	526-529	6	5	46
	WT8	530-533	6	5	7
	MUT1	D516V	0	0	0
	MUT2A	H526Y	0	0	0
	MUT2B	H526D	0	0	3
	MUT3	S531L	0	0	41
KatG					
	WT	315	5	1	19
	MUT1	S315T1	1	4	30
	MUT2	S315T2	0		0
inhA					
	WT1	-15/-16	3	5	32
	WT2	-8	6	5	49
	MUT1	C15T	3	0	17
	MUT2	A16G	0	0	0
	MUT3A	T8C	0	0	1
	MUT3B	T8A	0	0	0
* By conventional drug susceptibility testing using the Bactec MGIT 960 system					

2.5 DISCUSSION

This study reports the accuracy of the Genotype MTBDR*plus* assay for diagnosing MDR-TB in new and previously treated PTB patients in Cameroon. Our analysis showed that based on 49 MDR-TB cases, the Genotype MTBDR*plus* assay had a sensitivity and specificity of 94% and 100%, respectively. Such high sensitivity and specificity values make the Genotype MTBDR*plus* very suitable for use to diagnose MTB-DR in Cameroon. The specificity and sensitivity values reported in our study are similar to those reported in the two systematic reviews of the Genotype MTBDR*plus* (7,8). Our participant recruitment strategy led to both a high number of positive *Mtb* cultures and MDR-TB patients (22%; 49/225). This proportion of MDR-TB patients is higher than the population estimate of 2.2% (460/20882) among newly diagnosed TB patients and 11% (170/1575) among previously treated TB patients. However, a sensitivity analysis indicates that the high proportion of MDR-TB patients in our study does not affect the positive predictive value of the Genotype MTBDR*plus* in diagnosing MDR-TB due to the high specificity value (100%) obtained. Based on our data of 94% sensitivity and an estimated 630 new and previously treated cases of MDR-TB annually in Cameroon, the Genotype MTBDR*plus* assay would fail annually to diagnose 32 cases. These misdiagnosed cases would compromise the goal to identify every patient with MDR-TB in Cameroon. However, among the 3 MDR-TB cases not diagnosed as resistant to RIF and INH by the Genotype MTBDR*plus* assay, 2 were diagnosed as resistant to RIF and 1 as resistant to INH. As such, even if the Genotype MTBDR*plus* assay were to misdiagnose a few MDR-TB cases, the presence of resistance to either

INH or RIF among these cases would be detected. Overall, our data suggests that the Genotype MTBDR*plus* assay can be used to diagnose MDR-TB in Cameroon

The majority 44/48 (92%) of RIF-resistant isolates detected by the Genotype MTBDR*plus* assay had mutations at codon 531 41/48 (85%) and 526 (6%). These mutations are known to be the most prevalent RIF-resistance associated mutations (17–19), but their frequencies vary worldwide. The frequency of codon 531 mutation in our study was 85% which is higher than generally reported (20). A similar high frequency of codon 531 mutation among RIF-resistant isolates in Cameroon was also reported (18). As such, the codon 531 mutation may actually be the most prevalent RIF-resistant associated mutation among RIF-resistant isolates in Cameroon. The relevance of a predominant codon 531 mutations is unclear, but could reflect on-going transmission of isolates carrying this mutation. RIF-resistant associated mutation at positions 516 were not detected in this study, but have been reported in other studies in Cameroon (18,21).

In this study, four RIF-resistant isolates failed to hybridize with one or two of the wild type (WT) probes, and did not hybridize with any of the probes representing known mutations. These results suggest that there is either a technical problem or a new previously unreported mutation. The WT probes with no hybridization were mostly WT2, WT3, WT4 and WT8. First, Seifert and colleagues (2016) suggest that this type of result is likely due to failure of the mutant to hybridize with the mutation probe and is not due to the presence of a rare or new mutation. They concluded that in such situations, improved optimization of the Genotype MTBDR*plus* will demonstrate hybridization to the mutation probes (22). However, the absence of hybridization could also indicate that there is a mutation at positions 511 (WT2), 516 (WT3), 526 (WT4), and/or 533 (WT8).

Mutations at these positions have been reported, but not all mutations have been associated with RIF-resistance (23–26). In the current study, the four isolates that failed to hybridize to one or two WT probes were RIF-resistant in the MGIT DST, making it likely that unknown mutations associated with RIF resistance could be present. Unfortunately, DNA sequencing could not be done in our study to confirm or identify the mutations; however, since they were resistant by MGIT DST, further studies are warranted.

Besides identifying resistance to RIF or INH, the Genotype MTBDR*plus* assay provides information that is necessary for patient treatment and understanding the evolution of drug resistance. Mycobacterial isolates with the *KatG* codon 315 mutation have reduced ability to activate INH, but the isolate still has catalase and peroxidase activities. As such, isolates with this mutation can persist and be transmitted without any negative selection (27). Administration of a high-dose of INH (900mg) per day to patients harboring isolates with the *inhA* promoter mutations, might lead to better treatment outcome (2,28–30). Isolates with *inhA* promoter are also resistant to the anti-TB drug Ethionamide (ETH). As such, use of ETH will not be beneficial to the patient (31). A recent study suggests that routine evaluation of the frequency of the *inhA* promoter mutation can help predict progression to more severe forms of drug resistance. The authors of the study observed an increase in the frequency of *inhA* promoter mutations as isolates progressed to more severe forms of drug resistance, from MDR-TB, to pre-extremely drug resistant TB (pre-XDR-TB) and XDR-TB (30,32).

At present, the WHO recommends that the Genotype MTBDR*plus* assay be used directly on specimen without culturing if the specimen is smear positive and on Mtb

isolates obtained after culture (33). However, among smear positive specimens, a recent study showed that the Genotype MTBDR*plus* assay will perform best if specimens were graded ≥AFB2+ (34). In our study, 71% (192/270) of samples were ≥AFB2+. Gauthier and colleagues (2014) have proposed a diagnostic algorithm where the Genotype MTBDR*plus* assay should be used directly on ≥AFB2+ specimens (35), which is appealing because it helps accelerate diagnosis of drug resistance. In reality, a significant proportion of patients will be <AFB2+ and will not be eligible for direct testing according to the algorithm proposed by Gauthier and colleagues (2014). In our study, we had 29% (78/270) of such patients. As such, use of the Genotype MTBDR*plus* assay for direct testing might not be adequate. Furthermore, most laboratories are now equipped with the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) (11), that performs better than the MTBDR*plus* for direct testing of clinical specimens (36). However, the Genotype MTBDR*plus* assay offers great benefit when used on cultured isolates. First, results are available within 48hrs in contrast to the 7 days to weeks with culture-based DST. Secondly, the Genotype MTBDR*plus* assay is not affected by the presence of contamination with bacteria, fungi or non-tuberculosis mycobacteria as are most cultured-based DST systems (37,38). In our study, 29% (70/239) of the cultures were contaminated and had to be decontaminated and re-cultured before DST could be done. Among the 70 repeats, we were successful in obtaining pure cultures in only 57 cultures; whereas the other 13 remained contaminated and culture-based DST could not be performed. The presence of contaminants increases laboratory processing and reporting times that could be avoided if the Genotype MTBDR*plus* assay is used.

2.6 CONCLUSIONS

This study showed that the Genotype MTBDR*plus* assay has good accuracy for detecting resistance to RIF, INH and MDR-TB, showing it would be useful for the diagnosis of MDR-TB in Cameroon. At present, there are 3 functional TB reference laboratories in Cameroon with culture and molecular-based capacity to diagnose drug-resistant TB. Each laboratory serves health facilities in 3 to 4 regions of the country. The need to transport samples from health facilities to these reference laboratories, increases the turnaround time for obtaining results and the risk of having contaminated cultures. The Genotype MTBDR*plus* could be included in the diagnostic algorithm of MDR-TB and be used post-culture. Primarily, the Genotype MTBDR*plus* assay could be used to perform DST of *Mtb* positive-cultures especially for cultures containing contaminants for which culture based DST will be delayed. Additionally, the Genotype MTBDR*plus* assay could be used as a complementary test to confirm RIF and INH DST results obtained using the culture-based method. Lastly, the Genotype MTBDR*plus* assay can be used for epidemiological surveys to rapidly assess the type RIF and INH mutations present.

2.7 DECLARATIONS

List of abbreviations

MDR-TB: Multidrug-resistant tuberculosis; RIF: Rifampicin; INH: Isoniazid; Mtb: *Mycobacterium tuberculosis*; TB: Tuberculosis; PTB: Pulmonary Tuberculosis; EXPANDx-TB: Expand Access to New Diagnostics for TB; DST: Drug Susceptibility Testing; AFB: Acid-Fast Bacilli; BSL: Biosafety Level; WHO: World Health Organization; IUATLJ: International Union Against Tuberculosis and Lung Disease; NALC-NaOH: N-

acetyl-L-cysteine-sodium hydroxide; MGIT: Mycobacterial Growth Indicator Tube; MTBC: *Mycobacterium tuberculosis* complex; SIRE: Streptomycin, Isoniazid, Rifampicin, Ethambutol; CI: Confidence Interval; WT: Wild type: MUT: Mutation; ETH: Ethionamide; NIH: National Institute of Health, USA

Ethical approval and consent to participate

This study was reviewed and approved by the Institutional Review Board of the University of Hawaii (CHS #22381), USA, the National Ethical Committee of Cameroon and the technical committee of the Ministry of public Health Cameroon. The participants gave their written informed consent to be enrolled in the study.

Consent to publish

All authors have given their consent for publication.

Availability of data and materials

We have reported all the findings in the manuscript. Participant's questionnaire and consent form are available at the Immunology and Parasitology laboratory of the Biotechnology Center, University of Yaounde I, Cameroon. All *Mycobacterium tuberculosis* isolates analyzed in this study and test results obtained are available at the Mycobacteriology laboratory of Centre Pasteur du Cameroon. All microbial analysis were performed at Centre Pasteur du Cameroon. If anyone wants to look at or use our Mycobacterium isolates or our dataset, they should contact the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NNA, EL, EWY, WFM, GV, VMP, SIE, DWT, RGL, conceived, designed and coordinated the study. NNA, JYD and EWY enrolled participants and coordinated specimen collection. NNA and JYD processed samples and performed laboratory testing. NNA and EL analyzed the data. NNA, EL, DWT and RGL wrote the first draft of the manuscript. NNA, JYD, EWY, WFM, GV, VMP, SIE, DWT and RGL contributed to the writing of the manuscript and critically reviewed the manuscript. NNA, JVD, EWY, WFM, GV, VMP, SIE, DWT and RGL agreed with final manuscript results and conclusions.

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2.9 Tables and legends

Table 1: Demographic characteristic of 270 eligible participants					
	All Study participants N=270 (%)	New PTB patients N= 79 (%)	Previously treated PTB patients (N= 169)	Patients with unknown PTB history (N=22)	$^{+}$P-value
Variables					
Age (y); mean SD	37.7 ±13.4	36.6 ± 14	38.0 ± 13.5	39.1 ± 10.7	0.271
Gender					0.667
Male	179 (66)	50 (63)	113 (67)	16 (73)	
Female	91 (34)	29 (37)	56 (33)	6 (27)	
Cities					<0.0001
Bamenda	14 (5)	5 (6.3)	9 (5.3)	0 (0)	
Douala	133 (49)	65 (82.3	47 (27.8)	21 (95.5)	
Yaounde	123 (46)	9 (11.4)	113 (66.9	1 (4.5)	
HIV status					0.0008
Positive	63 (23)	13 (16.5)	50 (29.6)	0 (0)	
Negative	140 (52)	40 (50.6)	96 (56.8)	4 (18.2)	
Unknown	67 (25)	26 (32.9)	23 (13.6)	18 (81.8)	
Smear Status					0.0012

Smear negative	44 (16)	5 (6.3)	38 (22.5)	1 (4.5)	
Smear positive	226 (84)	74 (93.7)	131 (77.5)	21 (95.5)	
Grading of positive smears					
Smear positive (AFB Scanty)	17 (6)	5 (6.8)	10 (7.6)	2 (9.1)	
Smear positive (AFB 1+)	17 (6)	6 (8.1)	8 (6.1)	3 (13.6)	
Smear positive (AFB 2+)	46 (7)	22 (29.7)	22 (16.8)	2 (9.1)	
Smear positive (AFB 3+)	146 (54)	41 (55.4)	91 (69.5)	14 (63.6)	
Phenotypic DST results					<0.0001
Susceptible	152 (56)	53 (67.1)	82 (48.5)	17 (77.3)	
Mono-drug resistant	20 (7)	7 (8.9	11 (6.5)	2 (9.1)	
Polydrug resistant	5 (2)	2 (2.5)	2 (1.2)	1 (4.5)	
MDR	49 (18)	7 (8.9)	42 (24.9)	0 (0)	
*None	44 (16)	10 (12.7)	32 (18.9)	2 (9.1)	
Abbreviations: SD: Standard deviation; PTB: Pulmonary Tuberculosis; AFB: Acid-					

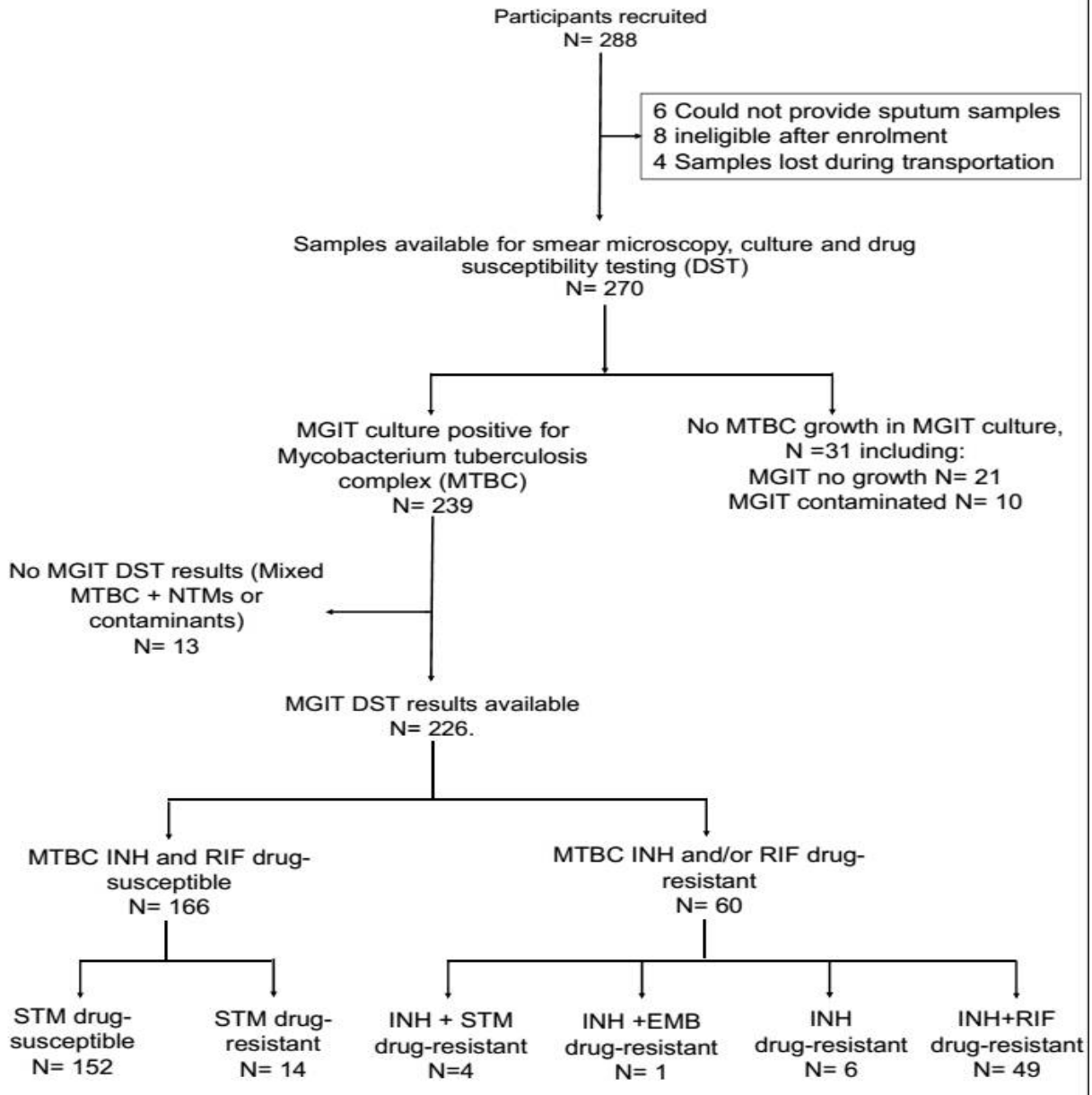
Fast-Bacilli; DST: Drug susceptibility testing: MDR: Multidrug resistant (Resistant to Rifampicin (RIF) and Isoniazid (INH).

[+] *The p-value was obtained from comparison between new and previously treated TB patients individuals*

**None implies no growth of Mycobacterium tuberculosis complex bacilli observed*

Figures

Figure 1. Schematic diagram of sample processing



Bactec MGIT 960 System used for cultures and DST. MTBC: *Mycobacterium tuberculosis* Complex; NTMs: Nontuberculous Mycobacterium; INH: Isoniazid, RIF – Rifampicin; STM: Streptomycin; EMB: Ethambutol

CHAPTER 3

*(*To be submitted to *Journal Clinical Microbiology*

Does the absence of Hybridization with the Wild-Type Probe in the GENOTYPE MTBDR*plus Assay Mean Mycobacterium tuberculosis is* Rifampicin Resistant?

Running title: Genotype MTBDR*plus* v2: no Wildtype probe hybridization

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3.1 ABSTRACT

Mutations that enable *Mycobacterium tuberculosis (Mtb)* to be resistant to Rifampicin (RIF) are located within the *rpoB* gene region from codons 507 to 534. However, not every mutation in this region is associated with resistance to RIF. The WHO-endorsed the Genotype MTBDR*plus* assay recommends that an *Mtb* isolate be assumed resistant to RIF solely on the basis of absence of hybridization to one or more wildtype probe on the assay strip. This recommendation suggests that an *Mtb* isolate with any mutation in the *rpoB* gene region from codon 507 to 534 should be assumed resistant to Rifampicin. Here, we screened 275 Mtb isolates with the Genotype MTBDR*plus* assay and identified 6% of *Mtb* isolates that fulfilled this recommendation. Sequencing of these Mtb isolates revealed that impaired hybridization to WT probes was due to the presence 'disputed' RIF mutations *i.e.* mutations that are sometimes associated with RIF resistance but not always. Mtb isolates bearing these 'disputed' RIF mutations may be responsible for causing resistance to RIF and have been associated with adverse treatment outcome in both naïve and previously treated TB patients. Thus, the recommendation of the Genotype MTBDR*plus* assay to assume resistance based solely on absence of hybridization to WT probe allows the identification of clinically important RIF-resistant Mtb isolates. This recommendation results in accurate and rapid diagnosis of RIF-resistant TB by the Genotype assay.

3.2 INTRODUCTION

The line-probe assay, Genotype MTBDR*plus*® version 2.0 (Hain Lifescience GmbH, Nehren, Germany) enables rapid and accurate detection of *Mycobacterium tuberculosis* (Mtb) and resistance to the anti-tuberculosis drugs, rifampicin (RIF) and Isoniazid (INH)(1–4). This assay is currently endorsed by the World Health Organization (WHO) and routinely used in TB endemic countries to diagnose and confirm resistance to RIF and INH (5–8).

The Genotype MTBDR*plus* assay detects resistance to RIF and INH by detecting mutations in gene regions associated with resistance to RIF and INH. The detection of mutations involves a series of steps. First, DNA is extracted from *Mtb*. Secondly, the region of the gene associated with either resistance to RIF or INH is amplified by multiplex PCR using biotinylated primers. Next, the PCR products are allowed to hybridize to specific oligonucleotide probes immobilized on a nitrocellulose membrane strip. Lastly, streptavidin-conjugated alkaline phosphatase is added and the hybridized probes are revealed by the appearance of a dark color precipitate on the strip (9). For the detection of mutations associated with resistance to RIF, the strip contains 8 wild type (WT) probes that hybridize *Mtb* DNA from codons 507 to 534 of the *rpoB* gene and 4 mutation probes that hybridize DNA that contains specific mutations at positions: 516 (D516V), 526 (H526Y, H525D) and 531 (S531L). Likewise, for the detection of mutations associated with resistance to INH, the strip contains probes that hybridized DNA at codon 315 of the *KatG* gene and positions -1 to -22 on the *inhA* promoter region. Mutation probes associated with INH resistance, bind DNA with mutations at

position 315 of the *KatG* gene and at positions 8, 15 and 16 of the *inhA* promoter region (1,9).

According to the Genotype MTBDR*plus* assay, an *Mtb* isolate could be classified as resistant to RIF and/or INH based on two alternative interpretations. The first interpretation recommends that if an isolate fails to hybridize to one or two of the WT probes on the strip but hybridizes to a mutation probe, the isolate is resistant to the drug (9). In such a case, a mutation is present and the mutation is known to be associated with resistance to the drug. However, the second interpretation recommends that if an isolate fails to hybridize to one or two of the WT probes and does not hybridize to any of the mutation probes, the specimen is resistant to the drug (9). In the second interpretation, lack of WT hybridization shows a mutation is present but makes the assumption that the Mtb isolate is resistant without identifying the mutation. Since not all mutations confer drug-resistance, it is not clear if the assumption is correct. For example, several mutations have been detected from codons 507 to 534 of the *rpoB* gene of *Mtb* (10), and some of these mutations could be silent mutations (11,12) or missense mutations with no or unclear association with resistance to RIF (13,14). As such, it is important to know what type of mutation is present in order to determine if the *Mtb* isolate is resistant.

In this study, we sought to evaluate the frequency of *Mtb* isolates that do not hybridize to *rpoB* WT probes and mutations probes on the Genotype MTBDR*plus* strip; and if impaired hybridization to WT probes is due to mutation associated with resistance to RIF or not.

3.3 MATERIALS AND METHODS

***Mycobacterium tuberculosis* isolates**. Two sets of *Mtb* isolates were used in this study. The first set of 225 isolates was from a recent study that sought to evaluate the accuracy of the Genotype MTBDR*plus* assay in diagnosing drug-resistant TB in Cameroonian TB patients. A detailed description of this study and findings were submitted for publication elsewhere (Dissertation Chapter 2). The second set of 50 *Mtb* isolates was obtained from the Mycobacteriology Unit at Centre Pasteur du Cameroon (Mycobact/CPC). Mycobact/CPC routinely archives drug-resistant *Mtb* isolates cultured from clinical specimen. Following a review of the laboratory records of Mycobact/CPC, the 50 *Mtb* isolates diagnosed as drug-resistant to RIF and/or INH by either the GeneXpert MTB/RIF assay® (Cepheid, Sunnyvale, CA, USA) or the automated BACTEC MGIT 960 system® (Becton Dickinson, Franklin Lakes, NJ) or by both systems between January 2015 and February 2016 were included in this study. Every *Mtb* isolate included in the study was isolated from a different patient.

Genotype MTBDR*plus* assay. For the first set of 225 Mtb isolates, the previously obtained MTBDR*plus* results were reviewed and isolates that failed to hybridized to *rpoB* wild type (WT) and mutation probes on the Genotype MTBDR*plus* test strip were selected. The second set of samples were screened using the genotype MTBDR*plus* according to the manufacturer's instructions (9). As with the first set of samples, *Mtb* specimens that failed to hybridized to one or more of the *rpoB* wild type (WT) probes and the mutation probe on the Genotype MTBDR*plus* strip were selected. The Genotype MTBDR*plus* assay was repeated for all *Mtb* isolates with this pattern of hybridization in order to confirm the results.

Determination of Minimum Inhibitory Concentration (MIC). MICs were only determined for the selected *Mtb* isolates with the Genotype MTBDR*plus rpoB* hybridization pattern (no hybridization to WT probes or mutation probes).

MICs were determined using the Bactec MGIT 960 system using the standard drug susceptibility testing protocol provided by the manufacturer. The drug concentrations tested for RIF included 0.125, 0.25, 0.5, 1, 2, 4, and 8µg/ml and INH concentrations of 0.1, 0.2, 0.4, and 0.8 µg/ml. Not all isolates were cultured with all drug concentrations. Based on the MGIT 960 DST results performed at critical concentrations of 1 µg/ml, RIF-resistant isolates were tested with concentrations of 1, 2, 4, and 8µg/ml and RIF-susceptible isolates were tested with concentrations of 0.125, 0.25, 0.5, and 1 µg/ml.

MIC values reported here as '>' (*e.g.* > 8 µg/ml) indicates that the Growth Unit (GU) of the drug-containing culture tube with 8 µg/ml was >100 when the 1:100-diluted drug-free control tube had reached a GU of 400. MIC values reported here as '<' (< *e.g.* 0.25 µg/ml) indicates that the GU of the drug-containing culture tube with 0.25 µg/ml was < 100 when the 1:100-diluted drug-free control tube had reached a GU of 400.

DNA extraction, PCR, and sequencing. The target *Mtb* isolates were grown in MGIT tubes using the Bactec MGIT 960 system. Following growth, the Mtb isolates were harvested and heat killed by boiling at 95°C for 20 min in nuclease free water to release DNA. Extracted DNA was used for PCR. The PCR primers and amplification conditions used to amplify the *rpoB* gene were as previously described (17). All PCR products were outsourced for sequencing (Genewiz, UK). Both primers used for

amplification and additional internal primers were used for sequencing as previously reported (17).

The Array Studio Suit version 9.0 software (Omicsoft Corporation, Weston Parkway, USA) on windows system was used to analyse the sequences. The . The OSA4 aligner was used to align the sample sequences to the reference *rpoB* gene sequence of Mtb H37Rv. After alignment, the summarize variant data module was used to generate a mutation report.

Ethical approval

This study was reviewed and approved by the Institutional Review Board of the University of Hawaii (CHS #22381), USA, Centre Pasteur of Cameroon, the National Ethical Committee of Cameroon and the technical committee of the Ministry of Public Health, Cameroon.

3.4 RESULTS

Identification of target samples. Figure 1 provides a detailed description of the Mtb isolates used in this study. A total of 275 Mtb isolates with known MGIT 960 drug susceptibility testing results and/or GeneXpert MTB/RIF results were screened by the Genotype MTBDR*plus* assay. Among the 275 samples, 16 (6%) *Mtb* isolates did not hybridize to one or more *rpoB* WT and mutation probes. Repeated screening of these samples with the Genotype MTBDR*plus* assay further confirmed the results. Of the 16 Mtb isolates, 3 were resistant by GeneXpert MTB/RIF, but susceptible by MGIT 960; whereas, 13 were resistant by both assays**.**

Sequencing, probe hybridization and minimum inhibitory concentration. To determine if a mutation was present and what type of mutation it was, the 81bp Rifampicin Resistance Determining Region (RRDR) of the *rpoB* gene was sequenced. Table 1 shows the mutations detected by sequencing of the 16 isolates. Mutations in the RRDR were detected in all isolates.

Table 1. Aligned sample sequences with Rifampicin Resistant Determining Region (RRDR) of the standard Mycobacterium tuberculosis strain H37Rv

Samples	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533
H37Rv	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S1	GGC	ACC	AGC	CAG	CTG	AGC	CCA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S2	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	TAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S3	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TGG	GCG	CTG
S4	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CTC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S5	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	AAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S6	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CTC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S7	GGC	ACC	AGC	CAG	CCG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S8	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	TAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S9	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	TAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S10	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	TAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S11	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CGC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S12	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CCG
S13	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CGC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S14	GGC	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CGC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S15	GGC	ACC	AGC	CAG	CTG	AGC	CCA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
S16	GGC	ACC	AGC	CAG	CCG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG

Interestingly, as summarize in Table 2, all of the mutations detected had been reported previously to be ‘disputed’ rifampicin-resistant mutations (14,18,19). These mutations are called ‘disputed’ RIF-resistant mutations because not all isolates bearing these mutations have a RIF-resistant phenotype when testing is done using the standard phenotypic assay MGIT 960 DST system. For example, isolate S4 and S6 were both detected to be RIF-resistant by GeneXpert and Genotype MTBDR*plus* assay. In the Genotype MTBDR*plus* assay, both isolates failed to hybridize to WT7. However, sequencing of both isolates identified the same mutation at position 526 that led to a change in amino acid from His to Leu (His526Leu). Phenotypic testing by the MGIT 960 DST assay classified S4 as RIF-susceptible and S6 as RIF-resistant. MIC results of S6 revealed the isolate had only a low level of RIF-resistance (< 4µg/ml).

Except S4, S7 and S16, all other isolates in Table 1 were associated with a high level of RIF resistance (MIC > 8µg/ml). Thus, making the assumption that isolates that failed to hybridize with the WT probe were resistant to RIF appears to be a valid assumption.

Only one discrepancy was found between the two genotyping assays. Sample S12 was detected as RIF-susceptible by the Genotype MTBDR*plus* as it hybridized to all WT probes and did not hybridize to a mutation probe. However, the isolate was detected as RIF-resistant by GeneXpert assay and sequencing revealed the presence of a disputed mutation Leu533Pro (Table 1).

3.5 DISCUSSION

Among our collection of 275 Mtb isolates, 16/275 (6%) of these isolates did not hybridize to the *rpoB* WT and mutant probes on the Genotype MTBDR*plus* strip assay. The frequency of isolates with such a hybridization pattern was lower than that reported in other published studies in other countries (>12%) (20,21). Interestingly, sequencing revealed that impaired hybridization to WT probes in these isolates was due to the presence 'disputed' RIF mutations. As earlier mentioned, isolates bearing these mutations can occasionally be detected as RIF-susceptible with the standard MGIT 960 DST assay (18). Disputed mutations are usually rare and assume to make up about 14% of all *rpoB* mutations (14). These disputed mutations can frequently cause important RIF resistance among patients both naïve and previously treated TB patients (13,18,19). As such, assuming resistance based solely on the absence of WT probe hybridization significantly contributes to the detection of RIF resistant *Mtb* isolates that could be missed by the conventional culture-based drug susceptibility testing.

At present, WHO recommends the molecular-based test GeneXpert MTB/RIF® as the initial diagnostic test in patients at risk of having drug-resistant *Mtb*(22). Considering the GeneXpert works on the same principle as the Genotype assay, by extrapolation, this test will detect patients with disputed RIF mutations as resistant to RIF(18). Once such a patient is diagnosed, the patient will immediately be placed on treatment for multidrug resistant TB. However follow up phenotype-based testing is required during the course of treatment and it will identify the bacteria as RIF-susceptible. This discord in test results might raise concerns on the reliability of the GeneXpert MTB/RIF test and affect its use.

The inability of the MGIT 960 DST assay to accurately classify all isolates with RIF-disputed mutations as resistant could be due to the critical concentration of 1µg/ml RIF used in this assay. Some authors have suggested that this critical concentrations is set too high and should be revised to 0.0625 µg/ml (23) or 0.125 µg/ml (24). As observed with the MIC of the isolates in our study and those of other published studies (13,18), if this critical concentration is revised to 0.625-0.125 ug/ml, most and if not all of these isolates with RIF-disputed mutations would become classified as resistant by the MGIT 960 DST assay.

Another interesting aspect about isolates with RIF-disputed mutations is that isolates with the same type of mutation have varying susceptibility to RIF. Certainly, there is a fitness cost associated with the acquisition of mutations in the *rpoB* gene (25,26). This gene codes for the β-subunit of the RNA polymerase that plays a key role in transcription. The mutation induced fitness cost might vary depending on the type and location of the mutation. For some mutations, the resultant fitness cost might be too high be so significant (26). For such mutants, the acquisition of a compensatory mutation may restore enzyme activity either partially or completely (27). Perhaps Mtb isolates that have a RIF-disputed mutation have a severe reduction in RNA polymerase activity and only isolates that acquire compensatory mutations have this activity restored. These compensatory mutations could arise either in the *rpoB* gene or genes that code other subunits of the RNA polymerase *i.e*., *rpoC* and *rpoA* genes as previously described (27)

The strengths of this study are the large number of Mtb isolates screened and selection of isolates based on phenotypic and genotypic testing, namely MGIT 960 DST

and GeneXpert MTB/RIF, respectively. Our study also has some limitations such as the absence of patient treatment and outcome data.

Drug-resistant tuberculosis is increasingly recognized as a threat to the global control of tuberculosis. The use of rapid molecular based test such as the Genotype MTBDR*plus* will enable the rapid identification of drug-resistant TB. In our study, we showed that the recommendation to classify an isolate as RIF resistant based solely on absence of hybridization to a WT probe allowed for the accurate identification of RIF-disputed isolates that could have been missed by relying on only the standard MGIT 960 DST assay for diagnosis.

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3.8 APPENDIX: Tables and Figures

Table 2. Phenotypic and mutation results of Mtb isolates classified as Rifampicin resistant by the Genotype MTBDR*plus* assay based solely on the absence of hybridization to Wildtype probes							
# Isolate	Isolate ID	MGIT 960 DST RIF	GeneXpert RIF	[β]Genotype MTBDR*plus* WT absent	MGIT 960 MIC Results (µg/ml)	*rpoB* Sequencing result	
						Nucleotide	Amino Acid
2	S7, S16	Sensible	Resistant	WT 2	< 0.25	CTG511CCG	Leu511Pro
1	S1	Resistant	Resistant	WT 3 & 4	>8	CAA513CCA	Gln513Pro
1	S15	Resistant	Resistant	WT 2 & 3	>8	CAA513CCA	Gln513Pro
4	S2*, S8 ,S9 ,S10	Resistant	Resistant	WT 3 & 4	>8	GAC516TAC	Asp516Tyr
3	S11, S13, S14	Resistant	Resistant	WT 7	>8	CAC526CGC	His526Arg

1	S4	Sensible	Resistant	WT 7	< 0.25	CAC526CTC	His526Leu
1	S6	Resistant	Resistant	WT 7	< 4	CAC526CTC	His526Leu
1	S5	Resistant	Resistant	WT 7	>8	CAC526AAC	His526Asn
1	S3	Resistant	Resistant	WT 8	>8	TCG531TGG	Ser531Trp
1	S12	Resistant	Resistant	None	>8	CTG533CCG	Leu533Pro

* GeneXpert MTB/RIF was not performed on this clinical specimen

[β] GeneXpert was performed directly on clinical specimens.

All other test were performed on cultured *Mycobacterium tuberculosis* isolates

Wild Type: WT; Rifampicin: RIF;

Figure 1. Flow chart of sample processing and identification of target samples.

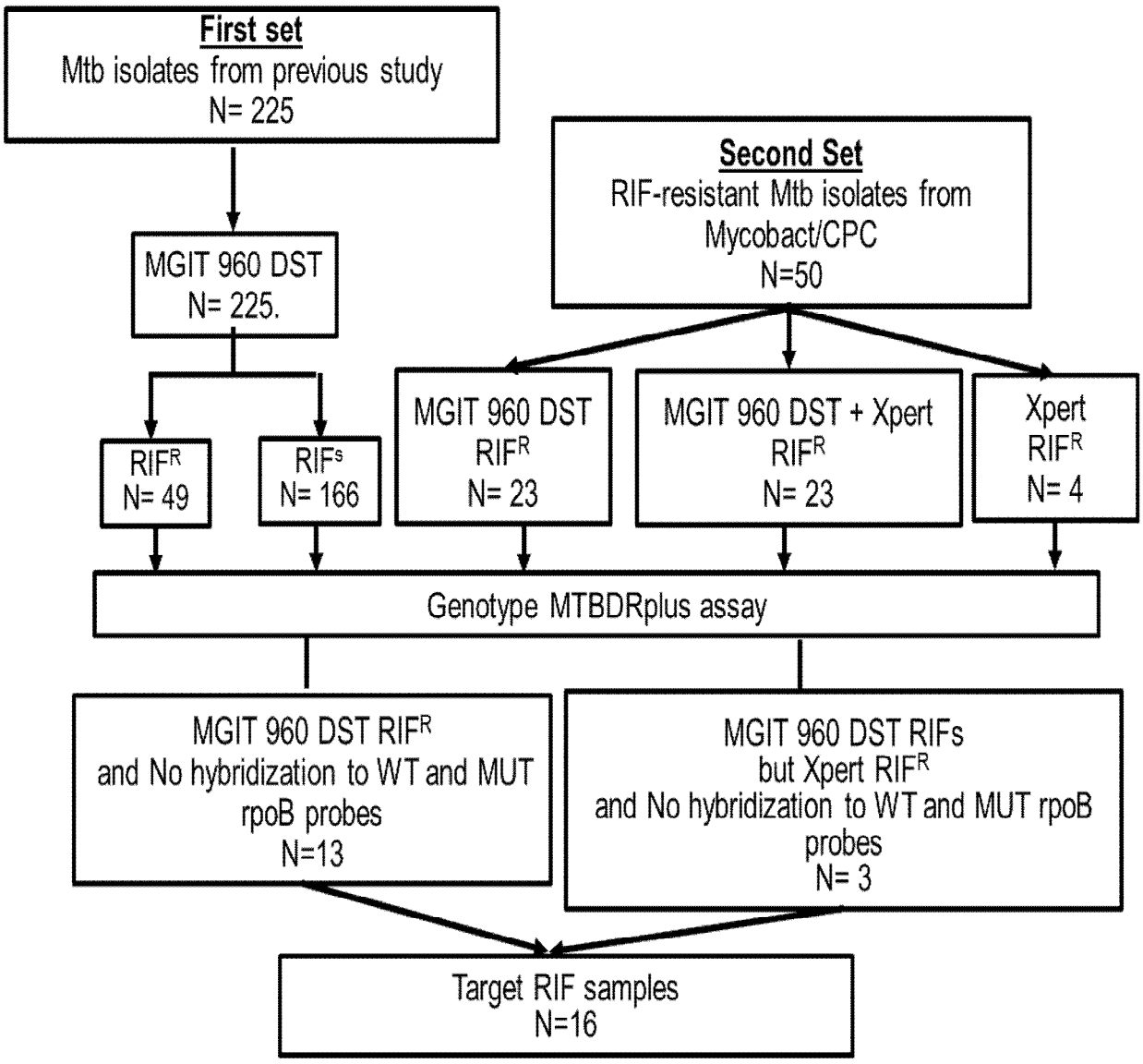

Figure 1.Flow chart of sample processing and identification of target samples

Bactec MGIT 960 System used for phenotypic drug susceptibility testing. cultures and DST. Mtb: *Mycobacterium tuberculosis*;, RIF^S : Rifampicin-susceptible; RIF^R : Rifampicin-resistant; WT: Wild Type; MUT: Mutation; Xpert: GeneXpert MTB/RIF assay

Bactec MGIT 960 system used for culture, phenotypic drug susceptibility testing

CHAPTER 4

(The following chapter was published in the Journal *Pharmaceutics* on February 2017)

Lobular Distribution and Variability in Hepatic ATP Binding Cassette Protein B1 (ABCB1, P-gp): Ontogenetic Differences and Potential for Toxicity

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4.1 ABSTRACT:

The ATP Binding Cassette B1 (ABCB1) transporter has critical roles in endo- and xenobiotic efficacy and toxicity. To understand population variability in hepatic transport we determined ABCB1 mRNA and protein levels in total liver lysates sampled from 8 pre-defined sites (n = 24, 18–69 years), and in S9 from randomly acquired samples (n = 87, 7 days–87 years). ABCB1 levels did not differ significantly throughout individual livers and showed 4.4-fold protein variation between subjects. Neither mRNA nor protein levels varied with sex, ethnicity, obesity or triglycerides in lysates or S9 (that showed the same relationships), but protein levels were lower in pediatric S9 ($p < 0.0001$), with 76% of adult ABCB1 present at birth and predicted to mature in 5 years. Pediatric total liver lysates were not available. In summary, opportunistic collection for studying human hepatic ABCB1 is acceptable. Additionally, ABCB1 may be lower in children, indicating differential potential for toxicity and response to therapy in this special population.

Keywords: development; pediatric; systemic toxicity; elderly; obesity

4.2 INTRODUCTION

Interest in active transporters, such as the ATP Binding Cassette (ABC) proteins, has peaked in recent years because they play crucial roles in drug, chemical, hormone, and nutrient disposition [1]. Clinical and environmental studies increasingly consider the effects of transporters on therapeutic failure, drug resistance, and chemical toxicity. Despite this, very few published studies have determined transporter protein dynamics in human liver tissue samples—a critical site of transport action and critical mediator of systemic endo- and xenobiotic levels. Primarily, this is due to: (i) difficulties in obtaining human liver tissue samples; and (ii) technical challenges working with ABC proteins in the laboratory [2–7].

One of the important ABC transporters is ABCB1 (EC 3.6.3.44, P-glycoprotein, P-gp). The ABCB1 protein is present on the plasma membrane of almost all tissues of the human body (reviewed [8–12]). In the liver, it is expressed on the apical surface of hepatocytes. First characterized in the 1970s, ABCB1 is a 170 kDa protein composed of two sub-units, each containing six transmembrane domains, a large cytoplasmic domain, and a nucleotide binding domain [12]. Although ABCB1 actions in the liver are necessary for elimination of substances from the body to maintain homeostasis, excessive expression (such as by induction) can also reduce the therapeutic effects of drugs and upset homeostatic balance of e.g., hormones and bile acids. An example of this is the drug-drug interaction between Rifampicin (an ABCB1 inducer) and Apixaban, where the therapeutic effect of Apixaban is decreased through greater elimination, increasing risk of stroke [13]. Conversely low expression or activity of ABCB1 may increase the risk of toxicity by compromising elimination. This is observed in the Apixaban/Ketoconazole interaction (ABCB1

inhibition by the latter), where there is an increase in total Apixaban exposure and risk of haemorrhage [13].

We hypothesized that hepatic expression of ABCB1 would be greater in areas near the portal and bile circulation due to its physiological role as an efflux transporter. Currently, there are no published studies of differential regional expression of ABCB1 in the human liver. Regional differences in ABCB1 expression would have implications for sample collection and for differentiating between localized and systemic effects on drug and chemical disposition due to ABCB1 expression. In addition to understanding regional variability in hepatic ABCB1, we were interested in determining whether ABCB1 expression is associated with demographic parameters. Although studies associating expression with age, sex, and ethnicity have been common with metabolic enzymes [14–22] and have revealed critical insight into chemical action and toxicity, they largely do not exist for transport proteins. Based on similar roles as metabolic enzymes, we hypothesized that ABCB1 in the human liver would vary with age, sex, ethnicity, or obesity, and tested this in both the lysates as well as a larger cohort of 87 liver S9 fractions (7 days–87 years).

A greater knowledge of the spatial and demographic ABCB1 expression can help with understanding liver function and disease etiology, as well as systemic drug, chemical, and hormone effects mediated by liver transport.

4.3. MATERIALS AND METHODS

All chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Tissue Samples

Liver S9 and lysate samples were released from the Hawaii Biorepository, with approval from the Institutional Review Board for Human Ethics at the University of Hawaii CHS15844 and the Review Ethics Board at the University of British Columbia H14-00092. The cohort was supplemented with samples purchased commercially from Cellzdirect (Durham, NC, USA), Puracyp (Carlsbad, CA, USA), and Xenotech (Lenexa, KS, USA). Table 1 summarizes donor demographics for the total liver lysates (*n* = 24). Table 2 summarizes the demographics of the S9 liver samples (*n* = 87). Lysates were prepared from approximately 5 g of frozen tissue. Tissues were thawed and weighed and homogenized 1:3 weight:volume in 0.1 M Tris-HCl containing 5 mM $MgCl_2$ and 2 mM freshly prepared Phenylmethylsulfonyl fluoride. Tissues were homogenized to total lysate in 50 mL falcon tubes using a Tissue Tearor electrical homogenizer (Daigger Scientific, Vernon Hills, IL, USA). Total lysates were aliquoted and frozen at −80 until use.

Extraction of mRNA and cDNA Preparation

Total RNA (up to 5 µg) was extracted from human liver S9 (100 µL at 10–30 mg protein/mL). RNA (up to 6 µg) was also extracted from human liver lysates (50 µL at 4–50 mg protein/mL) using the RNeasy Mini kit according to the manufacturer's instruction (Qiagen, Valencia, CA, USA). The RNA purity and concentration were determined by Nanodrop (ThermoFisher Scientific, Wilmington, DE, USA), then RNA was aliquoted stored at −80 °C until use. Only samples with 90% pure RNA or higher

(OD260/280 > 0.8) were used for reverse-transcription with an ABI High Capacity Reverse Transcription Kit (Life Technologies, Burlington, ON, Canada).

Table 1. Demographic information of the donors used to investigate regional ATP Binding Cassette B1 (ABCB1) expression in the liver.

ID	Number of Liver Samples Taken for Regional Study	Age (Years)	BMI (Kg/m^2)	Sex	Ethnicity
A	8	44	38.8	Male	Hawaiian or other pacific Islander
B	7	20	43.7	Male	Hawaiian or other pacific Islander
C	7	37	38.0	Male	Caucasian
D	8	48	31.6	Female	Asian
E	8	46	31.7	Female	Caucasian
F	2	57	22.7	Male	Asian
G	2	69	27.8	Male	Caucasian
H	2	62	35.5	Male	Caucasian
I	4	18	29.9	Male	Hawaiian or other pacific Islander

J	8	52	24.5	Male	Caucasian
K	1	61	21.1	Female	Japanese
L	1	51	29.3	Male	Asian
M	1	47	20.3	Male	Asian
N	1	32	25.5	Male	Caucasian
O	1	57	33.4	Male	Asian
P	1	58	24.7	Male	Asian
Q	1	40	15.7	Female	Caucasian
R	1	43	29.9	Male	Caucasian
S	1	59	28.3	Male	Asian
T	1	53	20.9	Female	Asian
U	1	52	22.3	Male	Caucasian
V	1	56	34.4	Female	Caucasian
W	1	39	52.4	Female	Asian
X	1	64	34.7	Male	Hispanic

Table 2. Demographic information of the liver cohort.

Group **Age Range**	**Age** **Mean ± SD** **(years)** ***n***	**Ethnicity**		**Sex**		**Body Mass Index (BMI)**	
Population 0.018–87 years	44.8 ±22.5 *n* = 87	Caucasian Asian Pacific Islander African American Hispanic Other/unknown	68% 14% 7% 5% 3% 3%	Female Male Unknown	33% 66% 1%	Underweight (BMI ≤ 18.9) Ideal weight (19–24.9) Overweight (25–29.9) Obese (30–39.9) Morbidly obese (≥40.1) Unknown/not included	3% 31% 16% 26% 9% 14 %
Pediatrics	4.6	African-	*n* = 2	Female	*n* = 2	Underweight (<5th	*n* = 1

7 days—18 years	±5.4 *n* = 12	American Caucasian Pacific Islander Hispanic Other/unknown	*n* = 6 *n* = 1 *n* = 1 *n* = 2	Male Unknown	*n* = 9 *n* = 1	percent) Ideal weight (6–85th percent) Overweight (86–94th percent) Obese (>95th percent) Unknown/not included	*n* = 3 *n* = 2 *n* = 2 *n* = 4
Adult 19—64 years	45.3 ±12.9 *n* = 60	Caucasian Asian Pacific Islander African American Hispanic Other/unknown	*n* = 39 *n* = 12 *n* = 4 *n* = 2 *n* = 2 *n* = 1	Female Male	*n* = 20 *n* = 40	Underweight (BMI ≤ 18.9) Ideal weight (19–24.9) Overweight (25–29.9) Obese (30–39.9) Morbidly obese (≥40.1) Unknown/not included	*n* = 2 *n* = 19 *n* = 10 *n* = 19 *n* = 5 *n* = 5

						Underweight (BMI ≤ 18.9)	*n* = 0
						Ideal weight (19–24.9)	*n* = 5
	74.6	Caucasian					
Geriatrics			*n* = 14	Female	*n* = 7	Overweight (25–29.9)	*n* = 2
	±6.7	Pacific					
65–87 years			*n* = 1	Male	*n* = 8	Obese (30–39.9)	*n* = 2
	n = 15	Islander					
						Morbidly obese (≥40.1)	*n* = 3
						Unknown/not included	*n* = 3

SYBR Green q-RT-PCR and Primer Selection

Primer sequences for ABCB1 were (F): 5′-CAC CCG ACT TAC AGA TGA TG-3′ and (R) 5′-GTT GCC ATT GAC TGA AAG AA-3′ with predicted amplicon length of 81 and can be retrieved from NM_000927 [23]. The primers for 18S rRNA were (F): 5′-CAC GGC CGG TAC AGT GAA A-3′ and (R): 5′-AGA GGA GCG AGC GAC CAA-3′, with a predicted amplicon length of 71 and can be retrieved from NR_003286.2 [24].

Real-time PCR was performed on an ABI Step-one-plus real time PCR system (Life Technologies) using cDNA template (2.5 ng RNA equivalent from S9 samples or 5 ng RNA equivalent from total liver lysates). First, the forward and reverse primers concentrations were optimized to give best signal-to-noise amplification and were 300 nM for both forward and reverse primers for 18S and 300 nM forward and 500 nM reverse for ABCB1. Detection was with PerfeCTa SYBR Green SuperMix for IQ (Quanta BioSciences, Gaithersburg, MD, USA). Cycling conditions were: 1 cycle 30 s at 95 °C, 40 cycles of 5 s at 95 °C, 15 s 60 °C, 10 s 70 °C then melt curve of 15 s 95 °C, 60 s 60 °C, 15 s 95 °C. The threshold value detection (C_T) was set in the exponential phase of amplification and quantified by normalization to 18S rRNA. Analysis was performed on StepOne™ software version 2.3 (Applied Biosystems, Foster City, CA, USA), with C_T values converted to fold change differences using the $2^{-\Delta\Delta CT}$ method for relative quantitation [25].

Immunoblotting for Relative Expression of Protein

For western blotting of ABCB1, SDS-page gels (7%) were used to resolve 20 µg of liver lysate or S9 and each sample was analyzed on at least 3 separate gels as previously described [19]. Primary antibody was rabbit polyclonal anti-ABCB1 (ab129450,

Abcam, Toronto, ON, Canada) incubated for 2 h at room temperature. Horseradish peroxidase conjugated donkey-anti-rabbit at 1:3000 was then incubated for 1 h at room temperature (Jackson Immunolabs, Westgrove, PA, USA). The membrane was developed for 1 min in enhanced chemiluminescence solution and detected on X-ray film for 60 min. Confirmation of even protein loading was by staining acrylamide gels with ponceau red and determining even loading. Additionally, a second variability control between blots run on different days was included. This second control was 20 µg of commercial human S9 from a pool of 200 individual livers, that was added to the left lane of every blot, and used to determine variability and to normalize expression. The inter-blot coefficient of variation (CV) of the XT200 was 14.6%, n = 9. Samples were semi-quantified with Image J 1.48v (http://imagej.nih.gov/ij) with background subtraction.

Total Triglyceride Liver Concentrations

The triglyceride colorimetric assay kit (Cayman Chemical Company, MI, USA) was used to determine the levels of triglycerides (mg/g of liver) as per manufacturer's instructions.

Statistical Analyses

All data sets were analyzed for normality with D'Agostino-Pearson tests. For sex or ethnicity (binary tests), student's *t*-tests (two-tailed) were performed between groups. For binned continuous data (age, BMI), one way ANOVA with Tukey's post hoc analysis was performed. Pediatric samples (<18 years) were not included in the BMI analysis, as BMI is not an appropriate measure for obesity in children. Where body weight was known, appropriate categories were assigned using the National Center for Health

Statistics weight-for-age growth charts for children: underweight, <5th percentile; ideal weight, 5–85th; overweight, 86–94th; obese, >95th percentile (http://www.cdc.gov/growthcharts [26], Table 2). Correlations between mRNA and protein were performed using Pearson's or Spearman's correlation according to the normality distribution. A one-phase association (not forced through zero), straight line, and sigmoidal fits were compared to predict development of ABCB1 protein expression from birth. The best fit was determined using Aikake's informative criteria (AIC), *F*-tests, sum of squares and residual analysis. All statistical analyses were performed using Prism 5.0 for Mac OsX (Graph Pad Prism, San Diego, CA, USA).

4.4 RESULTS

Expression ABCB1 mRNA and Protein in Hepatic Lysates: Regional and Demographic Associations

Transporter mRNA expression was determined in a cohort of 24 individual livers using multiple pieces from each liver obtained from 8 distinct regions (not all samples were available from all individuals, Figure 1a). There were no significant differences in ABCB1 mRNA regional expression (Figure 1b). The mean C_T values (± SD) for 18S and ABCB1 were 13.3 ± 2.2 and 32.6 ± 2.8, respectively. The ABCB1 mRNA levels had an average 30-fold variability in mRNA levels. Similarly, there were no significant differences in protein expression of ABCB1 throughout the liver (Figure 1c). The highest level of protein and greatest range of ABCB1 expression was detected in the samples taken adjacent to bile ducts. The levels of ABCB1 mRNA and protein did not correlate (Figure 1d), indicating that ABCB1 expression is not purely transcriptional.

Hepatic ABCB1 protein levels within the regional expression lysate cohort ranged from 3.4 ± 0.7 to 6.7 ± 3.0 area: density units (mean ± SD, Figure 1f). Lysates average five times more ABCB1 protein than S9 fractions. Intra-individual variability in hepatic ABCB1 protein expression, taken from eight different sites in the same liver, ranged from 1.2- to 4-fold with a mean value of 2.5 ± 0.9-fold (Figure 1f). In these total liver lysates, there was no significant correlation of ABCB1 protein with age, BMI, sex, or ethnicity (Figure 2a–d). However, for ethnicity it should be noted that statistical comparisons essentially compare Caucasians and Asians due to the small sample size for Hispanics and Hawaiians.

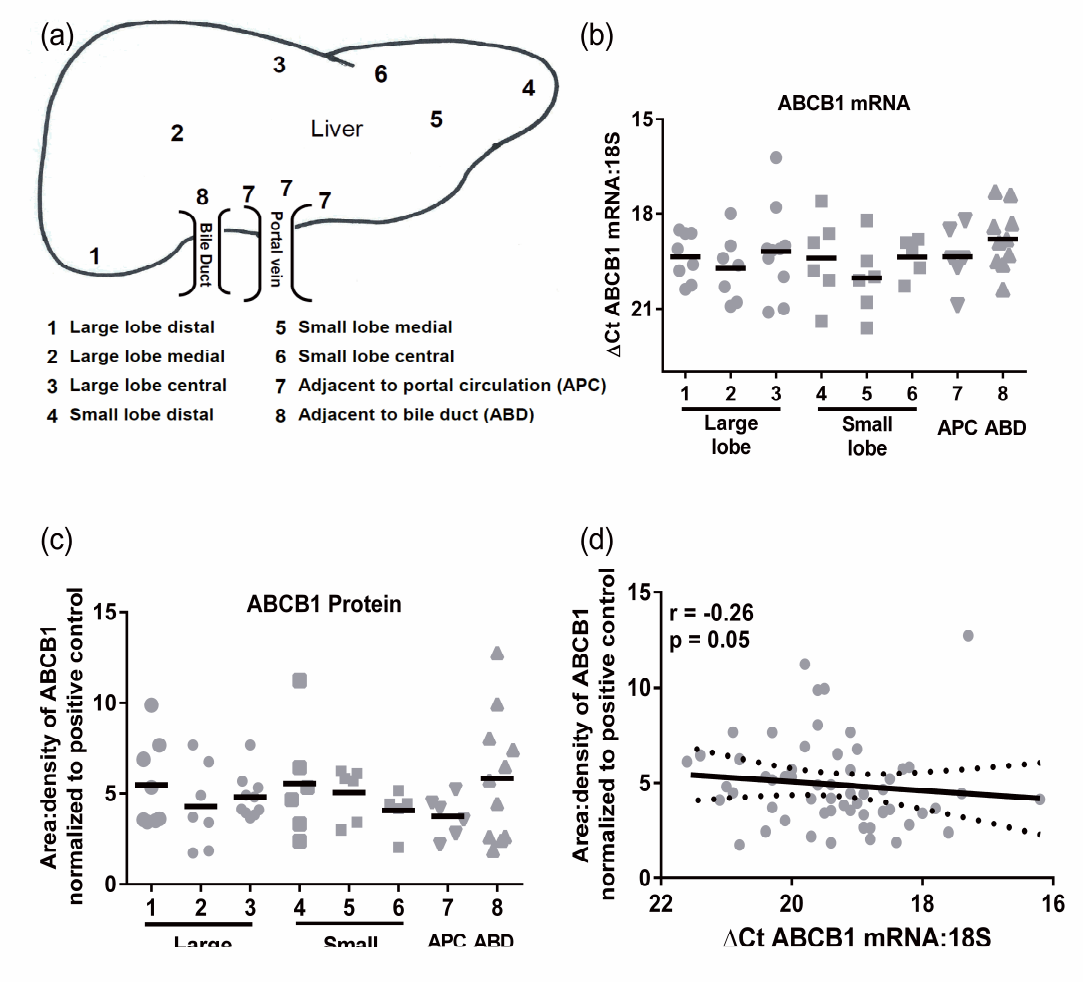

Figure 1. *Cont.*

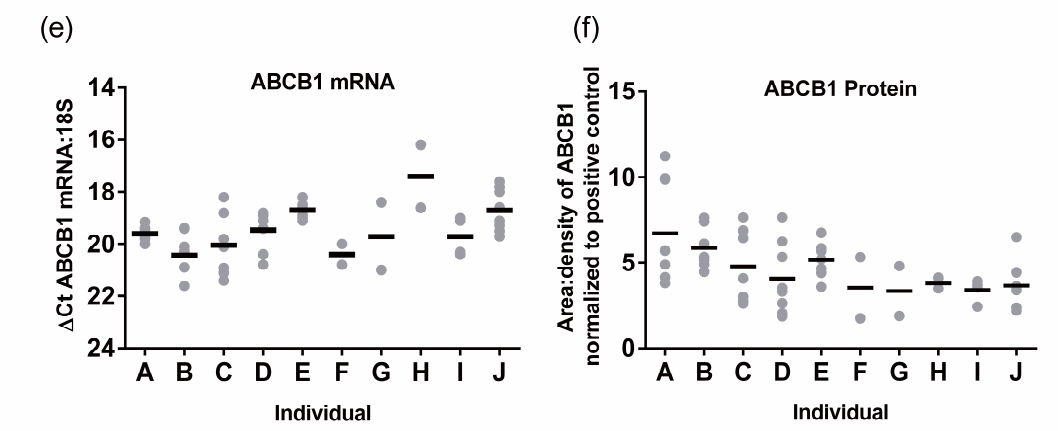

Figure 1. Expression of ABCB1 within different human liver regions. (**a**) Diagram of the different regions where samples taken within the same liver; (**b**) The mRNA

expression of ABCB1 was determined within different regions of 14 different livers; (**c**) Protein expression of ABCB1 within different human liver regions: (distal large lobe *n* = 8; distal small lobe *n* = 6), medial large lobe *n* = 7; medial small lobe *n* = 6, central large lobe *n* = 10, central small lobe *n* = 5, APC = adjacent to portal circulation *n* = 6 and ABD = adjacent to bile duct N = 11); (**d**) Correlation between mRNA expression and protein expression for ABCB1, with 95 % confidence intervals (dotted line), and data analyzed by Spearman's correlation; (**e**) ABCB1 mRNA expression (line = means) within the livers of 10 individuals; (**f**) The ABCB1 protein expression showing intra-individual variability of protein expression (8 regions for A, D, E, J; 7 regions for B and C; 4 regions for I and 2 regions for F, G and H).

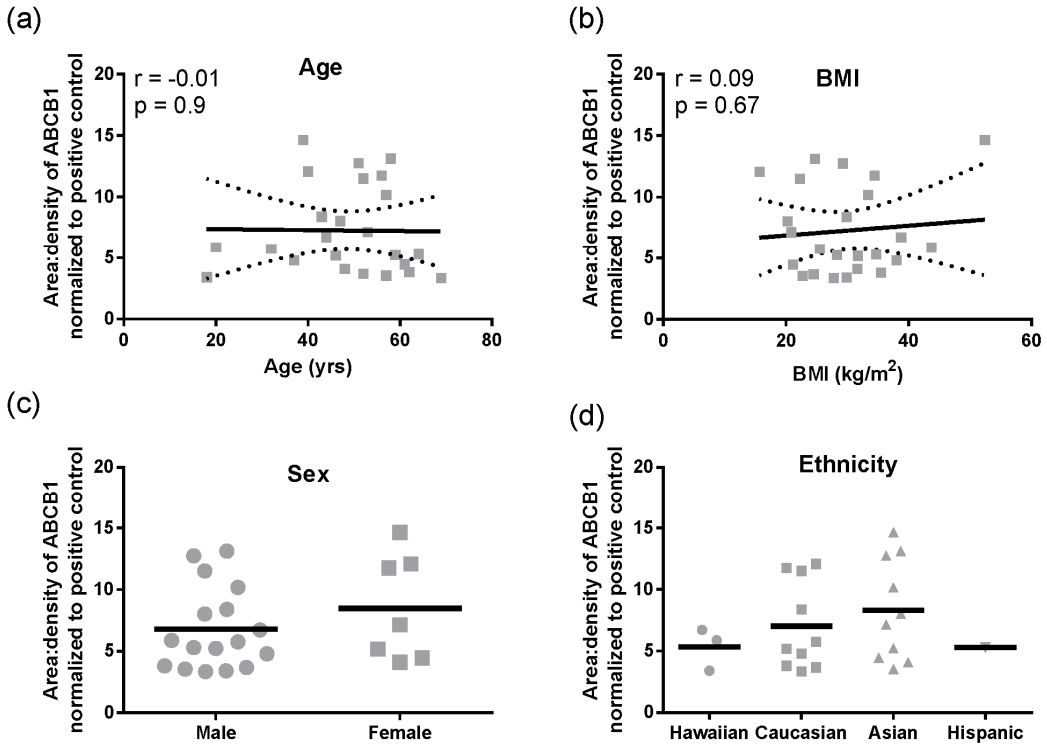

Figure 2: The protein expression of ABCB1 in a cohort of 24 liver lysates. The ABCB1 protein was detected by Western blotting and normalized to the ABCB1 levels detected in a pooled S9 liver sample (Xenotech, *n* = 200 individuals); (**a**) correlation between age and ABCB1 protein expression with dotted lines 95% confidence intervals; (**b**) Expression of ABCB1 protein compared with BMI; (**c**) The expression of ABCB1 proteins compared to sex; (**d**) Differences between ethnicity and ABCB1 protein expression.

Similarly, for triglycerides mean levels were 15.4 ± 1.2 mg/g of liver (mean ± SD). There were no regional differences in triglyceride levels (Figure 3a), but triglycerides varied significantly between individuals (Tukey $p < 0.0001$, Figure 3b). There was no correlation between hepatic triglyceride levels and age, BMI, sex, or ethnicity (Figure 3c–f). Triglyceride levels did not correlate with ABCB1 mRNA ($r = -0.004$, $p = 0.98$) or protein ($r = -0.23$, $p = 0.30$).

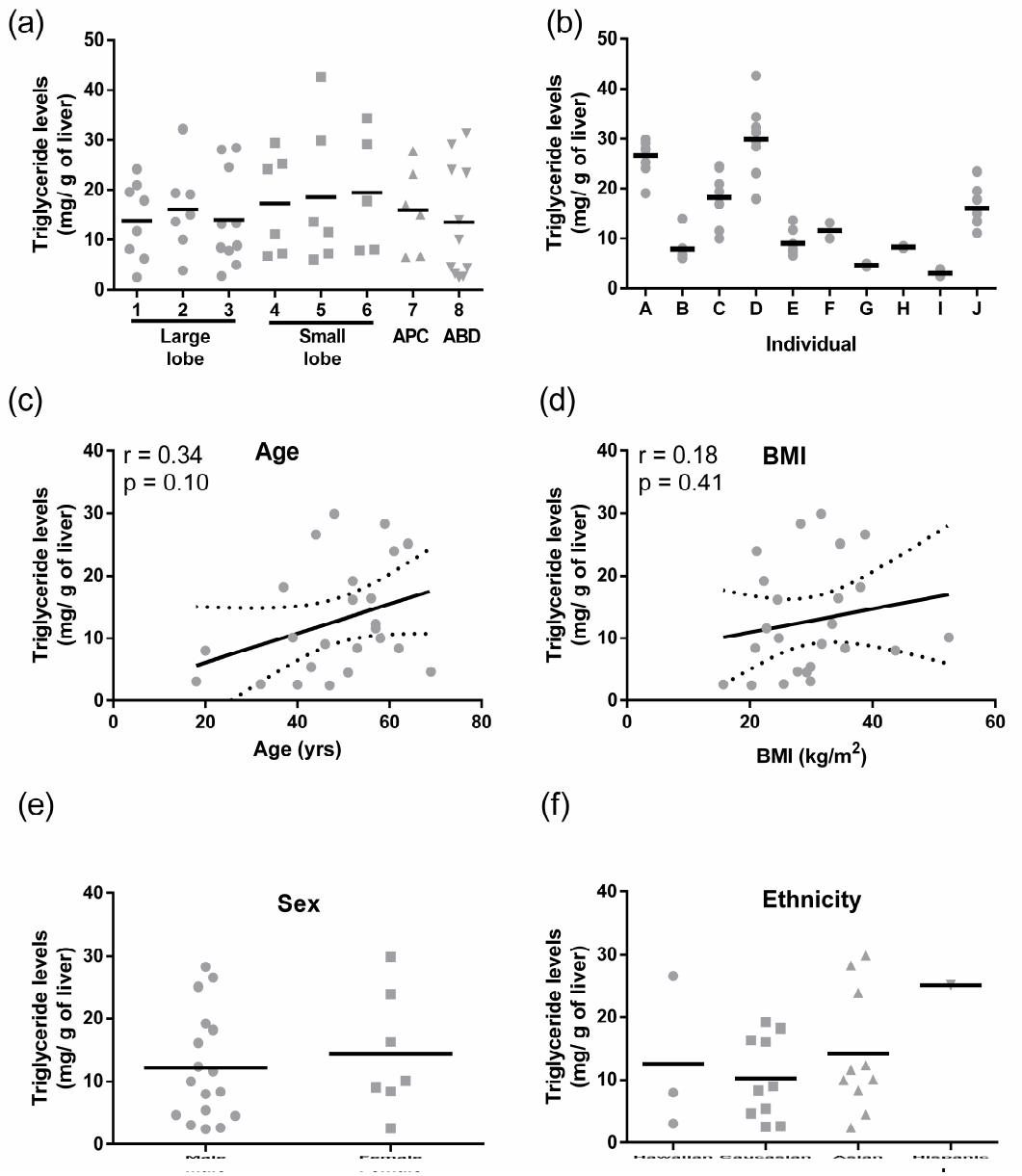

Figure 3: Triglyceride levels in human liver lysates. (**a**) The regional expression of hepatic triglyceride levels: 1 & 4 distal, 2 & 5 medial, 3 & 6 central, APC = adjacent to portal circulation and ABD = adjacent to bile duct, see Figure 1a for detailed liver regions; (**b**) The inter- and intra-individual expression of triglycerides levels. The correlation of triglyceride levels with (**c**) age and (**d**) BMI. The relationship between triglyceride levels and (**e**) sex and (**f**) ethnicity.

Expression of ABCB1 in Hepatic S9: Demographic and Ontogenetic Associations

Because we only had adult total liver lysates, to provide further insight into the ontogeny of ABCB1 we analyzed a cohort of liver S9 where pediatric, adult, and elderly samples were available (Table 2). The ABCB1 mRNA (n = 79) was detectable from birth, and a significant negative correlation with age was observed (Pearson r = −0.3, p = 0.02), although the correlation coefficient suggested that this was only a moderate association, with 8% of the change in mRNA expression being attributed to age as a covariate (Figure 4a). When grouped into categories, the elderly (>65 years, mean ΔC_T ± SEM of 22.7 ± 0.4) had lower ABCB1 mRNA levels than adults (mean ΔC_T ± SEM of 21.6 ± 0.2, Figure 4b). Although not statistically significant, this 2-fold difference in gene expression is driving the negative association. Exactly the same as in the case of the total lysates, there were no significant differences observed for BMI, ethnicity, or sex in ABCB1 mRNA expression levels in S9 (Figure 4c–e). Pediatric samples (<18 years) were not included in the BMI analysis, as BMI is not an appropriate measure for obesity in children; rather, percentile-weight-for-age (National Center for Health Statistics) was used.

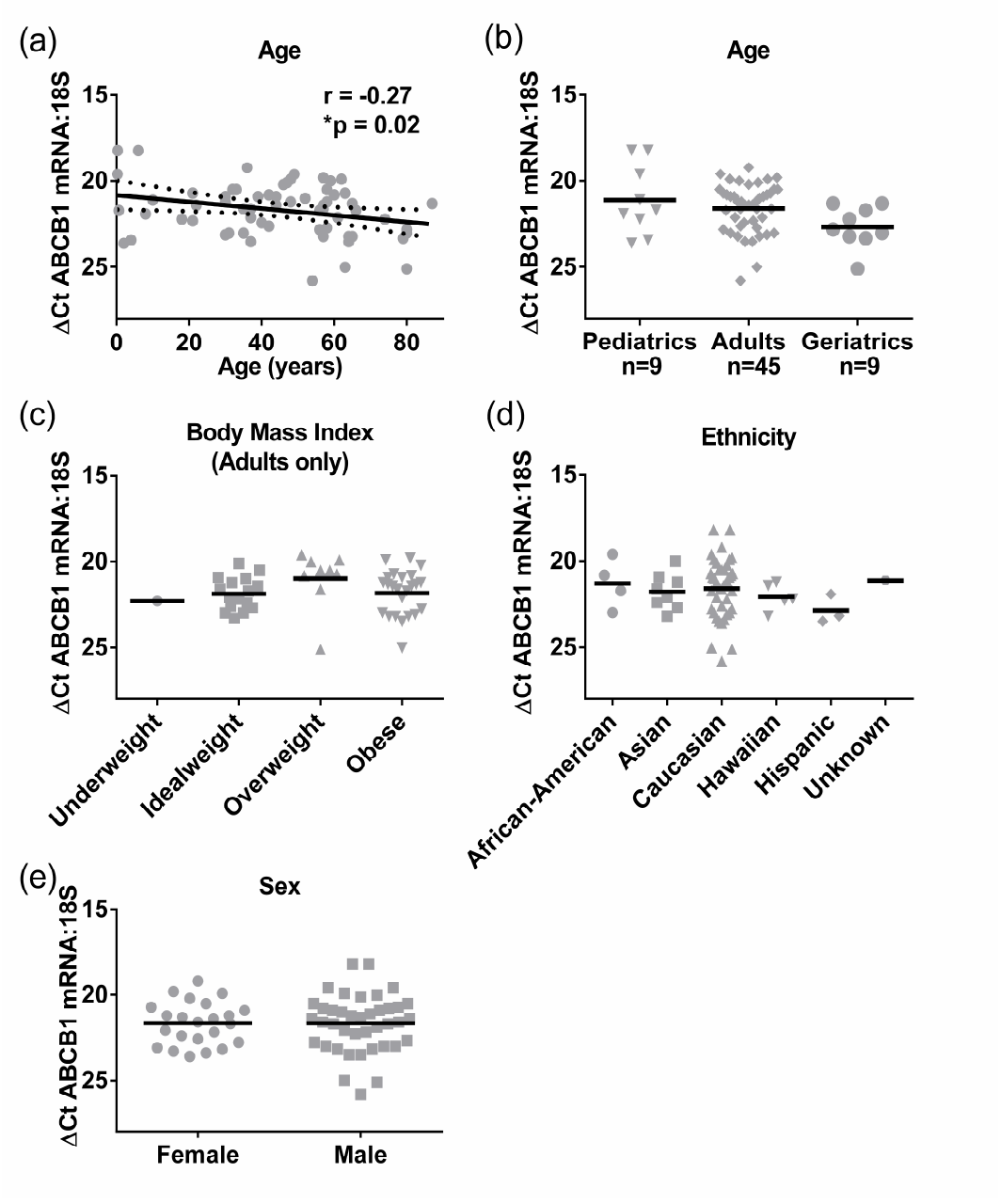

Figure 4. The mRNA expression of ABCB1 in a cohort of 80 liver samples Gene expression was measured using SYBRGreen detection and ABCB1 gene expression was normalized to 18S to give ΔC_T value. (**a**) Line graph shows linear regression with 95% confidence intervals, (dotted line), of age compared to gene expression. Data analyzed by Pearson's correlation (* $p < 0.05$); (**b**) Dot blot compare gene expression levels with age grouped into pediatric (≤18

years), adult (19–64 years), and geriatric (≥65 years) and data showing mean (horizontal line). The mRNA expression was also compared to (**c**) obesity, measured by BMI, (**d**) Ethnicity, and (**e**) Sex.

Subsequently, we performed the same analysis for ABCB1 protein levels (n = 87). A representative Western blot image for ABCB1 detection is presented in Figure 5a. There was a 2.9 ± 0.32 -fold variability for ABCB1 protein levels in the population sampled ranging from 0.44 to 1.26 area:density units. Again, the same relationships were observed in S9 as total liver lysates, with no significant differences in ABCB1 protein expression observed for obesity (BMI, range 15.8–57.6) ethnicity, or sex (Figure 5d–f). However, different to total lysates where no pediatric samples were available, children have lower ABCB1 than adults. The ABCB1 protein was detected from birth and increased with age following a mono-exponential rise-to-plateau relationship with best fit (F-test; and fit parameters of AICc, 15.56, Sum of Squares 0.77, Figure 5b). This model predicts protein levels are at 76% of adult levels at birth and reach adult levels (±10%) by 5 years of age. Using categorical data, children had a significantly lower ABCB1 protein levels than adults ($p < 0.001$, Figure 5c). Although the elderly population had a trend towards lower ABCB1 expression than adults, this was not significantly different.

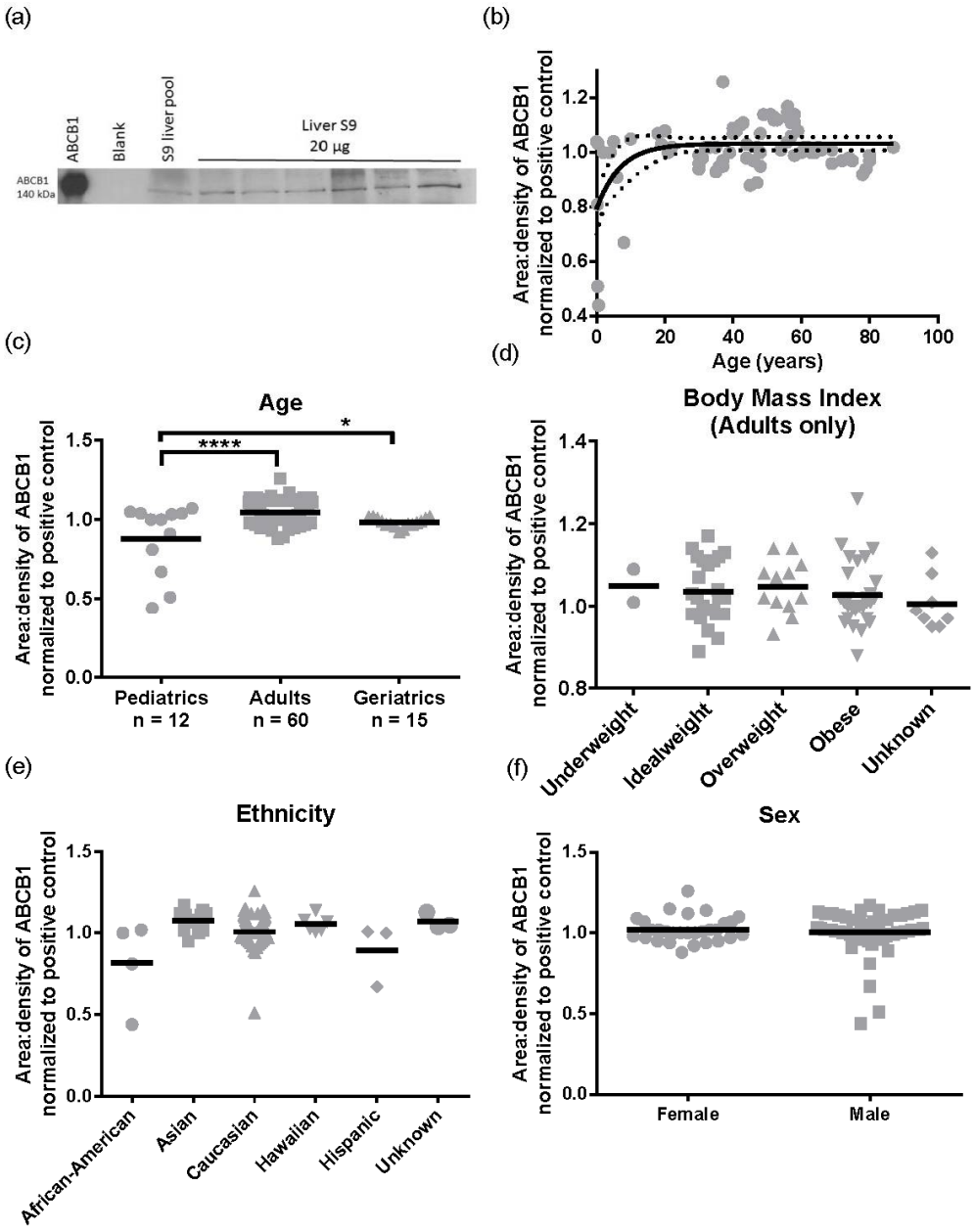

Figure 5. Protein expression of ABCB1 in a liver cohort. The ABCB1 protein was detected by Western blotting and normalized to the ABCB1 levels detected in a pooled S9 liver sample (Xenotech, *n* = 200 individuals). (**a**) Example of Western blot of 6 individuals with 20 µg liver S9 loaded compared with pooled S9 sample, ABCB1 = recombinant ABCB1 expressed in baculosome (5 µg), and blank = baculosome with no expression (5 µg); (**b**) Relationship between age and

ABCB1 protein expression with a one-phase association, with 95% confidence intervals (dotted line); (**c**) Protein expression compared to individuals grouped by age and analyzed by ANOVA ($p < 0.0001$) and ABCB1 expression in pediatrics was significantly lower compared to adults and geriatrics (Tukey's multiple comparison test, *** $p < 0.0001$, * $p < 0.05$); (**d**): Expression of ABCB1 protein in obese individuals, measured by BMI; (**e**): Differences between ethnicity and ABCB1 protein expression; (**f**): The expression of ABCB1 proteins compared to sex.

4.5 DISCUSSION

One of the key findings in this study was that ABCB1 protein expression does not differ significantly in different liver regions; this indicates that random collection of liver tissue is appropriate for studying ABCB1 ex vivo. The caveat to this finding is that in standardizing our assays on a per milligram of protein basis, we have assumed that there are equal numbers of hepatocytes per milligram of tissue in each of the 8 liver regions sampled. Although tissues did not differ visually in any way, but we did not confirm cell types histologically. No significant differences were associated with ethnicity, sex, or obesity (measured by both BMI and liver fat levels).

The BMI parameter is frequently used as a measure for obesity; however, this can be flawed [27,28], and many healthy individuals have high BMI but would have low internal fats (for example certain athletes) [29]. We therefore additionally evaluated liver triglyceride levels to understand if the pathological manifestation of obesity (liver fat)

could alter ABCB1 expression. Although the ABCB1 transporter is known to be involved in endogenous movement of cholesterol, phospholipids, and sphingolipids, and therefore plays a role in lipid homeostasis [30], neither triglyceride levels nor BMI were related to differences in ABCB1, a novel finding.

Finally, because we did not have pediatric or significant numbers of elderly liver lysates, we used S9 to determine ontogenetic differences in ABCB1. Children (≤18) have significantly lower and more variable expression of hepatic ABCB1 protein than adults, with ~76% of adult ABCB1 protein levels present at birth, reaching full maturity by five years of age. The strength of the data presented is that in every other category tested, S9 results were reflective of liver lysates, albeit with lower absolute levels of mRNA and protein present. In addition, all of the S9 samples were treated the same way i.e., similarly depleted of membranes, so the relative variability of the ABCB1 proteins should be preserved, even though absolute levels are not.

Relatively few studies of pediatric ABCB1 and ontogeny exist. Miki et al., 2005, reported no differences in hepatic ABCB1 mRNA but significant decreases in mRNA expression in the lungs of the elderly [4]. Moreover, several studies have investigated the ontogeny of intestinal ABCB1 in humans [5,31–33]. Immunohistochemistry techniques have demonstrated that the transporter is differentially expressed in children under the age of three: ABCB1 is present on both the apical and basolateral surfaces of enterocytes, while after three the protein is only detected apically [31]. Recently, lower neonatal intestine mRNA levels have been reported that reach adult levels in early childhood [5]. However, subsequent tests of hepatic and intestinal proteins by the same authors using LCMS showed stable expression of ABCB1 from fetus to adult, albeit in

fewer samples than presented here [33]. These studies suggest that organ-specific and ontogenetic regulation of ABCB1 protein is likely, albeit with some disagreement in the literature.

4.6 CONCLUSIONS

In summary, investigators who collect human liver samples opportunistically, as well as those who collect uniformly from a single site, likely have a cohort representative of population variability in ABCB1 that can be accurate for extrapolation. Additionally, children under five express less hepatic ABCB1 than the general population, which could cause xeno- and endobiotic toxicity including altered responses to therapy, but this requires confirmation in a larger study, preferably using total liver lysates. Better understanding of the natural expression patterns of ABCB1 in the human liver can assist in translating work from human tissues into understanding of the mechanisms of drug and chemical efficacy, toxicity, and resistance.

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 5

5.1 DISCUSSION

The availability of new anti-TB drugs in the 1950s and 60s (1) heralded an age of great optimism in the control of TB (2). Immediately after these drugs were introduced into clinical practice, the incidence of TB began to decline (3). Countries such as the United States reported a yearly decline of 6% in TB incidence (2). Unfortunately, by the late 1980s, this declining trend reversed and the incidence of TB once again began to rise (4,5). The growing worldwide TB incidence became so alarming that in 1993, the WHO declared TB a 'global emergency' (3). The resurgence of TB in the 1980s and 90s was due to the HIV epidemic, the emergence drug-resistant *Mycobacterium tuberculosis* (Mtb), increased immigration of people from TB endemic countries, lack of an effective global response to TB control, and the neglect of TB as a global priority (6). In response, to the public health threat posed by TB in the 1990s, the WHO called for the adoption of a series of recommendations. The main recommendations were the universal adoption of the current short-course (6 months) treatment regimen, supervised pharmacotherapy through the Directly Observed Treatment Short-course program, and the establishment of effective TB control programs in all TB endemic countries (3). Since then, TB incidence has been declining with some reports suggesting an average worldwide drop of 1.5% per year since 2000 (7). This new declining TB trend has once more nurtured new hope of eliminating TB. This renewed hope has attracted a lot of interest and there is now a push to put an end to the global TB epidemic as exemplified in the recently published WHO End TB strategy (8). The End TB strategy aims to reduce TB deaths by 95% and to cut new cases by 90% between 2015 and 2035 (8).

A central tenet of the End TB strategy is to treat every TB patient with the 6-month short course regimen composed of Rifampicin (RIF), Isoniazid (INH), ethambutol (EMB) and Pyrazinamide (PZA). Treatment of TB patients with this regimen is expected to lead to treatment success in all patients who adhere to the regimen (9) and minimize transmission of TB to other persons. Unfortunately, about 12 - 20% of TB patients fail treatment and another 3 – 10% relapse within two years of completing their medication regimen (7,10,11). In general, the successful treatment of TB patients with this standard 6-month regimen relies on a combination of microbial and host factors. TB patients infected with Mtb bacilli that are resistant to the drugs are likely to fail treatment (12–16). Likewise, patients with low plasma anti-TB drug levels are also likely to fail treatment (17,18).

This dissertation was undertaken to evaluate the accuracy of molecular based assays to diagnose of drug-resistant TB and to investigate P-glycoprotein as a determinant for low plasma concentration of Rif in TB patients. Specifically, the following questions were addressed 1) Does the Genotype MTBDR*plus* assay accurately distinguish RIF and INH drug-resistant from drug-susceptible *Mycobacterium tuberculosis* isolates in Cameroonian TB patients? 2) Does the recommendation to consider *Mtb* isolates that do not hybridize with the wildtype and mutation rpoB probes on the Genotype MTBDR*plus* strip as resistant, falsely diagnose resistance to RIF? According to the Genotype MTBDR*plus* assay, a *Mycobacterium tuberculosis* isolate could be classified as resistant if its DNA does not hybridize to a wild type probe and a mutation probe on the assay strip. This recommendation assumes that a mutation is present, the type of mutation is unknown and, the bacteria are resistant to RIF.

However, not all mutations in the rpoβ gene region assessed by the Genotype MTBDR*plus* assay are associated with resistance to RIF. 3) Does the expression of P-glycoprotein in the liver vary extensively among individuals especially individuals of different ages?

Does the Genotype MTBDR*plus* assay accurately distinguish RIF and INH drug-resistant from drug-susceptible *M. tuberculosis* in Cameroonian TB patients?

Of the two commercially available genotype-based assays used by most diagnostic laboratories in TB endemic countries, only the Genotype MTBDR*plus* assay is designed to detect resistance to both RIF and INH (19,20). The other genotype-based assay GeneXpert MTB/RIF, only diagnoses resistance to RIF (21). However, the GeneXpert MTB/RIF assay is preferred and used by most laboratories as the first line test to screen for RIF-resistant TB among patients (22–24). This preference could be attributed to three main advantages of the GeneXpert MTB/RIF assay. First the GeneXpert assay has a faster turnaround time (2 hours). Secondly, it can be used directly on clinical specimen. Lastly, the diagnosis of resistance to RIF also serves as a good diagnostic marker for resistance to INH. As such, patients diagnosed as RIF resistant by the GeneXpert MTB/RIF assay are immediately placed on multidrug resistant TB (MDR-TB) regimen (23,24).

Despite the excellent qualities of the GeneXpert MTB/RIF assay, it presents some limitations that could be resolved using the Genotype MTBDR*plus* assay. The GeneXpert MTB/RIF assay cannot diagnose resistance to INH; whereas, the Genotype MTBDR*plus* assay can (19). INH-resistant TB, that is not resistant to RIF, is common

and treatment of such patient with the standard 6 months regimen leads to poor treatment outcome (25). Furthermore, resistance to INH increases the risk of developing resistance to other drugs (25). As such, diagnosing INH-resistant TB that is not resistant to RIF is important and the Genotype assay can diagnose such cases (19). Direct testing of clinical specimens from previously treated TB patients may lead to false-positive results due to the presence of residual DNA from dead Mtb bacilli (26). Use of cultured specimens that are recommended for the Genotype MTBDR*plus* assay will prevent false-positive TB results but increase time to availability of results by about a week or more.

Our study showed that the Genotype MTBDR*plus* performed well in diagnosing RIF and INH drug resistant Mtb isolates cultured from sputum specimens from Cameroonian TB patients. We evaluated the Genotype MTBDR*plus* results to those of the standard method for drug susceptibility testing, Bactec MGTI 960 SIRE system. The Genotype assay correctly identified RIF resistance in 48/49 (sensitivity, 98% [CI, 89%-100%]), INH resistance in 55/60 (sensitivity 92% [CI, 82%-96%]), and resistance to both RIF and INH (MDR-TB) 46/49 (sensitivity, 94% [CI, 83%-98%]). The specificity for the detection of RIF resistant and MDR-TB cases was 100% (CI, 98%-100%), while that of INH resistant was 99% (CI, 97% -100%). The positive predictive value (PPV) and negative predictive value (NPV) of the Genotype MTBDR*plus* assay were high for RIF resistance, INH resistance and MDR-TB, ranging from 97%-100%. The degree of agreement to the standard drug susceptibility testing assay for the diagnosis of MDR-TB was very good (Kappa = 0.96 [CI, 0.92-1.00]).

Overall, our data shows that the Genotype MTBDR*plus* assay can be used to diagnose MDR-TB in Cameroon. The National Tuberculosis Control Program of Cameroon (NTCP) plans to use the Genotype MTBDR*plus* assay for the rapid diagnosis of patients with drug-resistant TB. This study provides the necessary scientific evidence to guide the NTCP on the accuracy of this assay if used. Furthermore, we propose that for routine diagnoses, the Genotype MTBDR*plus* assay be used to perform drug susceptibility testing of Mtb positive-cultures especially for cultures containing contaminants for which culture-based drug susceptibility testing will be delayed. The Genotype MTBDR*plus* assay has a faster turnaround time of 48 hours compared to the 2 weeks post-culture required for the standard culture-based drug susceptibility testing assay. Lastly, the Genotype MTBDR*plus* assay could be used as a rapid complementary test to confirm resistance to RIF detected by the GeneXpert MTB/RIF assay.

Does the recommendation to consider Mtb isolates that do not hybridize to wildtype and mutation rpoB probe as resistant over-diagnose resistance to RIF?

The Genotype MTBDR*plus* assay is highly accurate in diagnosing drug-resistant TB including resistance to RIF (27,28). The Genotype MTBDR*plus* assay detects resistance to RIF by detecting mutations from codons 507 to 534 of the *rpoB* of *Mtb*. Although 95% of RIF-resistant Mtb isolates have mutations within this region of the rpoB gene, not all mutations in this region are associated with resistance to RIF (29,30). However, the Genotype MTBDR*plus* assay recommends that an *Mtb* isolate be assumed resistant to RIF solely on the basis of absence of hybridization to one or more

wildtype probe on the assay strip(19,20). This recommendation suggests that an *Mtb* isolate with any mutation in the *rpoB* gene region from codon 507 to 534 be assumed to be resistant to Rif. Some of these mutations could be silent mutations (31,32) or missense mutations with no or unclear association with resistance to RIF (33,34). As such, it is important to know what type of mutations is present to determine if the Mtb isolate is resistant.

In our study, we show that only 6% (16/275) Mtb isolates fall into this category and have the potential for miss-classification using the Genotype MTBDR*plus* recommendation. Interestingly, sequencing of these Mtb isolates revealed that impaired hybridization to WT probes was due to the presence of 'disputed' RIF mutations. These mutations are referred to as 'disputed' because isolates bearing them may be detected as RIF-susceptible with the standard MGIT 960 DST assay (35). Mtb isolates bearing these 'disputed' RIF mutations may be responsible for causing resistance to RIF and have been associated with adverse treatment outcome in both naïve and previously treated TB patients (35–37). Thus, the recommendation of the Genotype MTBDR*plus* assay to assume resistance based solely on the absence of hybridization to WT probe is valid and allows the identification of clinically important RIF-resistant Mtb isolates. This recommendation leads to accurate and rapid diagnosis of RIF-resistant TB by the Genotype assay.

Unfortunately, the scope of our study did not allow us to investigate why disputed mutations resulted in varying susceptibility to RIF by the MGIT 960 DST assay. Perhaps Mtb isolates that have a RIF-disputed mutation have a severe reduction in RNA polymerase activity and only isolates that acquire compensatory mutations have this

polymerase activity restored. This compensatory mutation could arise either in the *rpoB* gene or genes that code other subunits of the RNA polymerase, such as the *rpoC* gene and *rpo*A as previously described (27).

Does the expression of P-glycoprotein in the liver vary extensively among individuals, especially individuals of different ages?

The bactericidal effect of RIF on *M. tuberculosis* is concentration-dependent (39,40). The plasma concentration of RIF varies extensively in TB patients with some patients having lower than expected plasma concentrations (41–44). Low plasma concentration to a single drug in the multidrug treatment regimen of TB is associated with treatment failure and development of bacterial resistance (45). Low plasma concentrations of anti-TB drugs could result from several factors (46–48), but the most likely is variation in the rate at which the drug is cleared from the blood (48,49). For RIF, a drug that is largely cleared from blood by the liver and excreted in bile (50), variation in plasma concentration could be due to differences in the rate of biliary excretion. The biliary excretion of RIF is mediated by the membrane transporter, p-glycoprotein (51,52). As such, variation in the plasma concentration of RIF could be due to inter-individual variability in the expression of p-glycoprotein.

Ideally, to investigate our study hypothesis, liver biopsies from TB patients would be required. Considering the challenges in obtaining such samples, we first opted to test if the protein levels of p-glycoprotein (p-gp) varied extensively across different age groups in a healthy population. A unique collection of 87 liver samples of different age individuals (7days – 87 years), ethnicity and gender obtained from organ donors who had met with accidental death were used. Contrary to our expectations, neither p-gp

protein nor mRNA-expression levels varied extensively between individuals. A 2.9 + 0.32 fold variability of p-gp protein levels was observed. However, with our unique set of samples, results showed that p-glycoprotein is expressed from birth and increases with age reaching 90% of adult levels by 5 years of age. Although functionality of p-gp was not tested, the early and high levels of expression of p-gp in the pediatric liver are likely to be functional. As such, the pediatric liver is capable of mediating biliary clearance of drugs and biomolecules that are substrates of p-glycoprotein. The rate at which the liver clears RIF from blood is determined by the rate of blood flow to the liver and the ability of the liver to extract, and excrete RIF from the blood. The relative liver weight and hepatic blood flow rate per unit liver weight is higher in children than in adults (53). Assuming the p-gp is fully functional in the livers of children, children may demonstrate a higher excretion rate when given a dose/kg of RIF similar to adults. Interestingly, this ability of children to rapidly eliminate RIF has been observed clinically, but the mechanism or reason was unknown (54,55). Consequentially, in 2009, the World Health Organization (WHO) revised and increased the daily dosage of RIF for children from 10 to 15mg/kg (56). Our data on p-gp protein expression levels provides a plausible explanation of why children, when given the same dose/kg of RIF as adults, have a lower plasma concentration.

Unfortunately, our data does not support our hypothesis that biliary clearance mediated by p-glycoprotein accounted for the variation in plasma concentration of RIF seen in TB patients. However, other transporters or metabolic enzymes involve in the systemic clearance of RIF may be involved. Recent evidence suggests that the uptake transporter SLCO1B1 could be responsible for the variation in the plasma concentration

of RIF. This uptake transporter is responsible for the uptake of RIF from blood into hepatocytes (52,57). Weiner and colleagues reported a 36% reduction in the bioavailability of RIF in individuals with a single nucleotide polymorphism (SNP) in the SLCO1B1 gene (57). Chigusta and colleagues reporting on another SNP in this uptake transporter, also reported a low bioavailability of RIF in a South African population (52). Although genetic polymorphism in the SLCO1B1 could account for the variation in plasma concentration of RIF; however, when adjusted for this transporter polymorphism other factors seemed to account for the variation of the plasma concentration of RIF (52,57). It is potentially possible that a polymorphism in p-glycoprotein could affect plasma concentration of RIF; however, it is most likely variation in plasma concentration in TB patients involves several factors such as poor absorption, pre-systemic and systemic clearance.

Besides our focus on low plasma concentration of RIF, our study also provides information on the developmental changes of p-gp with age, gender and ethnicity. P-glycoprotein is a major determinant of the pharmacokinetic, safety and efficacy of profiles of many drugs (58). As such, the information provided will be very useful in the development of drugs that are substrates of p-gp, such as HIV protease inhibitor saquinavir (59). Lastly, since the liver biopsies used in this study were from different regions of the liver, we investigated the regional expression of p-gp across the liver. Our data show the amount of p-gp is similar in different regions of the human livers and that collecting liver biopsies from any section of the liver for p-gp analysis is acceptable.

5.2 CONCLUSION

Each study aim sought to address a specific research question but overall, all the studies improved our understanding of why patients fail treatment and provide measures that could be used to improve treatment outcome of TB patients. First, our evaluation of the Genotype MTBDR*plus* assay showed that this assay can accurately distinguish RIF and INH drug-resistant from drug-susceptible *M. tuberculosis* isolates in Cameroonian TB patients and can be used diagnose MDR-TB in Cameroon. Secondly, we showed that the recommendation to assume resistant to RIF solely on the basis of absence of hybridization to one or more wildtype probe on the Genotype MTBDR*plus* assay strip allows accurate identification of clinically important RIF-resistant Mtb isolates. This recommendation leads to accurate and rapid diagnosis of RIF-resistant TB by the Genotype assay. Finally, we showed that the expression of P-glycoprotein in the liver does not vary extensively and probably does not account for the variation in the systemic clearance of RIF. Interestingly, the detection of high levels of expression of p-gp in the liver of children could provide a plausible explanation of why children, when given the same dose/kg of RIF similar as adults, have lower plasma concentrations of RIF.

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