University of Alberta

Effect of cardiometabolic syndrome on drug pharmacokinetics: obesity and hyperlipidemia

By

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This work is dedicated

to my great parents Ahmed and Lutfia
to my loving wife Enas, my two lovely sisters Amal and Essra

and to my wonderful brother Hatem

Thank you

Abstract

Cardiometabolic syndrome refers to a clustering of several risk factors for cardiovascular disease; obesity and hyperlipidemia are two underlying disorders associated with the syndrome. An often overlooked aspect of these conditions is the effect that they may have on the pharmacokinetics of drugs. In this thesis, the influence of obesity and hyperlipidemia on drug pharmacokinetic was explored using as test drugs azithromycin and cyclosporine. An LC-MS assay for azithromycin was developed and used in a pharmacokinetic study in obese patients. The results were comparable to literature data from lean subjects. Azithromycin bioavailability was reduced by one-third in gastric bypass subjects. Mean leptin and interleukin 6 levels were higher than previously reported for lean subjects. Although in rat hepatocytes lipoproteins had significant down-regulating effects on the mRNA levels of several genes, cyclosporine uptake was minimally affected. In conclusion, our findings could explain some variability in drug pharmacokinetic and unexpected dose versus effect outcomes in cardiometabolic syndrome that could contribute to both hyperlipidemia and obesity state.

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List of abbreviations and symbols

ABC	ATP-binding cassette
ABSORB	Absorption
ALT	Alanine transaminase
AM	Amidarone
ANOVA	Analysis of variance
AUC	Area under the concentration versus time curve
AZ	Azithromycin
β	Beta
BCS	The biopharmaceutical classification system
BCRP	Breast cancer resistance protein
BMI	Body mass index
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
Conc.	Concentration
CRP	C reactive proteins
CAP	Community acquired pneumonia
CHD	Coronary heart disease
CHOL	Cholesterol
CyA	Cyclosporine
CVD	Cardiovascular disease
CL	Clearance
cm	Centimetre
Cmax	Peak plasma drug concentration
Css	Steady state concentrations
CV	Coefficient of variation
CYP450	Cytochrome P450
dNTP	Deoxyribonucleotide triphosphate
DEA	Desethylamiodarone
DMEM	Dulbecco’s modified eagle's media
EGTA	Ethylene glycol tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Hepatic extraction ratio
ESI	Electron spray ionization
F	Oral bioavailability
FBS	Fetal bovine serum
Fu	Unbound fraction plasma drug unbound fraction
fg	Gastrointestinal availability
g	Gram
GFR	Rates of glomerular filtration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour
HCl	Hydrochloride acid

HDL	High density lipoproteins
HF	Halofantrine
HL	Hyperlipidemia
HPLC	High performance liquid chromatography
IL-6	Interleukin-6
IL-8	Interleukin-8
IP	Intraperitoneally
IS	Internal standard
I.V	Intravenously
KCL	Potassium chloride
KH2PO4	Potassium dihydrogen phosphate
Km	Affinity constant
Kg	Kilogram
L	Liter
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography -tandem mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LDL-r	Low density lipoprotein receptor
LLQ	Lower limit of quantification
Log p	Logarithm octanol/water partition coefficient
LP	Lipoprotein
MIC	Minimum inhibitory concentration
µg	Microgram
µL	Microliter
m	Meter
mL	Millilitre
mg	Miligram
µM	Micromolar
min	Minutes
MIC	Minimum inhibitory concentration
m/z	Mass-to-charge ratio
mM	Milimolar
$MgSO_4$	Magnesium sulfate
mRNA	Messenger RNA
CMS	Cardiometabolic syndrome
MDR	Multi drug resistant
MTT	3-(4,5- dimethylthiazol- 2-yl)-2,5- diphenyl tetrazolium bromide
Mrp	Multidrug resistance-associated protein
MS	Mass spectrometry
MSc	Master of Science
NR	Not reported
N	Normality
n	Number of samples

NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
ng	Nanogram
NL	Normal lipidemia
NR	Not Reported
O_2	Oxygen
OATP	Organic anion transport protein
OCT	Organic cationic transporter
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PLE	Pressurized liquid extraction
PK	Pharmacokinetics
P407	Poloxamer 407
Pgp	P-glycoprotein
TdP	Torsades de pointes or baseline
Ref	Reference
r^2	Correlation coefficient
rpm	Rotations per minute
RYGB	Roux-en-Y gastric bypass
R&D	Research and development
SIR	Selective ion chromatogram
SEM	Standard error of the *mean*
SD	Standard deviation
T2DM	Type 2 diabetes
TNFα	Tumour necrosis factor α
$T_{1/2}$	Terminal elimination phase half-life
TBME	Tertiary butyl methyl ether
TC	Total cholesterol
TG	Triglyceride
Tmax	Time of maximum concentration
TRL	Triglyceride-rich lipoproteins
USA	United states of America
UV	Ultraviolet
U	Units
V	Volt
V	Volume
Vd	Volume of distribution
VLDL	Very low density lipoprotein
VLDL-r	Very low density lipoprotein receptor
Vs.	Versus
V/V	Volume/volume
WBC	White blood cell
WHO	World Health Organization
^oC	Degree Celsius
%	Percentage
~	Approximately

1. Introduction

Cardiometabolic Syndrome

Cardiac metabolic syndrome (CMS), common in an estimated approximately 25% of the world's population [1-5], is defined by the clustering of several classic cardiovascular risk factors, such as type 2 diabetes (T2DM), elevated blood pressure, high triglycerides (TG) and low levels of high-density lipoprotein cholesterol (HDL). Recent estimates state a 23.7% CMS prevalence among adults in the United States (U.S.) [6], which will likely increase in the future, corresponding to the increase in the incidence of obesity and T2DM [7, 8].

Insulin resistance and abdominal obesity, the two underlying disorders of the syndrome, are thought to be the key physiological forces resulting in the adverse cardiovascular profile of patients with CMS [7]. Obesity is often associated with T2DM, hyperlipidemia and hypertension [9, 10]. Visceral obesity, which is characterized by accumulation of fat in and around the abdomen, is the main cause of metabolic abnormalities, and thus represents an important target in the treatment of metabolic syndrome. A strong mechanistic link exists between obesity and insulin resistance, and links with other aspects of the syndrome such as hyperlipidemia have been proposed. Patients with obesity and insulin resistance are generally resistant to the antilipolytic effects of insulin [11]. In addition, larger adipocytes and adipocytes present in visceral fat are more metabolically active and have a higher rate of lipolysis [12]. Also acceleration in hepatic synthesis of very-low-density lipoproteins (VLDL) in the CMS involves increased availability of free fatty acids derived from adipose tissue. However, a role for

hyperinsulinemia versus hepatic insulin resistance in driving hyperlipidemia is controversial because insulin may have dual effects, both enhancing lipogenesis and limiting Apolipoprotein B (apoB) secretion [11].

Individuals who are diagnosed with CMS have a significantly increased risk of developing complications of cardiovascular disease (CVD) and T2DM; additionally, non-diabetic subjects with CMS are markedly predisposed to the development of T2DM [13]. Diabetes mellitus, known to be one of the main risk factors for atherosclerosis [14], is associated with an increased cardiovascular morbidity and mortality [15, 16] as CVD is the leading cause of death in diabetic patients [17]. The hyperlipidemia seen in the metabolic syndrome is also commonly found in patients with T2DM. Recently, it has been reported that abnormalities in the lipoprotein profile such as mild hypertriglyceridemia can significantly increase the risk for myocardial infarction and stroke [18, 19], and low HDL cholesterol is among the major risk factors for CVD.

CMS patients are usually taking many medications for treatments. One of the most challenging aspects of the syndrome is understanding the cellular mechanisms that link the metabolic abnormalities with the pathophysiological effects that lead to disease symptoms. In this thesis we focus on the effect obesity and hyperlipidemia have on drug PK.

1.1. Obesity

Obesity is a chronic metabolic disease resulting from a chronic imbalance between energy intake and energy output. The prevalence of obesity (classified as a body mass index (BMI) of 30 kg/m^2) has dramatically increased in recent years and has reached epidemic proportions globally [20, 21]. Obesity is a well-recognized global health problem. In 2008, the World Health Organization (WHO) estimated that over 1.5 billion adults worldwide were overweight (BMI >25) and 500 million adults were obese (BMI >30), with an estimate that > 700 million people will be obese by 2015 [22, 23]. Not only have more and more adults become obese, but in Canada a review from 1981 to 2009 across all age groups from childhood to adulthood showed a significant increase in body weight [24, 25].

Obesity is often accompanied by excess fat storage in tissues other than adipose tissue, including liver and skeletal muscle, which may lead to local insulin resistance and may stimulate inflammation. In addition, obesity changes the morphology and composition of adipose tissue, leading to changes in protein production and secretion. Some of these secreted proteins include several proinflammatory mediators produced by macrophages in the adipose tissue. This excessive fat accumulation in adipose tissue, liver, and other organs strongly predisposes people to the development of metabolic changes that often accompany obesity, including hypertension, impaired glucose tolerance, insulin resistance leading to hyperinsulinemia, and dyslipidemia. Thus, there is an increase in overall morbidity risk [6, 26].

The interaction of multiple environmental (excessive caloric and food intake, social, economic, and insufficient physical activity) and genetic (family history of obesity, individual metabolism, and behavioral) factors can cause obesity [23]. Poor diets which include over ingestion of food that is rich in high caloric carbohydrates are contributing factors. Furthermore, lifestyles have changed, resulting in insufficient physical activity, which is the second major contributing factor for obesity. Worldwide there has been a large shift toward less physically demanding work, more use of public transportation, and changes in leisure activities requiring low to zero levels of physical activity (e.g. playing video games and television viewing) [26]. In addition, polymorphisms in various genes controlling appetite and metabolism may result in obesity.

Individuals with obesity (BMI > 30) have been reported to have higher mortality rates as compared to those who are not obese as obese subjects experience CVD at a higher rate than non-obese individuals [27-29]. Extreme obesity (defined as BMI > 40) is increasing at a greater rate than moderate obesity (classified as BMI between 30 to 40) [30]. Approximately 18 % of the Canadian population are obese [23, 31], and over 9 million people in the US alone suffer from extreme obesity [32]. The increased incidence of obesity represents a substantial risk to human health because it can predispose patients to a higher risk of metabolic disorders (HL and diabetes mellitus), CVD (hypertension, atherosclerosis, myocardial infarction and stroke), psychiatric disease, cancer, sleep apnea, osteoarthritis, respiratory diseases, cancer, non-alcoholic fatty liver disease, and myriad other health problems [6, 33-35].

Obese individuals typically require multidrug therapy for these various comorbidities. While many agents are available to treat these conditions, the current knowledge regarding their disposition in the obese remains limited. The physiological properties of this population differ from those of the non-obese population, including changes in regional blood flow and increases in cardiac output, fat mass, and lean mass. These pathophysiological changes can lead to alterations in the PK and pharmacodynamics (PD) of drugs [36].

1.1.1. Obesity management

The main treatment options for obesity consist of dieting and physical exercise [37]. Diet programs may produce weight loss over the short term, but maintaining this weight loss is frequently difficult and often requires exercise and a lower food energy diet [38-40]. One drug, orlistat (Xenical®) is approved for use, but there is little information on how this drug affects long-term complications of obesity, and it has a high rate of unwanted adverse effects [41]. The most effective treatment for obesity is bariatric surgery, reserved for patients who have failed dietary or medical weight loss methods or people with a BMI of 35 or higher with an obesity-related condition or for people with extreme obesity [42].

1.1.2. Bariatric surgery

In 2008, 220,000 bariatric surgeries were performed in the United States and Canada. More recently it was estimated that close to 350,000 bariatric operations were performed worldwide (63 % in USA and Canada) [43]. This rapid rise in the

conduct of these procedures is related to the rapidly increasing population of patients presenting with morbid obesity (defined as BMI > 40) [44]. Although this is a serious intervention, it is an effective way in which to alleviate many of the other serious comorbidities that these patients are afflicted with, notably the CMS comprising diabetes, hypertension and dyslipidemia [45, 46].

The surgical procedures used are typically either purely restrictive (gastric banding, gastroplasty), malabsorptive (biliopancreatic diversion, jejunoileal bypass), or combined restrictive and malabsorptive (gastric bypass) in nature [47]. The RYGB procedures are most common, accounting for more than 80 % of all bariatric surgical procedures [48]. Bariatric surgery usually results in sustained weight reduction (longer-term $\geq$ 5 year) with acceptable costs [43]. At 5 years post RYGB surgery, the mean average of the percent excess-weight loss was 75%. [49]. Patients experience improvements in weight-related comorbidities, quality of life, and mortality rates [42]. However, the reduced effective size of the stomach can place patients at a higher risk of adverse events associated with certain medications [50]. Such medications include non-steroidal anti-inflammatory drugs, salicylates, and oral bisphosphonates. Patients are also placed at an increased risk of nutrient deficiencies for essential dietary minerals (calcium, iron) and vitamins (fat-soluble vitamins). Vitamin and mineral supplements are therefore required after the surgery. The binding of vitamin B12 to intrinsic factor is reduced, and therefore vitamin B12 supplementation is also required to prevent anemia [42].

1.1.2.1. Roux-en-Y gastric bypass (RYGB)

Laparoscopic Roux-en-Y gastric bypass (RYGB), a procedure that circumvents the upper gut, is the most common bariatric procedure in USA and Canada (Figure 1) [51]. Indeed, RYGB surgery comprises about 80 % of bariatric surgeries performed by American surgeons [52] and it represents the gold standard in the U.S. for weight loss in morbid obesity. RYGB achieves not only significant, sustainable weight loss, but it has also been shown to resolve or improve other conditions associated with CMS [45, 46, 53].However, it results in malabsorption because the stomach capacity is reduced by 95 %: the proximal portion of the stomach is reattached to a more central part of the small intestine, bypassing the duodenum and 50-70 cm of the jejunum [54]. Since 2008 the use of gastric bypass in Europe has nearly quadrupled from 11.1 % to 39.0 %, while the use of gastric banding had decreased from 63.7% to 43.2% [51]. It has been documented to achieve up to 69 % long term weight loss [55, 56]. The laparoscopic RYGB approach is associated with a more rapid recovery, fewer pulmonary complications and wound infections, and less postoperative pain compared to open procedures [42].

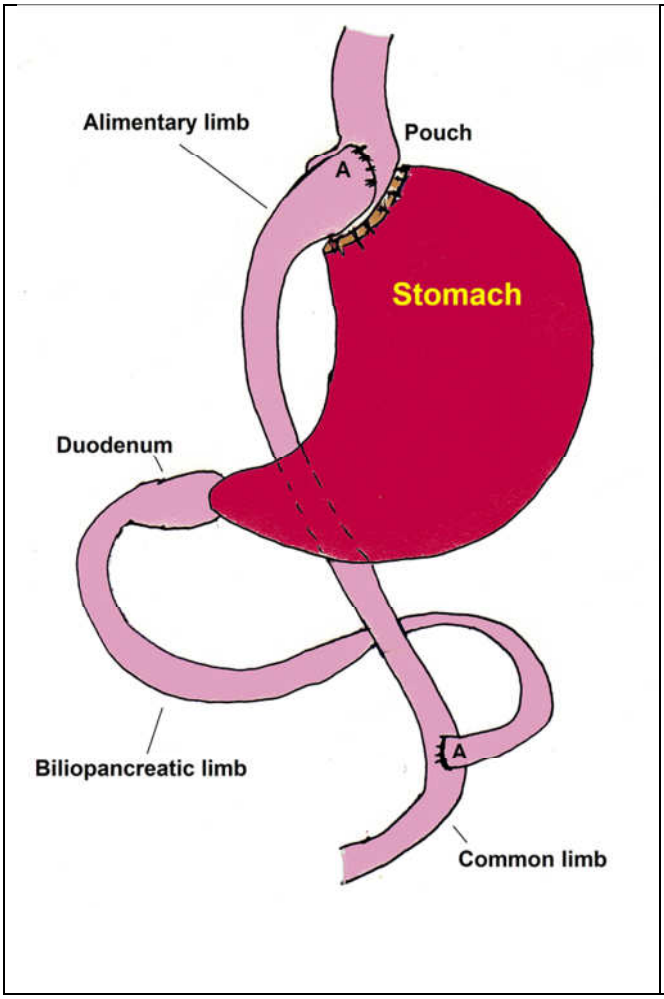


Figure 1 . Schematic diagram of Roux-en-Y gastric bypass. The proximal stomach is separated from the distal stomach using surgical staples to form a small, restrictive gastric pouch (20 to 30 mL capacity). The mid-jejunum is transected (site A) and the distal section is connected to the pouch. The length of intestine leading from the duodenum is reconnected at the jejunal-ileal region. The reconnected pouch forms an alimentary limb which bypasses immediate contact with biliopancreatic secretions; the outcome resembles a Y shape configuration (hence the name). The length of small intestine distal to the site at which the two limbs join is termed the common limb; this is where biliopancreatic secretions eventually mix with ingested food and most absorption occurs. The alimentary limb is typically 100 cm in length and the biliopancreatic limb is usually from 30 to 50 cm in length [43].

1.1.2.2. Effects of RYGB on drug disposition

RYGB surgery can clearly lead to direct alterations in gastrointestinal anatomy and physiology which can affect the absorption of not only nutrients (a basis for its therapeutic effectiveness), but also orally administered medications. An understanding of the effect of the surgery on the rate and extent of drug absorption and on bioavailability is just evolving. It is not surprising that there is not much information on this issue, given the relatively recent increase in the

prevalence of obesity and the associated increase in use of bariatric surgery for its treatment [57]. Currently there are no consensus guidelines for dosing of drugs to these patients, and there is some uncertainty regarding the prediction of how bariatric surgery may influence the PK of specific drugs [58]. One reason for uncertainty is that the outcomes are very drug-specific in nature, and furthermore, study design is an important criterion for assessing the data.

1.1.2.3. Summary of PK Studies after RYGB Surgery

Relatively few studies have focussed on the influence of bariatric surgery on PK. There are only 15 studies, involving 29 agents (Table 1). Of these, 5 were case reports evaluating 7 different drugs [59-63]. There were an additional 10 controlled studies evaluating 22 pharmacological agents and ethanol [42, 54, 64-72]. Solid dosage forms were used in all studies except one, in which ethanol was administered as an oral solution [72]. Sample sizes ranged from 1 to 32 patients. Pre-post designs were used in three studies. Comparisons of surgical cases with normal weight controls were performed in 7 studies and 2 studies used historical published data as the comparison group [67, 68]. Overall, the trend and evidence for altered rate or extent of absorption were found for 55 % of the studied drugs. The data did not show reduced absorption for 9/29 medications and ethanol (Table 1).

Evidence for an increase in magnitude of drug absorption was present for 3 medications [64, 66, 68]. Case reports or case series examining tamoxifen [59],

Table 1: Summary of studies in which the plasma/serum (unless indicated) pharmacokinetics (mean±SD, ranges in parentheses) of drugs were studied after RYGB surgery.

Drug	n	Control			Post RYGB Surgery		
		Cmax, mg/L	Tmax, h	AUC, mg·h/L	Cmax, mg/L	Tmax, h	AUC, mg·h/L
Atorvastatin [64]	12	0.028	1.6	0.075	0.013	1.8	0.05
Metformin [66]	32	1.8±0.61	3.0 (1.5-3.0)*	11.4±3.6	2.0±0.86	3.0 (1.5–3.0)*	13.7±6.0
Tacrolimus [67] (Blood)	6	0.023	2	0.26	0.016	1.3	0.071
Sirolimus [67] (Blood)	6	0.018±0.01	0.7±0.3	0.33±0.13	0.032±0.009	2.0±0.7	0.18±0.12
Moxifloxacin [68]	12	NR	NR	NR	3.38±1.41	1.75 (0.75–4.00)*	46.2±1.4
Sertraline [70]	10	0.048±0.01	3.4±1.1	0.31±0.12	↓0.019±0.007	3.9±0.9	↓0.12±0.05
Ethanol [72] (Blood)	24	577±112	0.5	910±170	↑741±211	↓0.16	935±213
Tamoxifen [59]	3	NR	NR	(0.003-0.021)	NR	NR	(0.0005-0.0021)
Phenobarbitone [62]	1	NR	NR	(1.25-3.41)	NR	NR	0.825
Phenytoin [62]	1	NR	NR	(0.41-0.83)	NR	NR	0.125

Arrows (↑ or ↓) indicate significant differences in the indicated direction from control subjects. * Median (range). NR= not reported.

temozolomide [60], nitrofurantoin, amoxicillin [61], phenobarbitone, phenytoin [62], and haloperidol [63]. Controlled studies evaluated atorvastatin [64], morphine[73], amoxicillin, acetaminophen, talinolol [74], tolbutamide,

omeprazole, oral midazolam and caffeine [75], metformin [66], tacrolimus, sirolimus and mycophenolic acid [67], moxifloxacin [68], levothyroxine [69], sertraline [42,70], venlafaxine, duloxetine, citalopram, escitalopram[42], cyclosporine A [71], ethanol [72].

After RYGB surgery, the bioavailability (assessed by AUC) of a few drugs was found to be reduced. These drugs (where known, general bioavailability in lean subjects in parentheses) include the antimicrobial drugs nitrofurantoin (87%) and amoxicillin (93%) [61, 65]; the immunosuppressive agents cyclosporine A (28-43%) [71], tacrolimus (25%), sirolimus (15%), and mycophenolic acid (94%) [4]; the replacement hormone levothyroxine (70%) [69]; and the β-adrenergic blocker, talinolol (55%) [74]. The circulating concentrations of the anticonvulsants phenytoin (90%) and phenobarbital (100%) [62], and the anticancer drug tamoxifen, were also reported to be lower after RYGB [59]. In some cases, no significant changes were observed in absorption parameters after RYGB. For example, no long lasting alterations were seen in the Cmax, tmax or AUC of the antidepressants of the selective serotonin reuptake and serotonin-norepinephrine reuptake inhibitory classes of drugs (sertraline, citalopram, escitalopram, venlafaxine and duloxetine,); the bioavailabilities of these drugs varies from less than 10% to over 80% in lean subjects. Each of these assessments was conducted in patients before and after surgery, with the PK being studied at different times (1, 6, and 12 months) after surgery [42, 70]. Although there was a transient decrease in oral absorption at 1 month in post RYGB individuals, there was a return back to the baseline at 6 and 12 months [42]. In some cases, inconsistent

findings were seen within the same study. For example, after RYGB it was found that in some patients atorvastatin bioavailability decreased by a factor of 3, whereas in others a doubling of AUC occurred. On average, the results showed no significant difference between pre and post RYGB surgery for this drug [64]. For other drugs, the observed changes fall in line with expectations. For example, highly lipophilic drugs such as cyclosporine and tacrolimus might rely on the presence of bile salts, and exposure to duodenal mucosa, for absorption. In bypassing a large absorptive area of proximal small intestine, and reducing direct exposure of drug to bile salts at this region, a combination of reduced solubility and loss of mucosa for optimal absorption could explain the observed decrease in bioavailability after RYGB. It is known that the duodenum is the primary site for tacrolimus absorption and for presystemic drug metabolism by CYP3A4/5[67, 76]. As in the case of metformin, one cannot exclude the possibility of changes in function or expression of membrane transporters, as they are involved in the clearance of each of these agents.

1.1.2.4. Physicochemical and physiological considerations

Disintegration is often the rate-limiting step for absorption of drug from solid oral formulations [57]. Gastric mixing, which is important in the disintegration process, is reduced by RYGB, as the truncated stomach only has a small volume (Figure 1) [43]. As such, a reduced rate of disintegration might be the case for certain formulations.

The Biopharmaceutical Classification System (BCS) allocates drugs according to their solubility and permeability characteristics. The small gastric pouch created after RYGB causes an increase in gastric pH due to its separation from other acid-producing cells in the more distal regions of the stomach [47]. Theoretically, increasing gastric pH should increase the solubility of weakly basic drugs, as a larger proportion would be in the unionized state, whereas the opposite would be expected for weak acids [77].

An invitro drug dissolution model of the gastrointestinal environment has shown that 10 of 22 psychiatric medications had a significantly lower dissolution in a simulated post-RYGB environment [78]. Drugs that depend upon an acidic environment for optimal dissolution (e.g. rifampin, digoxin and ketoconazole) seem more likely to be affected by the increased gastric pH post-RYGB [57, 79]. The rate of drug dissolution was more affected than the extent. However, this is an oversimplification of the in vivo situation, as for most drugs given orally the extent of absorption from the stomach is generally small due to its relatively small effective surface area. Rather, the small intestine is considered the major site of drug absorption owing to the presence of multiple levels of macroscopic and microscopic surface bends in the luminal surface introduced by the folds of Kerckring, villi and microvilli. These folds afford a substantial increase in absorptive surface and are most prevalent in the duodenum and proximal jejunum [80]. Thus, changes in gastric pH are less apt to cause changes in absorption than changes in intestinal pH.

Drugs with pH-dependent solubility like ketoconazole and limited water solubility are usually poorly absorbed, with the rate limiting step being drug dissolution. The solubility of highly lipophilic drugs is more likely to be influenced after RYGB surgery because there is a reduced direct interaction of the drug with bile acids. This interaction is critical for certain drugs, as the bile salts normally would enhance solubility by allowing for the formation of mixed micelles containing drug. There is evidence for the reduced absorption of the lipophilic drugs cyclosporine, phenytoin, thyroxin and tacrolimus after bypass surgery (Table 1).

The gastric emptying time after laparoscopic RYGB is variable [81], being short in some cases but longer in others. Although gastric emptying time may be altered after RYGB, this would not be expected to change the overall extent of drug absorption because most of the small intestine is still intact. Consequently, the area under the curve (AUC) levels would be expected to be mostly unchanged if the only change affecting a drug formulation is altered gastric residence time [47]. Small intestinal transit time can in particular influence the drug absorption of poorly soluble or extended release drug formulations [80]. Since the duodenum and the proximal jejunum are bypassed and passage through the intestine may be increased, leading to a decrease in transit time, it is conceivable that for poorly water soluble drugs and extended release formulations, inadequate transit time may be available for full dissolution and absorption of drug [80].

Gastric bypass procedures that markedly reduce mucosal exposure would seem to be most apt to cause changes in the oral absorption of drugs. In typical RYGB procedures, large parts of the stomach, the entire duodenum and a small portion of

the proximal jejunum are bypassed, thereby decreasing the surface area available for drug absorption [50]. Drugs that are known to be absorbed primarily in the proximal gut, and/or those that are intrinsically poorly absorbed, are most likely to be affected by the procedure.

Regarding the various surgical procedures, it is not clear if there is a difference in drug absorption between bypass of the proximal small intestine (gastric bypass) and the distal small intestine (jejunoileal bypass). Although the proximal small intestine has the largest overall surface area per unit length of the gastrointestinal tract, the intestinal transit time is slower in the longer distal small bowel [57].

1.1.3. Effect of obesity on drug pharmacokinetics

1.1.3.1. Absorption

Obesity is associated with a significant increase in subcutaneous fat, and the absorption of drugs administrated topically such as through the subcutaneous, transdermal, and intramuscular routes may be affected. To date only a few studies have characterized these extravascular routes of drug absorption in the obese population. Sanderink et al. assessed the subcutaneous absorption of a low-molecular-weight heparin, enoxaparin, in obese and non-obese volunteers [82]. They used anti-factor Xa and anti-factor IIa activity levels as surrogates for the PK of enoxaparin after once-daily subcutaneous administration and a single intravenous infusion. The rate of enoxaparin absorption after subcutaneous administration was slower in the obese volunteers. The median time to reach maximum activity level was1hr longer in obese subjects for both anti-factor Xa

and anti-factor IIa. However, the extent of absorption appeared to be complete in both groups. Similarly, using ^{125}I-labeled rapid-acting insulin in patients with T2DM, there was no effect of obesity on absorption rate from subcutaneous injection sites. Moreover, there was no correlation between the depth of the fat layer and the residual radioactivity measured at injection site [83]. The rate and the extent of drug oral absorption does not appear to significantly differ between obese and non-obese subjects for a number of drugs, including cyclosporine [84], dexfenfluramine [85], midazolam [86] and propranolol [87]. Obese subjects often have higher gastric emptying rate, higher cardiac output and an increased gut perfusion that could lead to an increase in bioavailability and rate of absorption [88], but limited data are available.

1.1.3.2. Distribution

Distribution to various tissues is dependent on the drug's physicochemical properties, as well as tissue makeup and quantity. In obesity the increase in adipose tissue is intuitively expected to cause changes in relative drug distribution in the body. Tissue perfusion may be reduced in obese individuals, and alterations in cardiac structure and function have been observed in the obese [89, 90]. These haemodynamic changes could potentially alter drug distribution and CL in obesity. The Vd, which reflects the ability of the drug to leave the blood and enter the tissues, is particularly susceptible to obesity. Protein binding is a critical determinant of Vd, with some of the major binding proteins being albumin, α_1-acid glycoprotein and lipoproteins. Obesity does not appear to have an impact on

drug binding to albumin. It has been reported that serum albumin is unaltered in both moderate and morbid obesity [22]. An understanding of how the Vd of a drug changes in obesity is of particular interest and critical because it is the principal parameter determining loading-dose selection. The ability of a lipophilic drug to penetrate into excess tissue stores of adipose is expectedly higher than a hydrophilic drug, which might increase the Vd. Distribution of many lipophilic psychotropic drugs, including benzodiazepines and tricyclic antidepressants, is increased in obese subjects [91, 92]. Lipoproteins, which can bind lipophilic drugs, are often increased in obese patients [93-95], and might also play a significant role in determining the Vd. Hyperlipidemia is often a comorbidity of obesity, and in such patients an increased level of binding to serum lipoproteins might limit the increase in Vd expected when only tissue penetrability for lipophilic drugs is considered. For hydrophilic drugs, the Vd based on total body mass would be expected to decrease because as a proportion of the total body mass, there is proportionately less tissue space available for the drug to penetrate. In general, Vd changes in obese subjects are not easily predictable based on the physiochemical properties of the individual drug. In addition to being drug specific, these changes are dependent on protein binding and the degree of obesity.

1.1.3.3. Metabolism

Pathophysiological changes associated with obesity, such as fatty liver infiltration, usually caused due to an increase in the amount of fat transferred to liver from

other parts of the body, can potentially alter drug metabolism and hepatic drug transport [22]. Depending on the extent to which the drug undergoes metabolism, the clearance in some cases is altered in obese patients [94, 96]. However, it is not altered in other subjects compared to normal weight subjects [91]. Obese patients are usually associated with an overall increase in liver size [97]. In morbidly obese persons, fatty liver infiltration resembles alcoholic hepatitis and may induce liver damage [98]. However, changes in function of liver enzymes are not routinely seen in obesity [93, 94]. In situations where fatty liver infiltration compromises hepatic function, concentrations of drug-binding plasma proteins may decrease, which might affect drug plasma protein binding and hence Vd and possibly CL of the total (bound+unbound) drug.

The hepatic cytochrome P450 (CYP) family of enzymes is important in the oxidative metabolism (phase I metabolism) of drugs. Hepatic metabolism by isozyme CYP2E1, which mediates the metabolism of fatty acids, ketones, and ethanol, plays a role in the development of non-alcoholic fatty liver disease. Chronic exposure to large amounts of these substrates can induce CYP2E1, leading to free radical formation, lipid peroxidation and consequently liver injury. Morbid obesity was found to increase CYP2E1 activity [22, 99]. Inhalational anesthetics (e.g. methoxyflurane, halothane, enflurane, sevoflurane, and halothane) that are substrates of CYP2E1 were found to have higher CL in obese individuals as compared to non-obese controls [100]. The effect of obesity on CYP-mediated metabolism appears to be isozyme-specific, with increases in

CYP2E1 and decreases in CYP3A4; its effects on other CYP isoenzymes are less clear [100]. With respect to phase II metabolism, obesity appears to preferentially increase the conjugative pathways of glucuronidation and sulfation. The clearance of oxazepam and lorazepam, each of which is excreted primarily as glucuronide conjugates, was enhanced in obese individuals compared to normal-weight individuals; when normalized to body weight, no difference was seen in clearance, however [101]. Acetaminophen, which has sulfation and glucuronidation as primary elimination pathways, was found to have higher clearance in obese volunteers than in the normal weight controls, although when corrected for body weight, no change was seen [102]. These studies raise an important point, in that outcomes of the evaluation may differ depending on how one chooses to report the PK data, be it normalized to body mass or not.

1.1.3.4. Excretion

Obesity may be most expected to affect renal CL by affecting either the rates of glomerular filtration (GFR) or tubular secretion. Tubular reabsorption, which is mostly dependent on urine pH and pKa of weak acids and bases, is expected to be less affected [93]. Henegar et al. reported a potential for altered renal excretion in obesity as a result of increased kidney weight, renal blood flow and GFR in an animal model of obesity [103]. Anastasio et al. reported evidence that GFR and perfusion of renal tissue appeared similar in obese and lean subjects, provided they were normotensive and did not have microalbuminuria [104]. In contrast, Janmahasatian et al. found that GFR was higher in obese subjects [105].

Regarding long-term effects of obesity and coexisting hypertensive status, Naumnik and Mysliwiec reported that visceral adipose tissue may physically compress the kidney and increase intra-renal pressure and tubular reabsorption[106]. Chronic obesity and hypertension could lead to renal injury which is manifested through continuous reduction in GFR, increase in arterial pressure and escalation of cardiovascular morbidity and mortality [93, 106]. GFR is normally estimated by calculating the creatinine clearance to predict drug elimination [94, 95]. Alterations in creatinine clearance have been observed in obese patients versus lean patients. Because serum creatinine is dependent on muscle mass, and in obesity the muscle mass to total body mass ratio decreases, in using the Cockroft-Gault equation to estimate creatinine clearance one must use instead of total weight the lean body mass or ideal body weight.

1.1.4. Obesity and Inflammation

A strong association exists between obesity and inflammation. The health risks of obesity are at least in part caused by the presence of a low-grade inflammatory state [107]. Obesity, specifically abdominal or visceral obesity, leads to high levels of most pro-inflammatory adipokines, such as leptin, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). In contrast, adiponectin, an anti-inflammatory adipokine, is decreased in obesity. The elevated inflammatory markers found in plasma of obese individuals appear to originate from adipose tissue [108]. There has been no attention paid to the impact that adipokines, or

their combination with cytokines, may have on altering other important aspects of drug disposition.

In addition, the biologic mechanisms by which bariatric surgery resolves the health risks associated with obesity (metabolic disorders) are not fully understood. One probable mechanism is the inflammatory hypothesis, which suggests that the health risks of obesity are substantially increased by the presence of a low-grade inflammatory state [107, 109]. Earlier work has shown variability in the response of inflammatory biomarkers after surgery, possibly resulting from differences in the type of weight loss treatment and the follow-up duration [110]. Although several studies have examined the effect of inflammatory markers after surgery, no studies have examined and measured the inflammatory markers longer than 1 year. During the early period, the biomarkers had either increased or showed no changes compared with baseline [111]. This was evident across a broad range of bariatric procedures, including RYGB.

1.1.5. Azithromycin (AZ)

Azithromycin is an azalide drug, a subclass derivative of macrolide antibiotics. It binds intracellularly to the 50s ribosomal subunit of bacterial cells and inhibits protein synthesis [112]. It differs from macrolide antibiotics in that it possesses two nitrogen atoms rather than one (Figure 2). This structure produces a dibasic molecule that has a superior therapeutic profile compared to older macrolides, including a broader spectrum of activity, better tolerability, slower clearance and a once daily dosing regimen [113].

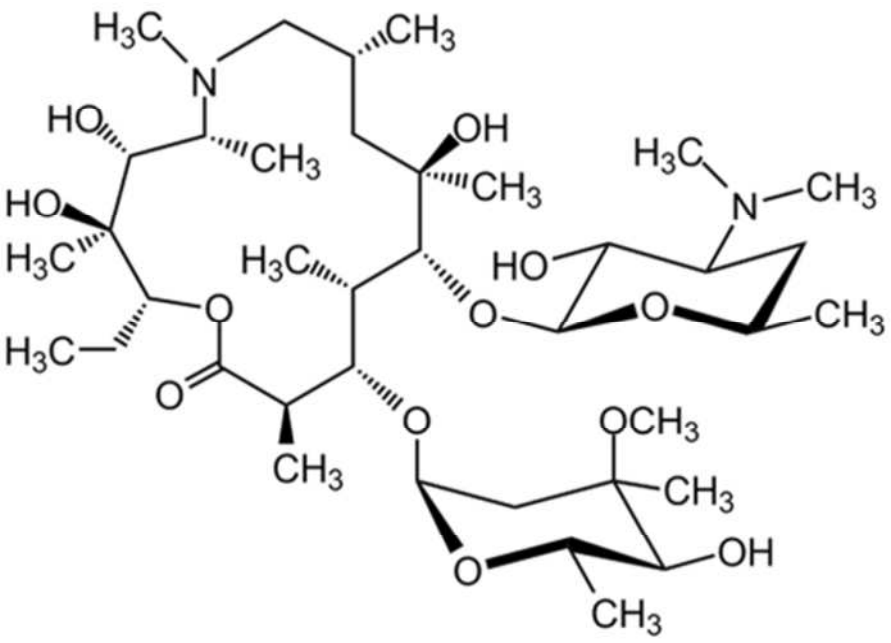


Figure 2. Chemical structure of azithromycin

1.1.5.1. AZ Pharmacokinetics and Pharmacodynamics

Typical pharmacokinetic parameters reported following a daily dose of AZ 500 mg oral include a Cmax of 0.40-0.45 mg/L, Tmax of 2.5-2.6 hours, low bioavailability of only 37 %, large volume of distribution of 23-31 L/kg, a long terminal phase half-life of up to 57 hours and $AUC_{0\text{-}24h}$ concentrations of 3.39 mg·L/h [112, 114, 115].

The drug is actively secreted into the biliary tract and is primarily eliminated fecally in unchanged form, with only 6% of an oral dose being eliminated through the kidney. AZ is also a known substrate for such membrane transporters as organic anion transporting polypeptides (OATPs), P-glycoprotein and multidrug

resistance-associated protein 2 (Mrp2), and these transporters play important roles in both AZ absorption and its biliary excretion [116, 117].

After oral administration, it is rapidly distributed to tissues, with concentrations significantly higher than those in plasma[118]. AZ is actively taken up by a wide variety of cells, including white blood cells (WBC) and fibroblasts, a pattern different from that of the classic macrolide agents [113, 119]. Tissue concentrations are often much higher than plasma levels. Particularly high drug concentrations are found in phagocytes (a 79:1 intracellular to extracellular ratio), and this is thought to facilitate AZ transport to inflamed and infected tissue [112, 120]. Drug accumulation is also high in the lungs, tonsils, and urological and gynecological organs and, thus, the drug is ideally suited to treat infections at these sites [115].

Community-acquired pneumonia (CAP) together with influenza, is the sixth leading cause of death in the United States, with an estimated four million cases occurring annually and 600,000 hospital admissions [65, 121]. For outpatients in North America, AZ is commonly prescribed as a first line mono-therapeutic agent for milder cases of CAP [112, 114, 122]. It is also used as combination therapy with a β-lactam antibiotic in hospitalized settings [122]. Studies have shown a link between obesity and infectious diseases such as pneumonia. Obesity may influence either the risk of getting an infection or the outcome of an infection once it is established [123, 124]. At the same time, more people worldwide are

putting on unhealthy amounts of weight, with more than 700 million globally expected to be clinically obese by 2015 [125]. No information is present in the literature about AZ PK in obese populations and no studies have examined the absorption of AZ post-gastric bypass.

Obesity can increase hospitalization [123, 124, 126, 127]. Indeed, 46–54% of hospital patients are overweight and 32% are obese with BMI > 30 [128]. A clear understanding of the effect disease states have on drug disposition can help to explain variability in response to drug therapy. A thorough understanding of the influence of obesity on antimicrobial drug dosing can be crucial to achievement of maximum safety and effectiveness [36, 121]. Obese subjects are often excluded from clinical trials during the drug development process and as a result information regarding the impact of obesity on the PK and PD of the majority of drugs is limited [36]. More studies including this population are required due to its high prevalence and because the obese commonly require medications for a wide range of comorbidities. Early AZ dosing in CAP patients is vital because mortality is lower when such patients receive antimicrobials within 4-8 hours of assessment in the hospital [129]. In addition, rapid initiation of therapy in adequate doses is essential for achievement of therapeutic success. The first day of therapy is a critically important period in determining success or failure of treatment of CAP [65].

The efficacy of AZ is well correlated with the area under the plasma concentration vs. time curve (AUC) to minimum inhibitory concentration (MIC) ratio [118]. Antibacterial action is optimized when this ratio is maximized, and increases with the initial dose ingested [118, 130]. The AZ MIC for streptococcus pneumonia, the commonest cause of CAP, is ≤ 2 mg/L [115]. The drug is most commonly administered at a dose of 500 mg once daily, for a period of between 3 to 5 days. Trough values are typically above 25 ng/mL after usual clinical doses [130-133]. Thus, a sensitive analytical assay is needed in order to determine AZ concentrations in plasma.

1.2. Hyperlipidemia

Hyperlipidemia (HL) is identified as a major risk factor of cardiovascular diseases, specifically atherosclerosis, and is associated with increased risk ofT2DM and a greater risk of coronary heart disease (CHD) for a given level of low-density lipoprotein (LDL) cholesterol [6, 33-35]. There is strong correlation between dyslipidemia and abdominal obesity [6]. HL has been shown to change the PK of drugs that are bound to plasma lipoproteins. It can limit their uptake into the cells, thereby decreasing their metabolism. It usually decreases the unbound fraction (fu) of drugs through increasing their lipoprotein binding, and it is expected to decrease the metabolism and hepatic CL of drugs with moderate and low hepatic extraction ratios (E) [134-136]. In general, it has been observed that hyperlipoproteinemia leads to a decrease in the fraction of the drug unbound in plasma, and in the clearance (CL) and volume of distribution (Vd) of the

bound+unbound drug [136-143]. Therefore, in HL patients, depending on the extent of hepatic extraction, a decrease in the plasma drug fu could potentially lead to an increase in plasma concentrations and/or decrease in tissue concentrations of drug, and therefore their pharmacological (therapeutic and toxic) activities.

The liver is the major organ involved in the metabolism of endogenous compounds and xenobiotic. The rate of drug biotransformation is dependent on several factors including the drug concentration and availability at site of metabolism, the levels of enzymes, availability of cofactors and drug protein binding. It is known that only free drug is available for metabolism. Liver, where most drug metabolizing enzymes are expressed, is considered as a terminal site of catabolism for lipoproteins containing apoB (CM, LDL and VLDL) through their recognition through LDL receptors [144, 145]. HL, like many other diseases, can potentially affect the expression of drug metabolizing enzymes and transporter proteins in the liver and other tissues. Lipoproteins can reduce the metabolism of drugs through reduction and inhibition of CYP enzymes or through decreased fu, which in turn results into decreased load of drug into tissues. As a result, metabolism of drugs in the presence of lipoproteins would be reduced. For highly metabolized drugs with lower estimates of hepatic extraction ratio, this would be expected to cause no significant changes in the amount of the drug present in tissues, or in the clearance of the unbound drug. When assayed for drug concentrations in individual tissues, however, this has not been the case. Drug concentrations have been seen, in a tissue-specific manner, to either be the same,

higher or lower than in normolipidemic (NL) animals [146-153]. An understanding of a disease state like the HL condition and of lipoprotein's effects on the blood levels of drugs, tissue uptake and drug metabolism can help to explain variability between individuals in their response to drug therapy.

1.2.1. Lipoproteins

HL is associated with chronic intrinsically elevated lipoprotein levels [135, 136, 154, 155]. Lipoproteins can be classified based on their density and content of lipid protein into five groups going from (lowest to highest density); very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), high-density lipoproteins (HDL), and chylomicrons (CM), [137, 156, 157]. They are produced entirely by the liver except for CM which is primary synthesized by the enterocytes[134]. The LDL receptor (LDL-R) family is composed of more than ten receptors [158], which are extensively expressed by the liver and adrenals [156, 158]; they mainly bind to Apolipoprotein B and Apolipoprotein E containing lipoproteins including VLDL, IDL and LDL [158, 159]. The major role of the LDL-R is to regulate plasma cholesterol (CHOL) by mediating uptake and catabolism of plasma LDL, the major carrier of plasma CHOL [144]. Lipoprotein receptor-mediated transport of the bound drug fraction into the liver counteracts such an effect by making more drugs available to the liver metabolizing enzymes [148, 160].

1.2.2. Lipoprotein-bound drugs

For the highly lipoprotein-bound drugs amidarone (AM), halofantrine (HF) and cyclosporine A (CyA), in the liver of HL, concentrations were found to be significantly higher than in NL controls [146, 148, 149]. In addition, the HL state increases the drug binding to the lipoprotein fraction [134, 161]. Lipophilic drugs are known to associate with circulating lipoproteins in the plasma [136, 148, 162]. In HL, most of the drug is bound to the VLDL followed by the LDL then the HDL fraction [161]. CyA was mostly found in the HDL fraction in NL patients but in the VLDL/LDL fraction in HL patients; its percentage in the VLDL/LDL fraction moved from 32 % in NL patients to 46, 54 and 55 % in hypercholesterolemia, hypertriglyceridemia and mixed HL patients, respectively [163]. In rat plasma CyA was mostly found in the LPDP fraction in NL rats but shifted to be mostly found in the VLDL fraction in P407 HL rats. In the HL state CyA concentrations increased by 5.3 and 2.2 fold in the VLDL and HDL fractions, respectively. However, more CyA was found to be associated with LDL particles, with a 19 fold increase in concentration. In HL human and rat plasma, AM was found to shift from the LPDP fraction to the VLDL, LDL and HDL fractions, with LPDP AM concentrations decreased by 3.5- and 55- fold in human and rat plasma, respectively [161]. The HF (+)-enantiomer was found mostly in the LPDP and HDL fractions in NL rat plasma while the (-)-enantiomer was mostly found in the LPDP fraction [134]. In P407 HL rat plasma, both (+) and (-)-enantiomers were mostly found in the VLDL fraction [134]. Thus, the contribution of the lipoprotein receptors in the transport of lipoprotein-bound

drugs into liver is clinically relevant and can possibly explain why liver concentrations for these drugs are high in HL state. However, the binding affinity of the drug to the lipoprotein fraction, the type of lipoprotein fraction the drug is bound to and the expression, regulation and type of LDL-R in tissues are all factors affecting such transport.

CyA is an immunosuppressive drug, extensively metabolized by the liver, and because it possesses a low hepatic extraction ratio, its clearance is expected to be directly related to the plasma fu. The available data for CyA, however, are not consistent, and in some cases directly in conflict with the outcome expected based on the commonly accepted belief that plasma protein binding restricts the cellular access to drug. Factors that increase plasma lipoprotein concentrations, such as ingestion of a high fat meal, or familial hyperlipoproteinemia, might be expected to give rise to reduced fu in blood or plasma and reduced CL, and higher blood or plasma concentrations. For example, in transplant patients with higher plasma CHOL or TG levels, CyA showed a decrease in plasma fu [8] and associated decreases in Vd and/or CL of the drug [9–13]. Similarly, CyA Vd was 3.4 fold lower in the Poloxamer (P407) induced HL rat model [135, 142, 154] than in the NL state. In contrast, in healthy volunteers, high fat content meals cause decreases in blood and plasma CyA concentrations compared to those attained in the fasted state [14]. CyA concentrations in heart and spleen decreased whereas, kidney, plasma, blood and liver showed higher levels in HL rats after a single iv dose [146]. Indeed, increased concentrations of drug in organs such as liver could be

due to a combination of increased lipoprotein-containing drug particles, an increase in drug uptake mediated by transporter proteins, or to a decrease in drug metabolism. It has been reported that CyA binds more with LDL particles in HL rat plasma. The binding of drug to the plasma lipoproteins facilitates uptake of LDL-bound CyA by hepatic and extra-hepatic LDL receptors, perhaps explaining these findings. The increased transport of CyA could be associated with lipoproteins across cell membranes through the action of lipoprotein receptors. Thus, the effect of LDL-R mediated transport for this drug is more important and outweighs the decrease in fu and the down regulation of the enzymes, leading to a net increase in metabolism and CL of the drug in HL state. Furthermore, lipoprotein receptors in turn may be up or down-regulated by HL.

AM is a highly lipophilic class III antiarrhythmic drug used in the treatment of life-threatening ventricular and supraventricular arrhythmias[164]. A moderately hepatically extracted drug, it showed 23-fold lower Vd in the Poloxamer 407 (P407)-induced HL rat model, [135, 142, 154] and significantly higher plasma, heart, spleen, and liver concentrations and lower lung, kidney, and brain concentrations in rat post iv AM dosing [160]. AM coadministered with high fat meals caused greater increases in AM concentration compared to its active metabolite desethylamiodarone (DEA) in healthy subjects[165]. Similarly, racemic HF was also shown to exhibit stereoselective decreases in Vd of its enantiomers in HL animals [136, 166]. The (+)-HF enantiomer increased in plasma, liver, lung and spleen and decreased in heart of HL rat after a single iv

dose of HF racemate [148]; however, the (-)-enantiomer concentration increased in plasma, lung, and spleen and decreased in brain and kidney in the same animal model [148].

In the HL section, we will describe our purpose to initially examine the influence of HL and the role of LDLr on CyA uptake by using freshly isolated rat hepatocytes. The primary cultured hepatocyte model is a useful tool with which to investigate drug uptake and metabolism, changes in drug metabolizing enzymes and transporter protein expressions, and the screening of cytotoxic and genotoxic compounds [167]. We used the antihyperlipidemic drugs fenofibrate (FF) and atorvastatin (Atv) (5μM) as probes. These two drugs are thought to increase the expression of LDLr, which then are hypothesized to increase CyA uptake into the cells when bound to LDL particles in serum. In addition, further investigations have been done on the effect of serum lipoprotein on the hepatic mRNA expression levels of some relevant transporter proteins and metabolizing enzymes involved in the hepatic uptake and metabolism of the clinically important lipoprotein-bound drugs CyA and AM in the HL state using primary rat hepatocytes.

1.3. Rationale

One of the most challenging aspects of CMS is understanding the cellular mechanisms that link the metabolic abnormalities with the pathophysiological effects that lead to disease symptoms. Mechanistically strong links between obesity and insulin resistance, diabetes, and other aspects of CMS such as, dyslipidemia, have been proposed. Visceral obesity, which is characterized by excess fat storage in and around the abdomen, is the prime cause of the metabolic abnormalities, and therefore represents an important target in the treatment of CMS. Unfortunately, obese subjects are often excluded from clinical trials during the drug development process and, as a result, information regarding the impact of obesity on the PK and PD of the majority of drugs remains limited. A clear understanding of the effect of disease states on drug disposition can help to explain variability in response to drug therapy.

Bariatric surgery is recommended and considered the most suitable treatment option for morbidly obese patients in whom other dietary or medical weight loss modalities have failed [42]. Bariatric surgery achieves not only significant, sustainable weight loss, but has also been shown to induce resolution or improvement in CVD risk factors, including diabetes, hypertension and dyslipidemia [45, 46]. The RYGB procedures are most common used, accounting for more than 80 % of all bariatric surgical procedures [48]. Currently there are no consensus guidelines for dosing of drugs to these patients, and there is some uncertainty regarding the prediction of how bariatric surgery may influence the PK of specific drugs [58]. In RYGB bypassing the stomach and much of the small

intestine could lead to changes in drug PK. Recent studies have shown contrasting results, with only 17 medications plus ethanol studied. A strong association exists between obesity and inflammatory cytokines [107]. Obesity, specifically abdominal or visceral obesity, leads to high levels of most pro-inflammatory biomarkers. Previous studies have followed the changes in proinflammatory cytokines, but only up to one year after RYGB.

AZ is an azalide antibiotic commonly prescribed as a first line monotherapy agent for milder CAP [112, 122]. No information is present in the literature about AZ PK in the obese population and post RYGB surgery. AZ dosing can be crucial to the achievement of maximum safety and effectiveness. Thus, if the antibiotic is not optimally absorbed, the ramifications of treatment failure for infections such as CAP could be severe and may pose a significant risk in terms of respiratory complications or death.

There is a strong correlation between dyslipidemia and abdominal obesity [6]. HL has been identified as a major risk factor of CVD [6, 33-35]. HL is associated with chronic intrinsically elevated LP levels and increases in drug binding to the LP fraction [134-136,154,155, 161]. For drugs that bind to lower density LP, this could mean an increase in the drug unbound fraction, with an increase in Vd and possibly an increase in CL. For the highly LP-bound drugs AM, HF and CyA, concentrations in the liver of HL subjects were found to be significantly higher than in NL controls [146,148, 149]. The contribution of the LP receptors to the transport of LP-bound drugs into liver is clinically relevant and can possibly explain why liver concentrations for these drugs are high in the HL state. Results

are conflicting and unclear, but the binding affinity of the drug to the LP fraction, the type of LP fraction the drug is bound to and the expression, regulation and type of LDL-R in tissues are all factors affecting such transport. HL can also modify drug effects by altering the tissue uptake of drugs facilitated by LP receptors and/or other drug transport proteins or metabolizing enzymes. There are limited data in literature regarding on the effect of HL on hepatic mRNA expressions.

1.4. Hypotheses

1. An alternative simple, selective and rapid LC-MS assay for AZ can be developed with properties of high sensitivity and specificity with suitability for assaying the drug in human plasma samples.
2. AZ absorption will be altered and increased in obese subjects compared to lean subjects.
3. Cumulative 24-hour AZ plasma concentrations will be reduced following RYGB surgery.
4. Pro-inflammatory cytokines (leptin and IL-6) will be higher in obese patients and will return to normal level 25 months post RYGB surgery
5. Hyperlipidemia will down regulate the hepatic protein mRNA expression of drug metabolizing enzymes and membrane transporting proteins
6. CyA hepatic uptake will increase when bound to LDL particles in serum mediated by LDLr.

1.5. Objectives

To address our hypotheses, the specific aims of this project:

1. To develop a simple, selective, rapid and valid LC-MS method for the determination of AZ in human plasma using a liquid-liquid extraction technique and C18 column.
2. Using the LC-MS method to determine whether AZ PK parameters are altered in obese volunteers compared to healthy lean subjects.
3. Using the LC-MS method to determine whether gastric bypass (RYGB) results in clinically significant reductions in AZ absorption.
4. To examine the effect of weight loss and changes in biomarkers of inflammation (leptin and IL-6) 25 after months of RYGB and in morbidly matched obese individuals
5. To assess the effect of serum LPs on the gene expression of hepatic metabolizing enzymes (CYP3A2, CYP2C11, CYP2D1 and CYP1A1), LP receptors (LDLr, VLDLr), and transporter proteins (OATP2B1and MDR1A) using primary cultured rat hepatocytes.
6. To assess the role of LDLr on CyA hepatic uptake using primary cultured rat hepatocytes.

2. Experimental

2.1. Materials

AZ (> 95%; MW =749) and imipramine HCl (> 97%; MW=280.4) were purchased from Sigma Aldrich (St. Louis, MO, USA). Diethyl ether (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methanol, water, ammonium acetate and ammonium hydroxide (all HPLC grade) were purchased from Caledon Laboratories Ltd (Georgetown, ON, Canada).). Enzyme linked immune sorbent assay kits (high sensitivity for leptin and IL-6) were from R&D Systems (Minneapolis, MN).

Rat normolipidemic (NL) serum was purchased from Innovative Research (Novi, MI, USA). AM HCl, poloxamer 407, heat inactivated new born fetal calf serum, fetal bovine serum, recombinant human insulin, HEPES sodium salt, collagenase, trypsin inhibitor, percoll and collagen from rat tail, and trypan blue solution (0.4%) were obtained from Sigma Aldrich (St.Louis, Mo, USA). Heparin sodium injection, 1000 and 10,000 U/mL, was purchased from Leo Pharma, Thornhill, ON, Canada), and dexamethasone sodium phosphate injection, USP from Sabex (Boucherville, QC, Canada). Normal saline sodium chloride (9 mg/mL) was obtained from Hospira Healthcare Corporation (Montreal, QC, Canada). Isoflurane USP was purchased from Halocarbon Products Corporation (River Edge, NJ). 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide (MTT) was purchased from Sigma Aldrich (St.Louis, Mo, USA). For assay of lactate dehydrogenase from cells, a kit was used (Cytotoxic-ONE™, Promega, and Madison WI). Calcium chloride and magnesium sulphate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Penicillin-streptomycin, dexamethasone

phosphate, Dulbecco's Modified Eagle Medium (DMEM) and RIzol reagent was purchased from Invitrogen Corporation (Carlsbad, CA, USA). High capacity complementary DNA (cDNA) Reverse Transcription Kits and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA, USA). SYBR Green Super Mix was purchased from Applied Biosystems (Warrington, UK). Real time PCR primers were synthesized and supplied by Integrated DNA Technologies, Inc. (Coralville, IA, USA) based on previously published sequences in the literature (Table 2).

2.2. Methods

2.2.1. Development of analytical method for AZ using Liquid Chromatography-Mass Spectrometry (LC-MS)

2.2.1.1. Chromatographic Conditions

The LC-MS analyses were performed using a single quadrupole system comprising a Waters Micromass ZQ™ 4000 spectrometer equipped with electron spray ionization (ESI) source coupled to a Waters 2795 pump and autosampler (Milford, MA, USA). The instrument was operated in positive ion and selected ion recording (SIR) acquisition modes. The ions utilized for quantification of AZ were m/z 749.6 $(M+H)^+$ and the major fragment, m/z 591.4. For the internal standard (IS), imipramine, 281.1 m/z was used for quantitation. Chromatographic separation of AZ and IS was performed using a Kinetex XB-C18 (Phenomenex, Torrance, CA, USA) column (2.6 μm particle size, dimensions 2.1×50 mm) at

room temperature. The mobile phase consisted of a 75:25 (v/v) mixture of methanol: 0.2 % ammonium hydroxide and 0.1 % ammonium acetate in water, pumped isocratically at 0.2 mL/min. The other parameter settings were gas source temperature of 150°C, capillary voltage of 3.6 kV and cone voltages set at 30 and 20 V for the drug and IS, respectively. The injection volume was 10 μL and gas flow of desolvation and cone gas flow were set at 550 and 110 L/h, respectively. The desolvation temperature was 275°C.

2.2.1.2. Stock Solutions

A stock solution of 1 mg/mL AZ was prepared in methanol. The IS was also prepared as a stock solution of 0.1 mg/mL in methanol. Various working concentrations of 0.1, 1, 10 and 100 μg/mL were prepared for generation of standard curves. An IS working solution of 1 μg/mL was similarly prepared by dilution of the stock solution with methanol. The stock solutions were stored at –20ºC.

2.2.1.3. Extraction Procedure

AZ was extracted from heparinized human plasma using a one-step liquid–liquid extraction step. A 50 μL volume of IS working solution was added to 0.5 mL of plasma. The samples were alkalinized with 20 μL of 10 M NH_4OH. The analytes were extracted by adding 4 mL diethyl ether. The mixture was vortex mixed for 60 s and then centrifuged at 3000 g for 10 min. Each supernatant layer was transferred into a clean glass tube and then evaporated to dryness *in vacuo*

unheated. The residues were then reconstituted with 130 μL of mobile phase. A volume of 10 μL was injected into the LC-MS system.

2.2.1.4. Solvent Extraction Recovery

Recoveries were determined with AZ concentrations of 50 and 500 ng/mL, using six replicates for each concentration. The extraction efficiency was determined by comparing the extracted peak heights of analyte in samples to the peak heights of the same amounts of analyte directly injected into the instrument without extraction.

2.2.1.5. Calibration, Accuracy and Validation

Using appropriate dilutions of the working solutions with methanol, samples for calibration curves were freshly prepared to provide AZ concentrations of 10, 50, 100, 250, 500 and 1000 ng/mL; each tube also contained IS as described above under extraction procedure. For constructing the curves, data were weighted by a factor of concentration^{-1}.

For validation assessment, concentrations were selected at 10, 50, 100, 500 ng/mL. Three days of assessment were undertaken with replicates of six samples per selected concentration. From this, estimates of interday accuracy and precision were made. For each daily run, a set of calibration samples separate from the validation samples were prepared to permit quantification of the peak height ratios of AZ in the validation samples. Precision and accuracy were

assessed using the coefficient of variation (CV %) and percentage error, respectively.

2.2.1.6. Assessment of Matrix Effect

To determine the effect of plasma on the process of ionization of the analytes the post-extraction spike method proposed by Matuszewski et al. (Matuszewski, 2006; Matuszewski et al., 2003) was utilized. Briefly, analyte-free plasma (0.5 mL per tube) or HPLC water was extracted using the procedure described above. The supernatant residues were transferred to clean tubes and dried as described above. Thereafter, to each dried and extracted residue was added sufficient analyte from stock solution to provide AZ concentrations of 10, 50, 100 or 500 ng/mL, or IS (1000 ng/mL) in 130 µL of total volume per tube. A total of 6 replicates per concentration of AZ, or IS, were used for each of the plasma and water extracts. The samples were injected into the LC-MS and responses of peak height m/z recorded at the retention times reflective of each analyte (AZ or IS). The responses of the analytes in the plasma-containing extracts were compared with those of the matrix samples extracted from water.

2.2.2. Applicability of the Assay

To demonstrate applicability, the method was used to determine AZ concentration in plasma samples from 14 obese women (mean age 44 years and BMI of 36.4), after oral administration of 500 mg AZ as a tablet. These subjects were matched to a treatment group that received RYGB surgery. Blood AZ levels were sampled

for up to 24 hours after a single dose. The study was performed in the University of Alberta Hospital Clinical Investigation Unit in collaboration with other investigators, led by Dr. Raj Padwal. It was approved by the University of Alberta Health Research Ethics office, with informed written consent requested from patients. After dosing, the blood samples were centrifuged for 10 min and plasma separated and frozen at -70 $^{\circ}$C until assayed.

2.2.3. ELISA Assay

Fasting plasma samples (pre-dose samples) were assayed from the 28 patients enrolled in the AZ ABSORB study (14 obese volunteers and 14 patients who underwent the RYGB surgery; see the method, applicability of assay section) [14]. Fasting plasma concentrations of leptin and IL-6 were determined using enzyme linked immunosorbent assay kits (high sensitivity for IL-6) from R&D Systems (Minneapolis, MN). All samples were measured in duplicate, and the average of the 2 values was used for data analyses. Samples with values greater than the maximal detection limit were diluted and reanalyzed. The assay employs the quantitative sandwich enzyme immunoassay technique. Monoclonal antibodies specific for leptin and IL-6 have been precoated onto a micro plate. Standards and samples are pipetted into the wells and any leptin or IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for leptin or IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substance solution is added to the wells and color develops in

proportion to the amount of leptin or IL-6 bound in the initial step. The color development is stopped and the intensity of the color is measured using a microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 570 nm.

2.2.4. Determine the effect of rat serum on hepatic mRNA expression using primary rat hepatocytes *in vitro*

2.2.4.1. Animals and Ethics

The protocols involving animals were approved by the University of Alberta Health Sciences Animal care and use Committee (ACUC). Sprague-Dawley rats (Charles River, QC, Canada) were used for the studies, with body weight ranging from 250-350 g. The animals were housed in temperature controlled rooms with 12 h light per day and were fed standard rodent chow containing 4.5% fat (Lab Diet® 5001, PMI nutrition LLC, Brentwood, USA). Free access to food and water was permitted prior to experimentation.

2.2.4.2. Collection of NL and HL serum

Rats were administered either single intraperitoneal doses of saline (NL rats) or 1 g/kg P407 (HL rats) in saline (0.13 g/ml) [168, 169]. This dose of P407 is known to lead to major increases (up to 40 to 90-fold higher) in triglyceride and CHOL plasma concentrations [136,148, 161]. The rats were allowed free access to water and food for 36 h. Then, while the rats were under anesthesia with isoflurane, blood was collected from NL and HL rats by cardiac puncture followed by

collection in unheparinized glass test tubes. Immediately, the blood was kept at 4^{o}C for 30 min to allow for blood cells to clot and then centrifuged for 10 min at 2500 g and the supernatant serum layer was collected and stored at -20°C until used.

2.2.4.3. Hepatocytes

2.2.4.3.1. Hepatocyte Medium preparation

DME medium with additives was prepared by adding 10% fetal bovine serum, 1% penicillin/streptomycin antibiotic (10 µg/ml), 1 µM insulin, and dexamethasone (50ng/ml) to 500 mL medium. The pH was adjusted to 7.4 using 10 N HCl or 10 N NaOH before adding insulin and penicillin/streptomycin antibiotic and DME medium was filtered through bottle top filters after which fetal calf serum was added.

2.2.4.3.2. Preparation of perfusion solutions

Perfusion solutions for the isolation of hepatocytes were prepared as previously described [170, 171]. Briefly, stock solutions of A_1 (prepared with 115 mM sodium chloride, 5 mM potassium chloride and 1 mM potassium dihydrogen phosphate in 500 mL of water), A_2 (25 mM Hepes sodium salt dissolved in 100 mL of deionized water), A_3 (0.5 mM ethylene glycol tetra acetic acid (EGTA) dissolved in 50 mL of deionized water plus 1 crystal of NaOH to dissolve EGTA), 1 mM calcium chloride solution and 1.2 mM magnesium sulphate solution, were prepared one day before the experiment. On the day of experiment, the fresh

working solutions A*, A, B and C were prepared with appropriate proportions of stock solutions as follows: Solution A* was prepared by mixing 100 mL of solution A1, 100 mL of solution A2, 0.8 g of glucose and 0.8 mL of heparin in a final volume made up with 500 mL of autoclaved water. Solution A was prepared by mixing 250 mL of solution A* and 50 mL of solution A3 and completed to 400 mL with autoclaved water. Solution B was prepared by mixing 203 mL of solution A*, 325 µL of 1 mM calcium chloride, 162.5 µL of trypsin inhibitor and 162.5 mg of collagenase (collagenase was added just before perfusion) and completed to 325 mL with autoclaved water. Solution C consisted of 25 mL of solution B supplemented with 100 µL of 1.2 mM magnesium sulphate and 75 mL of DME medium. All the solutions were adjusted to pH 7.4 (by using 10 N HC1 or 10 N NaOH) and filtered through a 22 µm membrane prior to use.

2.2.4.3.3. Isolation of rat hepatocytes

Isolation and preparation of the hepatocytes was accomplished using a two-step liver perfusion method described previously [171]. Briefly, on the day of experiment, a midline laparatomy was performed under isoflurane anaesthesia for the cannulation of the portal vein and the suprahepatic inferior vena cava. During the course of experiment, all tubing and solutions were maintained at 37°C and saturated with 95 % O_2. Following the cannulation, solution A was perfused through the portal vein into the liver at a flow rate of 35 mL/min for 8-10 min using a peristaltic pump till all the blood was out. Immediately after the perfusion of solution A, the pump was switched to solution B with a flow rate of 30 mL/min

for 10-15 min until the liver appeared completely blanched and softened. The liver was externally washed with normal saline (0.9 % sodium chloride solution) during the entire period of isolation. After perfusion with solution B, the liver was carefully excised and placed in a Petri dish containing 100 mL of solution C and the capsule was stripped away from one side of the liver and cells dissociated by brushing the liver with a plastic comb.

The cells were then filtered through cotton filter into a funnel and kept in a shaking water bath for 5 min at 37°C and supplemented with 95 % O_2 with gentle shaking. After 5 min incubation, the cell suspension was filtered again through a 100 µm filter into 50mL sterile cell culture plastic tubes (VWR International, Mississauga, ON, Canada) and placed on ice until it was 4° C. Thereafter, the cells were centrifuged at 1000 rpm for 5 min at 4° C and the supernatant was aspirated and cells were resuspended in the DME medium (additive-free). This step was repeated twice, after which the supernatant was aspirated and the cells were suspended in DME medium. This cell suspension was added to the Percoll gradient (prepared by adding 6.75 mL of 2X phosphate-buffered saline, 8.25 mL of lx phosphate-buffered saline and 10 mL of Percoll) and centrifuged at 4000 rpm for 10 min at 4° C. Finally, the supernatant was aspirated and cells were resuspended in DME medium containing additives and the cells were adjusted to 0.5 million/mL.

2.2.4.3.4. Preparation of primary cultures

The hepatocytes were seeded on a 6 or 24 well plastic culture plates (VWR International: Mississauga, ON, Canada) precoated with collagen (50 µg/mL) on the day before the experiment. Collagen stock solution was prepared by dissolving 25 mg collagen in 12.5 mL of 100 µL acetic acid in 87 mL of autoclaved water. Out of this 1 mL of collagen stock was diluted to 40 mL with 100 µL acetic acid in 87 mL of autoclaved water and 1 mL was added to each well of 6 well plates. After viability assessments, the cell suspension was diluted to 0.5 million/mL with DME medium containing 10 % fetal bovine serum and penicillin/streptomycin antibiotic (1 %). 1 mL cell suspension containing 0.5 million cells was added per well and the plates were incubated for 6 h at 37°C in a humidifier with 95 % O_2 and 5 % CO_2. After 6 h, medium containing the dead cells was removed and the medium was changed.

2.2.4.4. Determining hepatocyte viability

After isolation of hepatocytes, the cell viability was determined by using the trypan blue exclusion method. For this purpose, 50 µL of cell suspension in DME media with additives was added to 50 µL of 0.2% trypan blue solution after which the cells were counted in 16 microscopic squares. The viable cells were expressed as million/mL, excluding the dead cells. To assess the effect of time and treatments on hepatocyte viability, both MTT and LDH tests were used as described previously [172].

2.2.4.4.1. MTT assay

The assay measures the reduction of MTT into insoluble blue coloured formazan crystals by the action of mitochondrial succinate dehydrogenase enzyme present in the viable cells. Briefly, freshly isolated rat hepatocytes were kept in complete medium for 36 h in presence of 10 % FBS. For LP and drug treatments, hepatocytes were treated for 24 h in the presence of 5 or 10 % NL/HL rat serum or 5µM antihyperlipidemic drugs (Atv or FF). Thereafter, after 24, 36 h of incubation, the cell viability was measured with MTT assay. First, the medium was removed and replaced with cell culture medium containing 1.2 mM MTT dissolved in phosphate-buffered saline (PBS) (pH 7.4). After 2 h of incubation, the formed crystals were dissolved in isopropanol. The intensity of the color in each well was measured at a wavelength of 550 nm using the Bio-Tek EL 312e 96-well microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The percentage of cell viability was calculated relative to control wells and designated as 100 % viable cells. Three independent experiments were performed (3 rats and n of 8 for group).

2.2.4.4.2. Lactate dehydrogenase (LDH) leakage assay

The effects of lipoproteins and antihyperlipidemic drugs on the integrity of cell membranes were determined flurometically by assessing the leakage of LDH from the cytoplasm of damaged cell membrane into the surrounding culture media using Cyto-Tox-ONE kit (Promega, Madison, WI, USA). Primary rat hepatocytes were treated for 24 h in a 96-well cell culture plate with tested compounds, 10 %

NL or HL rat serum or with 5 µM of the antihyperlipidemic drugs Atv or FF. LDH release was measured with a 10-min coupled enzymatic assay that results in the conversion of resazurin into florescent resurofin. The fluorescence produced was then recorded with an excitation wavelength of 560 nm and an emission wavelength of 590 nm according to manufacturer's instructions (Promega). The amount of fluorescence produced is proportional to the number of cells with a damaged membrane. The extent of LDH leakage was calculated relative to controls designated as 100 %. Triplicate reactions for each experiment and three independent experiments were performed (3 rats and n of 8 for group).

2.2.4.5. Lipoproteins treatments

Hepatocytes isolated from rats were divided into three main groups: (1) Control group containing 10 % FBS in media (no rat serum was added). (2) Incubated in the presence of 5 % NL rat serum. (3) Incubated in the presence of 5 % HL rat serum. The hepatocytes were preincubated for 24 h at 37 °C. Thereafter, the medium containing serum was removed, and total RNA was isolated.

2.2.4.6. RNA Extraction and cDNA Synthesis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. The quality of the isolated RNA was determined by measuring the 260/280 ratio. Thereafter, first strand cDNA was synthesized using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA)

according to the manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was added to a mix of 2.0 μL of 10x reverse transcriptase buffer, 0.8 μL of 25x dNTP mix (100 mM), 2.0 μL of 10x reverse transcriptase random primers, 1.0 μL of MultiScribe reverse transcriptase, and 3.2 μL of nuclease-free water. The final reaction mix was kept at 25°C for 10 min, heated to 37°C for 120 min, heated for 85 °C for 5 seconds, and finally cooled to 4°C.

2.2.4.7. Quantification by Real-Time PCR

Quantitative analysis of specific mRNA expression was performed by real-time PCR by subjecting the resulting cDNA to PCR amplification in 96-well optical reaction plates using the ABI Prism 7500 System (Applied Biosystems, Foster City, CA, USA). The 25 µl reaction mix contained 0.1 µl of 10 µM forward primers, 0.1 µl of 10 µM reverse primer, 12.5 µl of SYBR Green (Applied Biosystems, Warrington, UK), Universal Mastermix, and 11.05 µl of nuclease-free water and 1.25 µl of cDNA sample. The primers used in the current study were chosen from previously published studies and are listed in Table 2. No-template controls were incorporated to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Melting curve (dissociation stage) was performed by the end of each cycle to confirm the specificity of the primers and the purity of the final PCR product.

Table 2: Primer sequences used for real-time PCRs

Genes	Forward primers	Reverse primers
CYP3A2	GCT CTT GAT GCA TGG TTA AAG ATT TG	ATC ACA GAC CTT GCC AAC TCC TT
CYP2C11	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
CYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
CYP2D1	GGTCATTTGTCTTTGGGAGCC	GCAAGGATCACACCTTGGGA
LDLr	CAACGGTGGCTGCCAGTAC	GAACTTGGGTGAGTGGGCAC
VLDLr	CGAGGTCAACTGTCCTTCTCG	TGCCATGGATACAGCTACCG
MDR1A/B	GACAGGACATCAGGACCATCAAT	GACGTTTTCTCGGCCATAGC
OATP1B2	CTCACCCCTCTACCTGGGAAT	TGAGGCTACCCAGCCCATAG
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
18S	GCCGCTAGAGGTGAAATTCTTG	CTTTCGCTCTGGTCCGTCTT

2.2.4.8. Real-time PCR data analysis

The real time-PCR data were analyzed using the relative gene expression (ΔΔCT) method as described previously [173]. Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene and relative to a calibrator. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA (18S) were used as the endogenous control and the control group (10 % fetal bovine serum, no lipoproteins were added) was used as the calibrator when the change of gene expression by LPs was being studied.

2.2.5. Role of low density lipoprotein receptor (LDLr) in CyA hepatic uptake using primary rat hepatocytes

2.2.5.1. Cyclosporine Uptake Study

Drug uptake studies were conducted to study the role of LDLr in CyA hepatic uptake. Hepatocytes isolated from NL rats were divided into two main groups: pre-incubated group and coincubated serum group. Pre-incubated groups were subdivided into four main groups: 1.) Incubated with 10 % normolipidemic rat serum. 2.) Incubated with 10 % hyperlipidemic rat serum. 3.) Incubated with 5 µM Atv in 10 % NL rat serum 4.) Incubated with 5 µM FF in 10 % NL rat serum subgroups. Hepatocytes were preincubated for 24 h at 37 °C. Thereafter, medium containing rat serum or drugs was removed and treatment was initiated with drug incubated with rat serum medium. For the rat serum coincubation groups, CyA was preincubated with NL and HL rat serum for 1 h at 37 °C in a shaking water bath. This was carried out to promote the association of CyA with serum lipoproteins. This preincubated mixture of drug and serum was further diluted to 2 % in medium and added to the wells containing hepatocytes. Thereafter, the entire media from the wells were dumped and the amount of drug accumulated inside the cells at different time points [0, 0.08, 0.25, 0.5 and 1 h] was measured (n of 6 wells for each time point for three independent experiments using primary hepatocytes from three rats). For each time point, cells were washed twice with 500 µL of ice cold PBS 1X. Then, volumes of 20 µL of methanol, 80 µL of 1N NaOH and 300 µL HPLC water were added to the well contents. Then, the wall

contents were transferred and collected into Eppendorf tubes and frozen at -20°C until the LC-MS analysis was performed for the CyA remaining.

2.2.5.2. Assaying the hepatocyte samples for CyA content

A validated LC-MS method was used for the analysis of CyA in hepatocytes with a modification regarding IS concentration [174]. All test and standard samples were extracted with ether: methanol liquid extraction method. Briefly, 40 µL (10 µg/mL) of AM IS was added to the samples before the extraction. CyA and IS were extracted by adding 4 mL diethyl-ether. All samples were vortex mixed for 60 seconds, centrifuged at 2500 g for 10 min, and the organic layers were transferred to clean tubes and dried under vacuum. The calibration samples were prepared by spiking the desired amount of each working standard solution of CyA to 0.25 million/0.5ml of analyte-free cells in DMEM with additives followed by the addition of solvents. The extraction was performed as mentioned previously. Standard curves were highly linear (r^2 = 0.999), and in all analytical runs, quality control samples were included to verify the precision and accuracy of the measures. Drug-free hepatocyte cells from NL rats were spiked with known amounts of CyA and used for the construction of standard curves. Standard curve samples of 0, 50, 100, 250, 500 and 1000 ng/mL concentrations of known amounts of CyA were prepared in both blank cell matrices and the concentrations of drug which were accumulated inside the cells were measured using peak height ratios. Using LC-MS the amount of CyA present was determined.

2.2.6. Statistical and Data analysis

2.2.6.1. Pharmacokinetic studies

Analyses were performed using Microsoft Excel® (Version 2010, Microsoft Corp.), and InStat® (Version 3.1a, GraphPad Software, San Diego, CA). Non-compartmental analysis was used for calculation of PK parameters. The linear trapezoidal rule was used to calculate the AUC from the time of dosing to the 24 h concentration. The maximal concentration (Cmax) and the time to achieve it (Tmax) were determined by visual inspection of the data. Dose-normalized AUC_{0-24} levels were calculated in each patient by dividing the AUC_{0-24} by the dose/kg. Between-group differences in the arithmetic means of continuous variables were analyzed using unpaired t-tests. Mann-Whitney U tests were used if t-test assumptions were violated. Two tailed p-values were considered significant at the 0.05 threshold, α was set at less than 0.05.

2.2.6.2. Pro-inflammatory Marker Concentrations

The data were reported as mean ± SD and were tested for statistical significance using the unpaired Student's t-test. The results were considered statistically significant when $p < 0.05$. Data analysis was performed using Sigma Plot 12.

2.2.6.3. Hepatocytes

The gene expression data was assessed without log transformation; if normality was present the data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. Non-normal data was analyzed using

a Kruskal Wallis one-way ANOVA followed by Dunn's method of pairwise multiple comparison procedure. Data are presented as mean±SD unless indicated; for all comparisons, α was set at 0.05.

For the CyA uptake study, the corresponding AUC of concentration versus time in the wells after incubation with CyA was calculated. The AUC of percent of CyA accumulated versus time in cultured primary rat hepatocytes was also calculated using the linear trapezoidal rule from the time of adding the drug to the last measured time point up to 60 min. Data are presented as mean ± standard deviation of the mean of three independent experiments (n of 6 in each group). Data analysis was performed using Sigma Plot 12. For each time point, comparisons across group's analysis were done using a one-way analysis of variance followed by Duncan or Dunn's post hoc comparison. The Bailer's method of assessing for significance of comparisons between AUC values was used with Bonferroni correction; analysis of variance followed by Dunn's post-hoc test was used to assess the significance of differences between means of the treatment groups at each time point; for all comparisons, results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Development of analytical method for azithromycin using Liquid Chromatography-Mass Spectrometry (LC-MS)

3.1.1. LC-MS chromatograms

The chromatographic retention times were 6.9 min for AZ and 3.4 min for IS. The total analytical run time of this method was 9 min. The method provided specificity, with baseline resolution of IS and AZ and a lack of interference between endogenous components and the analytes (Figure 3). The molecular ions of AZ and IS were represented by peaks with m/z of 749.6 $(M+H)^+$ and m/z 591.4 (fragment) as the monitored ions for AZ, and 281.1 m/z for IS (Figure 4).

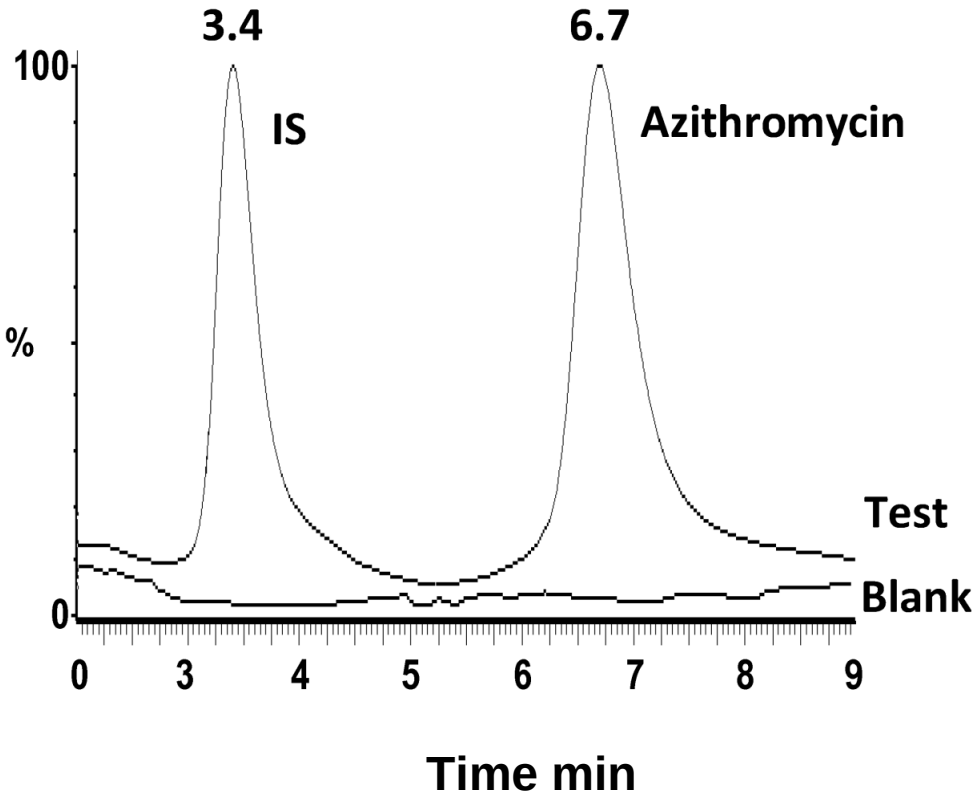


Figure 3. Selective ion chromatogram (SIR) of AZ and imipramine (IS). (A) Blank human plasma, (B) Test human plasma sample after an oral administration of a 500 mg AZ tablet and spiked with IS

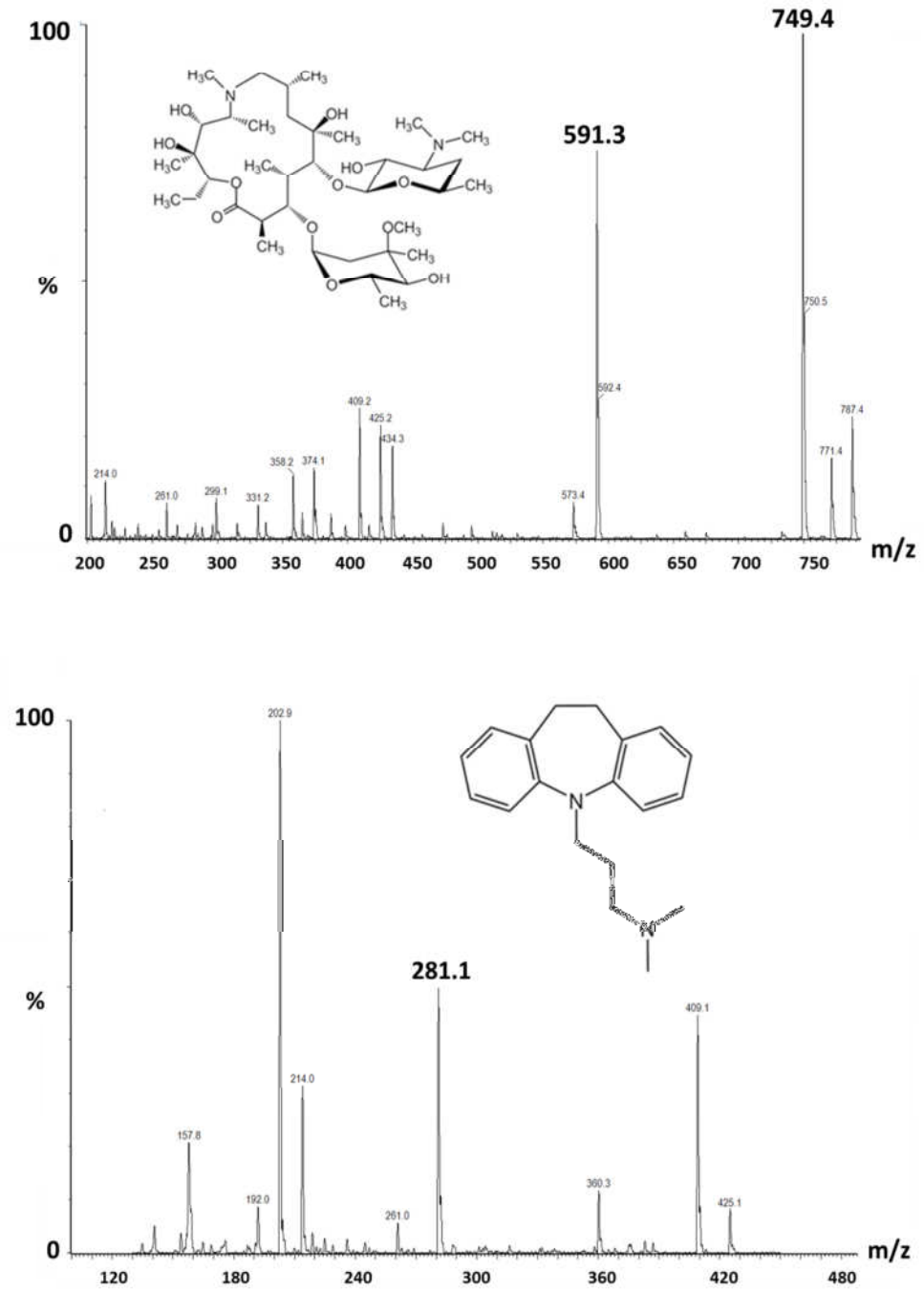


Figure 4. Mass spectra and chemical structures of AZ (upper panel), and the IS, imipramine (lower panel)

3.1.2. Solvent Recovery, Assay Linearity, and Matrix Effect

The mean recoveries for 50 and 500 ng/mL AZ and 1 µg/mL IS from the plasma were 75.9, 77.5% and 94.7 %, respectively. There were excellent linear relationships (r^2 >0.999) noted between the peak height ratios of the drug to IS and AZ concentrations over the range of 10-1000 ng/mL based on 0.5 mL human plasma. The calibration curves had typical regression equations of y=0.0007x-0.0021, where y is the peak height ratio of AZ to IS and x is the concentration curve parameters. Regarding the matrix effect, there was no significant matrix effect on the ionization of either AZ or IS. The assessment of matrix effect showed a small negative influence of the matrix on measurement of the analytes. AZ peak heights were reduced by mean values of 8% at the lowest concentration (10 ng/mL), but less than 1% at higher concentrations. The IS peak height was reduced by 6% by matrix components.

3.1.3. Precision, Accuracy & Validation

CV values of intra-and inter-day assessments were less than 11% and mean inter-day error was less than 6% (Table 3). Based on the inter- and intra-day CV and mean error, it could be determined that the lower limit of quantitation (LLQ) of AZ was 10 ng/mL based on 0.5 mL of human plasma.

Table 3: Three-day validation data for the assay of azithromycin in human plasma, n=6

Intraday precision				Interday precision		
Nominal Conc. ng/mL	Mean ± SD (intraday CV %)			Mean± SD, ng/mL	CV%	error %
10	9.68 ± 0.99 (10.22)	9.33 ± 0.83 (8.89)	9.52 ± 0.78 (8.19)	9.51 ± 0.86	9.11	--5.17
50	53.64 ± 4.65 (8.66)	49.61 ± 3.70 (7.47)	54.67 ± 4.39 (8.02)	52.64 ± 4.25	8.07	4.85
100	89.14 ± 8.28 (9.28)	110.19 ± 6.79 (6.17)	111.17 ± 5.88 (5.29)	103.50 ± 6.99	6.75	2.37
500	458.68 ± 30.68 (6.57)	485.45 ± 40.41 (8.32)	524 ±5 1.30 (9.78)	489.55 ± 40.63	8.30	–2.44

3.2. Applicability of the Assay

Compared to published literature data from lean subjects [115, 132, 175] (Table 4) , the mean $AUC_{0\text{-}24}$ in our obese subjects was slightly lower with an average ± SD of 2.07± 0.75 µg·h/mL and ranging from 2.36 to 2.67 µg·h/mL for lean subjects. Mean peak AZ concentrations were 0.36 µg/mL in our obese volunteers compared to lean data ranging from 0.4 to 0.44 µg /mL and were reached at 2.36 ±0.99 h for obese subjects and ranged from 2 to 2.8 h for the lean control group. The mean partial terminal phase half-life extending from 5 to 24 h post-dose was 10.57±2.5 h and oral clearance was 251.4±98 vs. 147 to 212 L/h mean range for lean subjects. All AZ PK data for the comparison were extracted from published literature studies on lean subjects [115, 132, 175]. Figure 5 shows the mean concentration time profile for AZ in plasma of 14 obese female volunteers after oral administration of a 500 mg immediate release tablet.

Table 4: Azithromycin pharmacokinetics outcomes in obese patients and after RYGB surgery.

Variable	Obese n=14 Mean ±SD	Bypass n=14 Mean ±SD	Lean Subjects Mean[$] ,Reference [115, 132, 175]
$AUC_{0\text{-}24h}$ (µg·h/ml)	2.07±0.75	1.41±0.51*	2.36 -2.67
Dose-normalized $AUC_{0\text{-}24h}$ (g·hr/L)**	0.40 ±0.13	0.27±0.12*	NA
C_{max} (µg /ml)	0.36 ±0.2	0.26±0.12	0.40-0.44
T_{max} (h)	2.36 ±1.17	2.14±0.99	2-2.8
CL/F (L/h)	251.45±98.3	382±192*	147-212
$t½^{\wedge}$ (h)	10.57±2.5	10.7±3.1	NA

*Statistically significant compared to obese control group using Mann-Whitney U test. ** $AUC_{0\text{-}24h}$ normalized for dose and body weight, $^{\wedge}$ = partial terminal phase half-life from 5 to 24h. **$** =** range of mean. NA= not applicable.

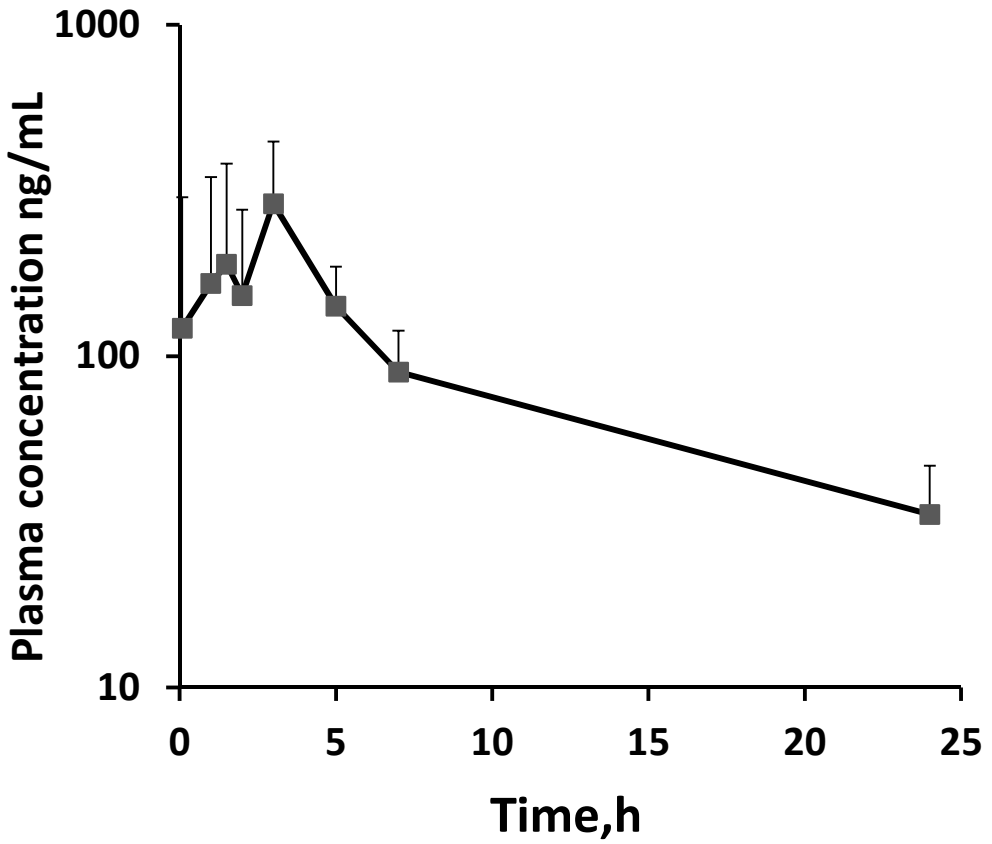


Figure 5. The 24-hour mean plasma AZ concentration versus time profiles after oral administration of a 500 mg AZ tablet to 14 female obese subjects. The data were reported as mean ± SD.

After RYGB surgery, our results indicated that overall, AZ mean concentrations were lower in gastric bypass patients compared to our control obese group throughout the entire duration of sampling up to 24 h. Compared to controls, the AUC_{0-24} was reduced in gastric bypass subjects by 32 % and dose-normalized AUC_{0-24} was reduced by 33 % (Table 4, Figure 6). Peak AZ mean concentrations were 28 % lower in bypass subjects and were reached slightly faster compared to the control group. Compared to control obese subjects, the mean partial terminal phase half-life extending from 5 to 24 h post-dose was 10.7 ± 3.1 h vs. 10.57 ± 2.5 h and oral clearance was 382 ± 192 vs. 252 ± 98 L/h for lean subjects.

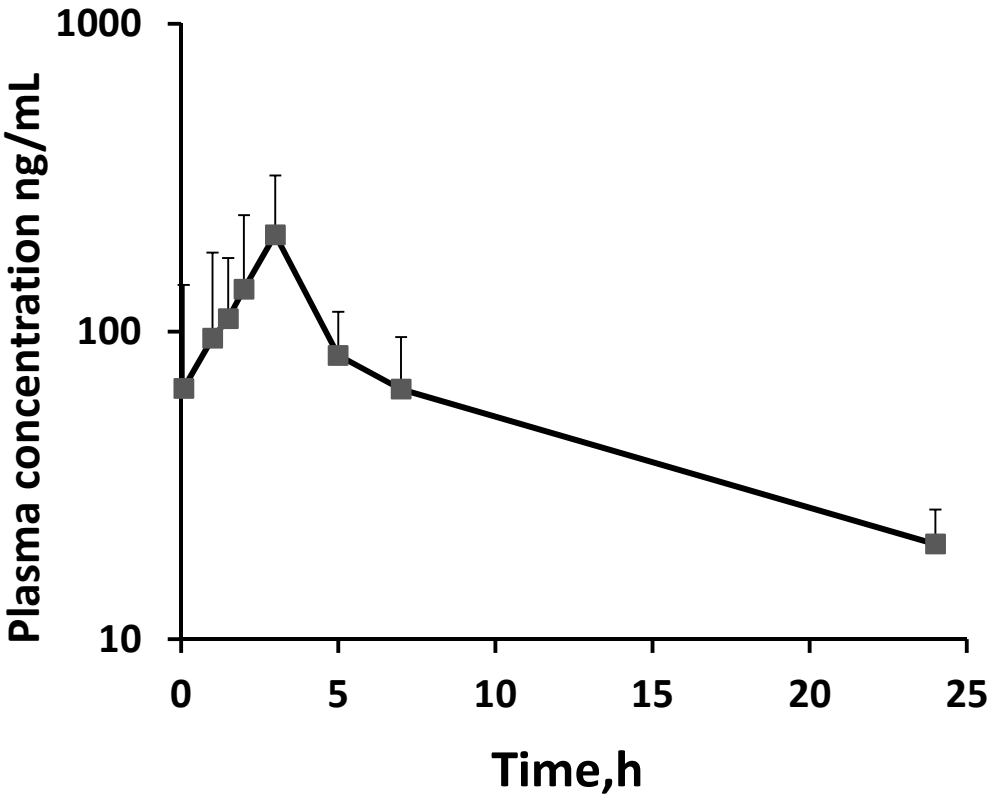


Figure 6. The 24-hour mean plasma AZ concentration versus time profile after oral administration of 500 mg azithromycin to 14 post RYGB patients. The data were reported as mean ± SD.

In addition, lipid levels had returned to the normal level. Fasting total cholesterol (TC) and triglyceride (TG) levels were significantly reduced by 20% and 31%, respectively. High density lipoproteins (HDL) levels were increased 11 %. The bad lipid (LDL) CHOL levels were significantly lower by 32% in the bypass group compared to obese matched subjects (Figure 7).

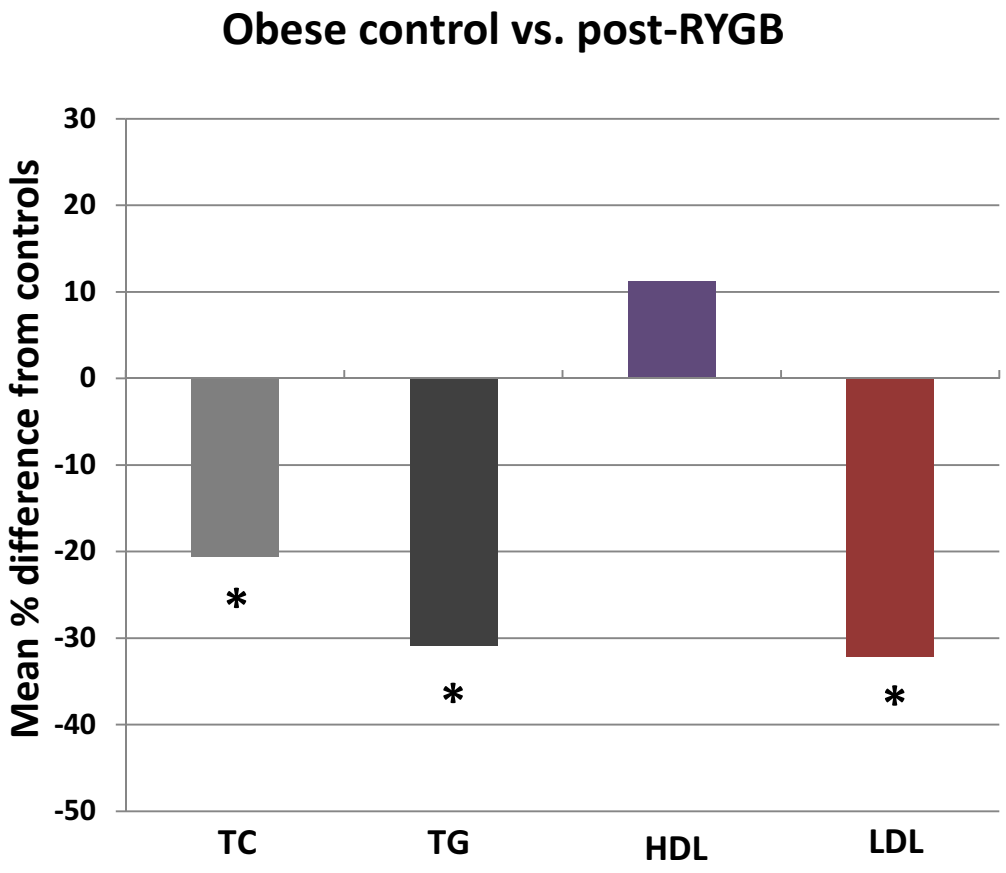


Figure 7. Outcomes of plasma lipid profiles as a result of bariatric surgery. Asterisks show values which were significantly altered by the intervention. *Statistically significant using unpaired Student t-test.

3.3. Pro-inflammatory Cytokine levels in obese and post RYGB patients

Mean leptin levels were higher at 25 month postoperative follow-up compared with the normal reference mean of 20.7, but within the range of values expected in normal subjects (3.9-77.3 ng/mL). In the surgical patients there were significantly lower levels of leptin compared to obese control subjects. No significant differences were found between the obese control group and the surgical group in the IL-6 concentrations at 25 month point (Figure 8).

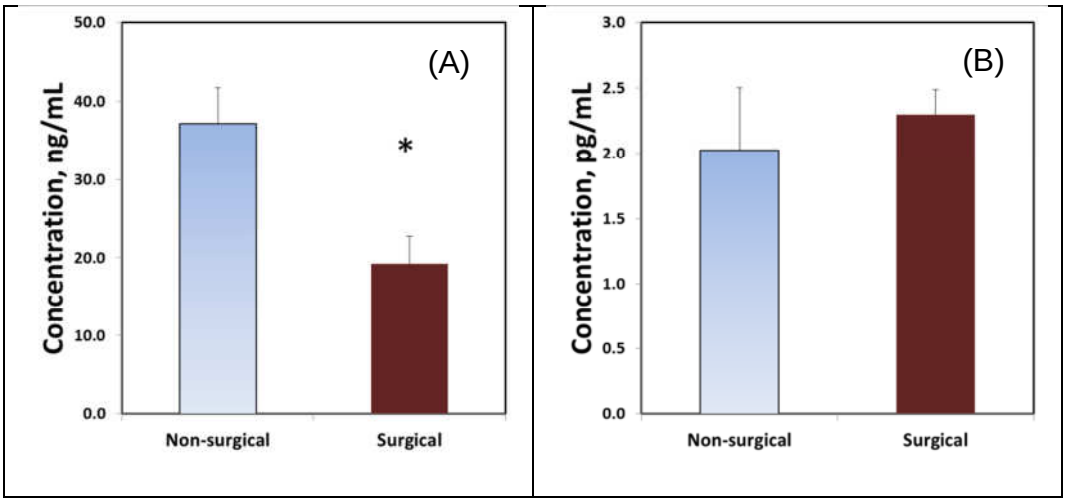


Figure 8. Leptin and interleukin-6 plasma concentrations (mean±SD) 25 months after RYGB surgery. Pre-dose fasting samples were assayed using kits from R&D Systems (Minneapolis, MN,USA). Data were compiled from 28 subjects enrolled in a recent trial [65]. Comparisons were made between the weight matched non-surgical obese control and post-bariatric surgical patients. Left panel (A), leptin, Right panel (B), IL-6. *Statistically significant using unpaired Student t-test.

3.4. Determine the effect of rat serum on hepatic mRNA expression using primary rat hepatocytes in vitro

3.4.1. MTT assay results

Compared to zero time point, no significant change was observed in the cell viability up to 36 h in the presence of 10 % FBS, showing that hepatocytes were viable throughout the study with cell viability of over 93 % (Figure 9, Table 5).

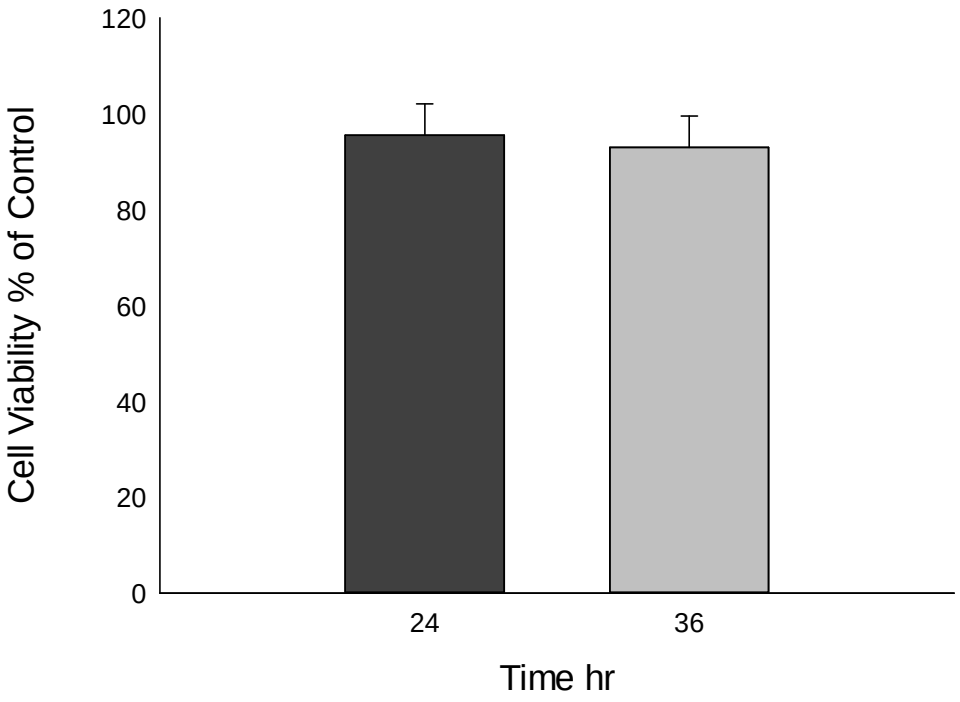


Figure 9. Effect of time on cell viability in isolated rat hepatocytes. Freshly isolated rat hepatocytes were kept for 36 h with 10 % FBS. Cell viability was determined using MTT assay. The data are presented as mean ± standard deviation of 3 independent experiments (3 rats and n of 8 for each time point).

Table 5: The percentage of cell viability in isolated rat hepatocytes (up to 36 h incubation with 10 % FBS).

Time	% Cell Viability
0 h	100 %
24 h	95.5 %
36 h	93 %

To investigate the effect of rat serum on cell viability, hepatocytes were incubated with 5% NL/HL rat serum and the viability was measured with the MTT assay. In rat serum-containing media, the loss of cell viability was less than 8% over a 36 h period. When rat serum was added, mean viability was similar to media (within 91-94%) with no significant difference being noted between incubations with either NL or HL rat serum (Figure 10, Table 6).

Table 6: Effect of lipoproteins on cell viability in isolated rat hepatocytes after 24 h incubation with 5 % NL/HL rat serum

Treatments	% Cell Viability
Control (10 % FBS)	100 %
Control + 5% NL	93.84 %
Control + 5% HL	91.1 %

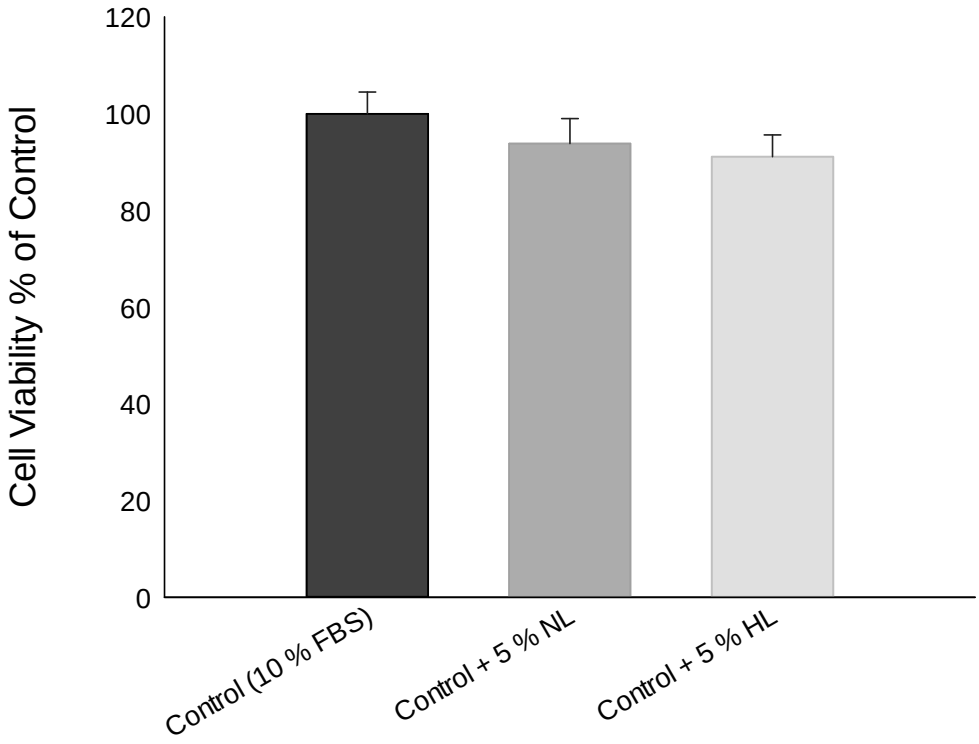


Figure 10. Effect of lipoproteins on cell viability. Freshly isolated rat hepatocytes were treated for 24 h with 5 % NL rat serum or 5 % HL rat serum. No significant changes were observed in the cell viability in the presence of NL or HL rat serum. Cell viability was determined using the MTT assay. The data are presented as mean ± standard deviation of 3 independent experiments (3 rats and n of 8 for each group).

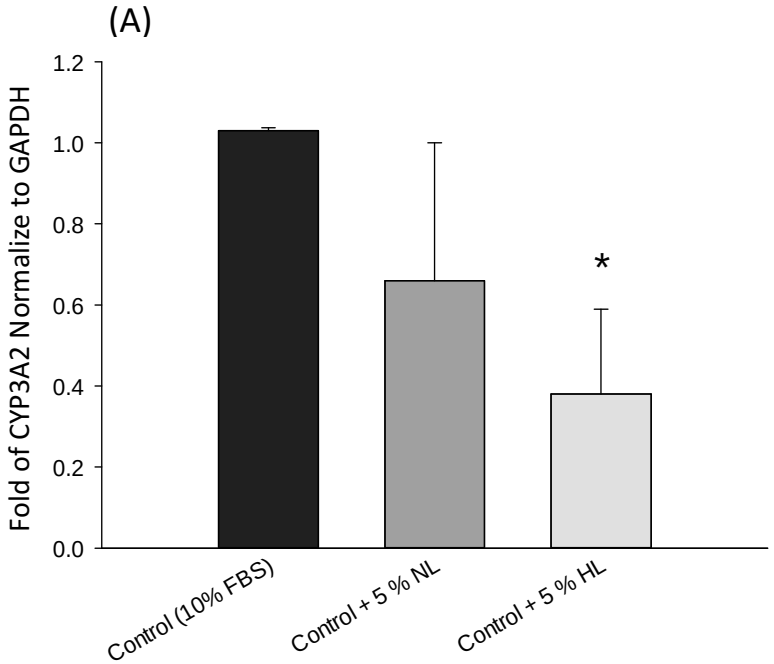
3.4.2. Effect of rat serum on hepatic mRNA expression

In general, the addition of NL rat serum did not affect the expression of the mRNA examined, although it did cause some decrease in hepatic mRNA of CYP3A2, CYP2D1 and OATP2B1. No effect was seen on the genes for CYP2C11, CYP1A1, LDLr, VLDLr or MDR1A (Figure 11 to 12 and Table 7).

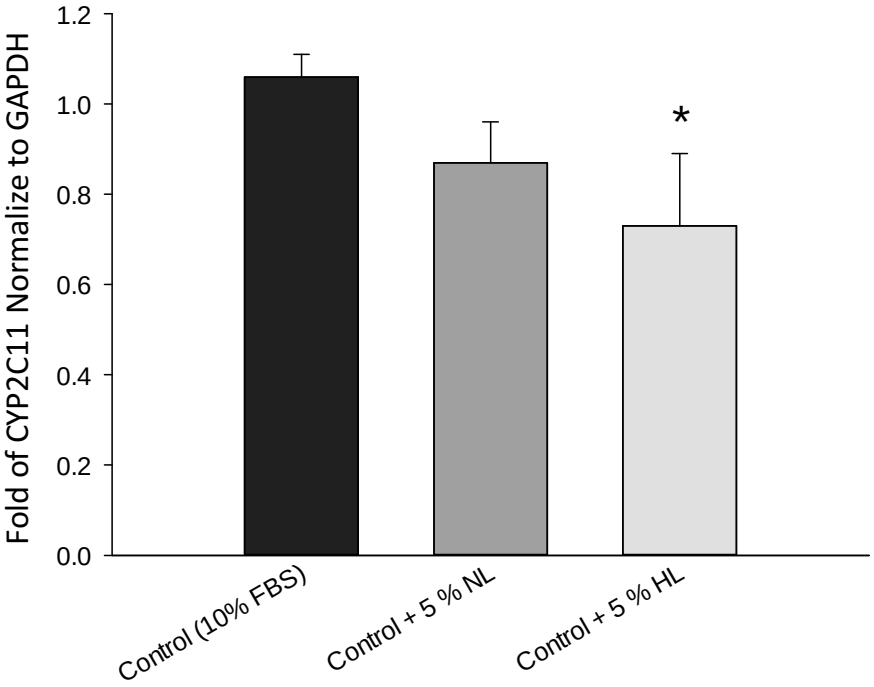
Table 7: The effect of rat serum on metabolizing enzymes and transporting gene expression by isolated rat hepatocytes. Data shown are the mean ± SD based on three independent experiments using hepatocytes from 3 rats (6 replicates per group).

Genes	Control	NL	HL	Ranking
CYP1A1	1.04±0.03	0.92±0.15	0.19±0.18	[A=B]>C
CYP3A2	1.03±0.007	0.66±0.34	0.38±0.21	[A=B]>[B=C]
CYP2C11	1.06±0.05	0.87±0.09	0.73±0.16	[A=B]>[B=C]
CYP2D1	1.07±0.10	0.68±0.27	0.16±0.14	A>B>C
LDLr	1.00±0.01	0.97±0.12	0.52±0.16	[A=B]>C
VLDLr	1.06±0.04	1.04±0.10	0.33±0.22	[A=B]>C
MDR1A	1.05±0.03	0.89±0.11	0.24±0.12	[A=B]>C
OATP2B1	1.03±0.028	0.60±0.030	0.19±0.13	A>B>C

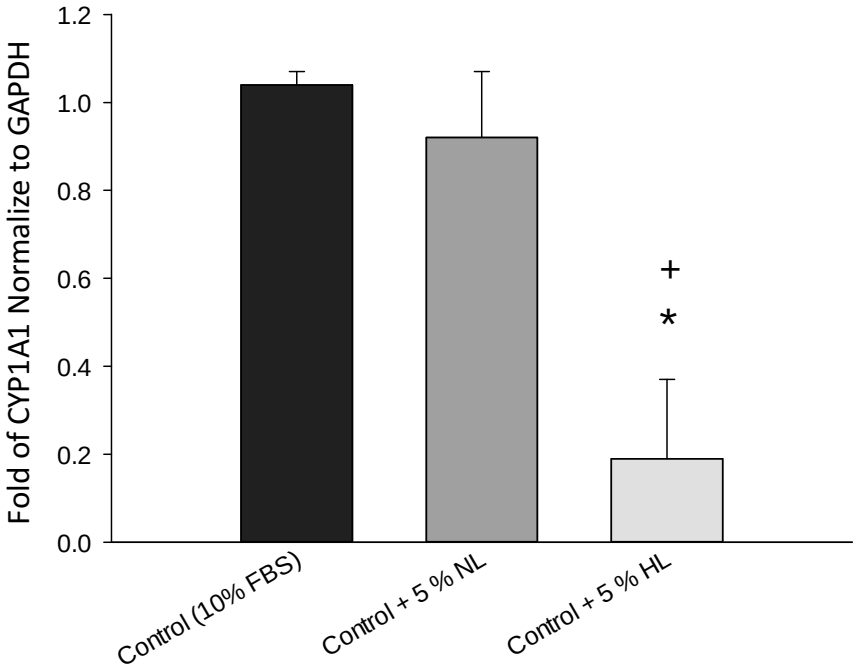
In contrast, compared to both rat serum-free media and NL rat serum incubations, the addition of HL rat serum had significant down-regulating effects on the hepatocyte mRNA expressions of each of the genes assessed. The least amount of down regulation was seen for CYP2C11, and the most for CYP2D1, CYP1A1 and OATP2B1 (Figure 11 to 12 and Table 7).



(B)


Fold of CYP2C11 Normalize to GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control (10% FBS)
Control + 5 % NL
Control + 5 % HL

(C)


Fold of CYP1A1 Normalize to GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+
*
Control (10% FBS)
Control + 5 % NL
Control + 5 % HL

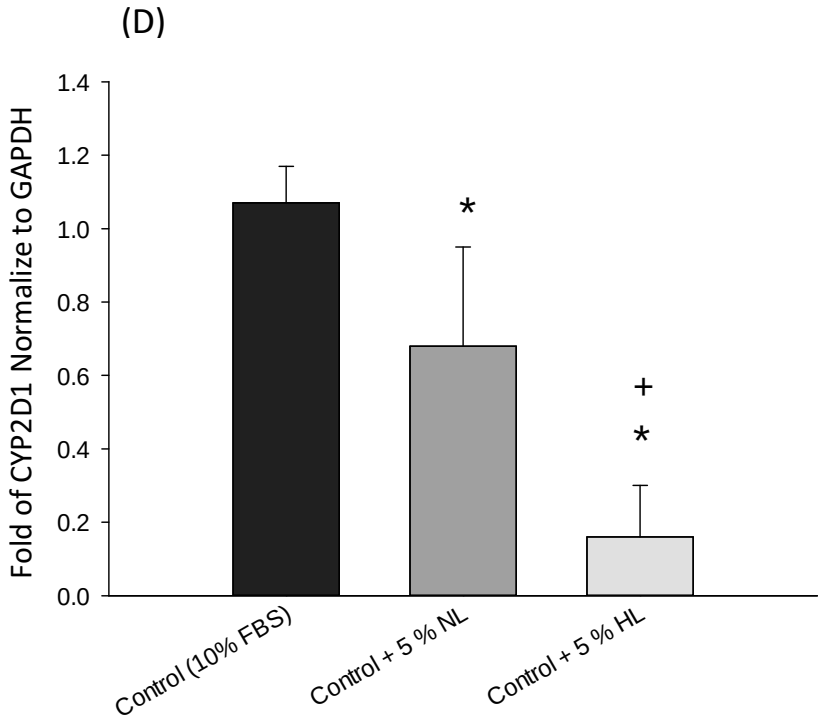
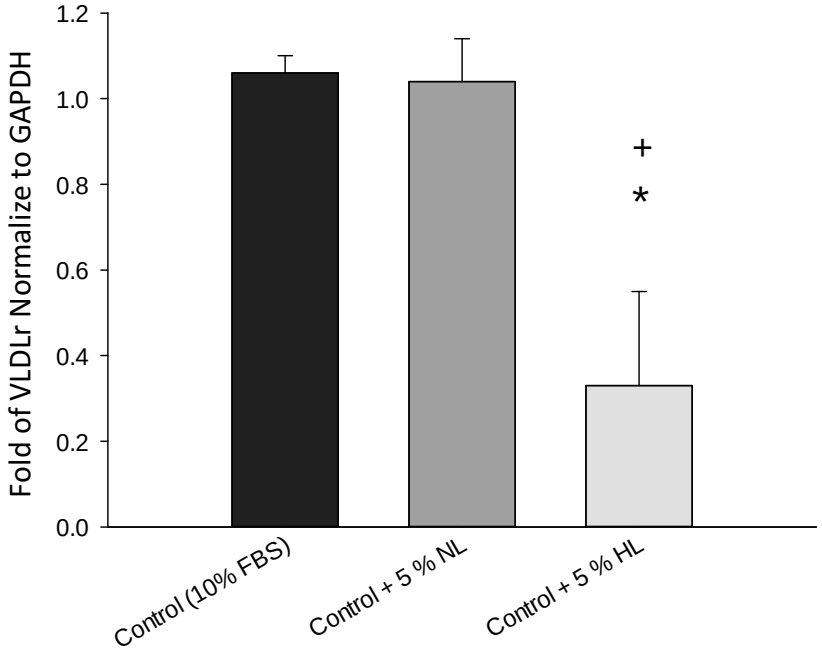
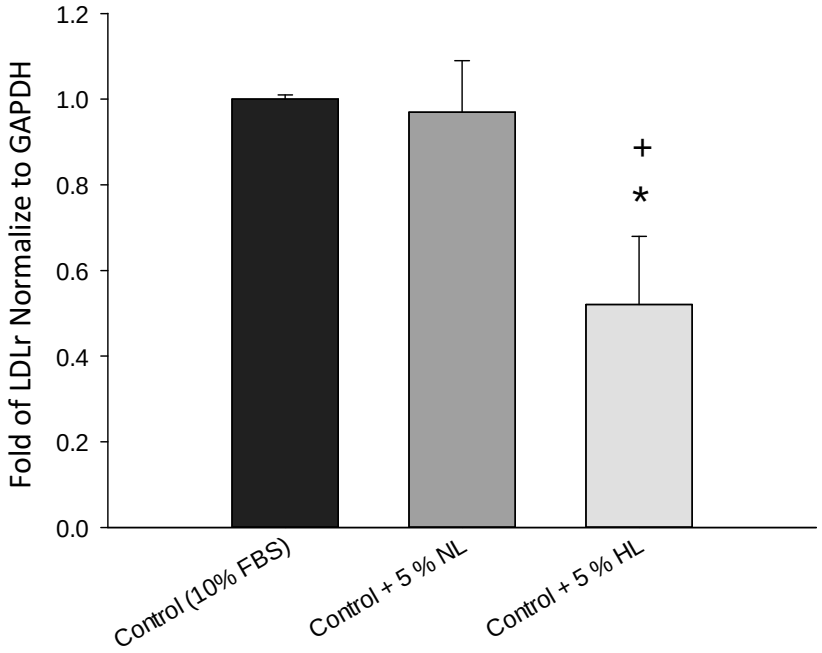


Figure 11. Effect of lipoproteins on metabolizing enzyme mRNA expression in isolated rat hepatocytes. Freshly isolated rat hepatocytes were treated with 5 % NL rat serum or 5 % HL rat serum for 24 h. (A) CYP3A2, (B) CYP2C11,(C) CYP1A1 and (D) CYP2D1 mRNA levels were quantified using RT-PCR and normalized to GAPDH. Duplicate reactions were performed for each experiment, and data are presented as mean ± standard deviation of 3 independent experiments (3 rats and n of 6 for each group). (*) $P < 0.05$, compared to control (10 % FBS, no lipoproteins were added); (+) $P < 0.05$, compared to Control + 5 % NL treatment.

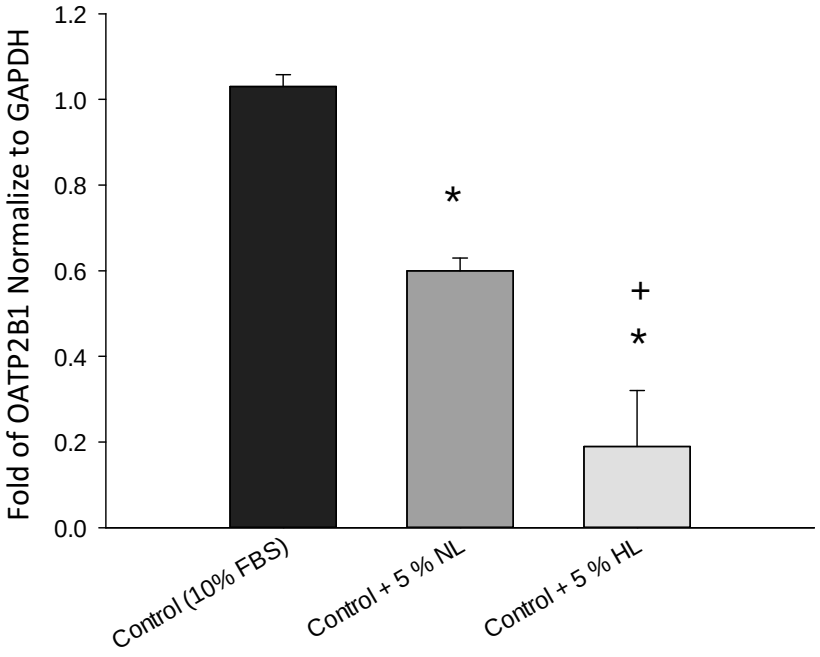
(A)


Fold of VLDLr Normalize to GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+
*
Control (10% FBS)
Control + 5 % NL
Control + 5 % HL

(B)


Fold of LDLr Normalize to GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+
*
Control (10% FBS)
Control + 5 % NL
Control + 5 % HL

(C)


Fold of OATP2B1 Normalize to GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
+
*
Control (10% FBS)
Control + 5 % NL
Control + 5 % HL

(D)

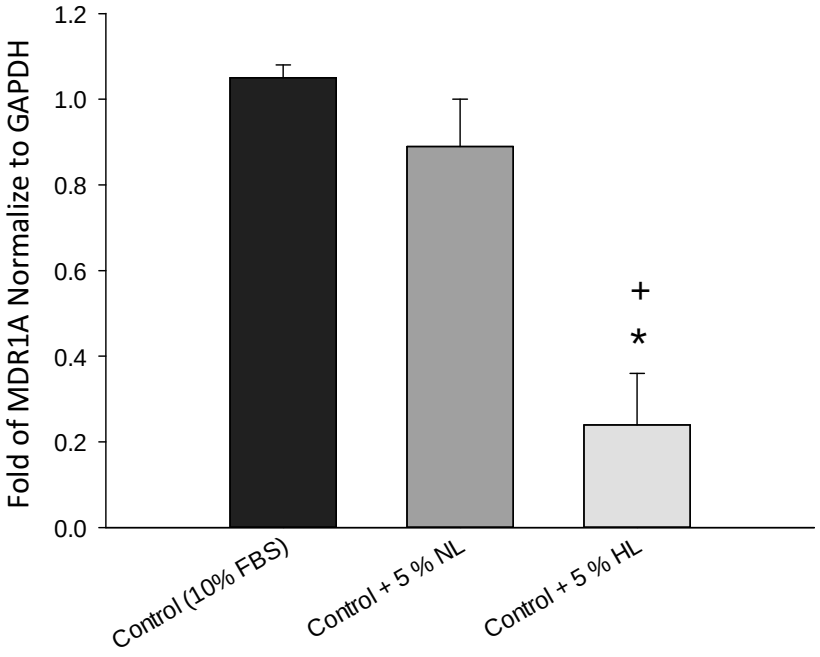

Fold of MDR1A Normalize to GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+
*
Control (10% FBS)
Control + 5 % NL
Control + 5 % HL

Figure 12. Effect of lipoproteins on transporters' mRNA expression in isolated rat hepatocytes. Freshly isolated rat hepatocytes were treated with 5 % NL rat serum or 5 % HL rat serum for 24 h. (A) VLDLr, (B) LDLr,(C) OATP1B2 and (D) MDR1A/B mRNA levels were quantified using RT-PCR and normalized to GAPDH. Duplicate reactions were performed for each experiment, and data are presented as mean ± standard deviation of 3 independent experiments (3 rats and n of 6 for each group). (*) $P < 0.05$, compared to control (10 % FBS and no lipoproteins); (+) $P < 0.05$, compared to control + 5 % NL treatment.

3.5. Role of LDLr in CyA hepatic uptake using primary rat hepatocytes

3.5.1. Cell viability

3.5.1.1. LDH Results

To determine if the concentrations of 10 % LP or 5µM antihperlipidemic drugs utilized in the current study were not toxic, hepatocytes were treated for 24 h with the tested compounds. Thereafter, cytotoxicity was assessed using LDH leakage assays. Figure 13 shows that LPs and drugs were non-toxic to cells at all concentrations used. Percent of LDH leakage was not significantly altered after the treatment of cells with 10 % NL or HL rat serum or with 5 µM Atv or 5 µM FF in 10 % NL rat serum for 24 h compared to serum-free control group (table 8).

Table 8: The percentage of cell toxicity using LDH leakage assays after 24h incubation (3 rats and n of 8 for each group).

% of cell toxicity mean ± SD	
Serum free	5.8 ± 0.2
10 % NL serum	5.7 ± 0.1
10 % HL serum	5.8 ± 0.2
5 µM Atv in 10 % NL serum	5.9 ± 0.1
5 µM FF in 10 % NL serum	5.9 ± 0.1

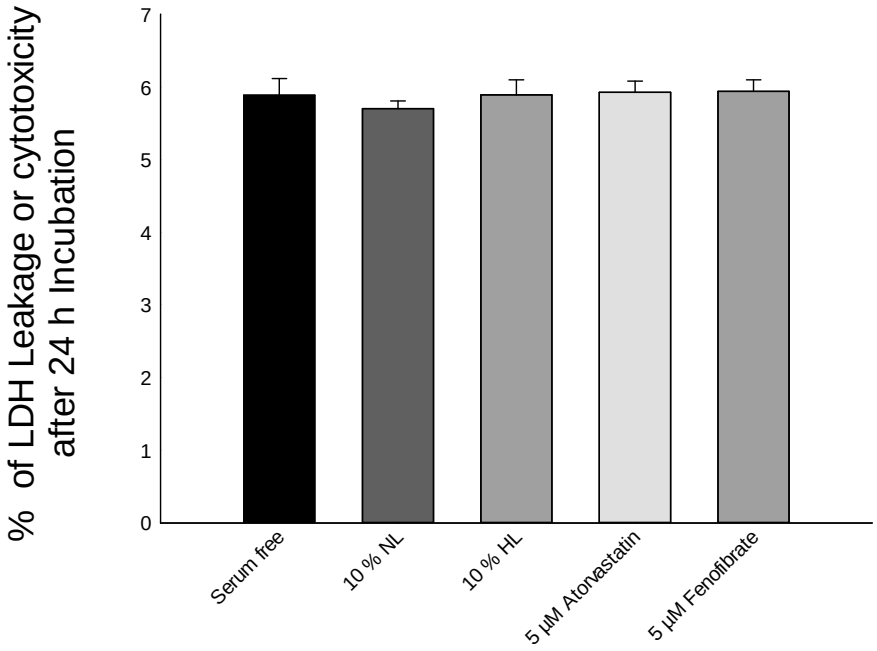


Figure 13. Effect of lipoproteins and drug treatment on cell viability. Freshly isolated rat hepatocytes were treated for 24 h with 10 % NL rat serum or 10 % HL rat serum or with 5 µM antihyperlipidemic drugs (atorvastatin or fenofibrate). Cell cytotoxicity was determined using LDH leakage assays. The data are presented as mean ± standard deviation of the mean of 3 independent experiments (3 rats and n of 8 for each group).

3.5.1.2. MTT assay results

To investigate the effect of rat LPs and drug treatment on cell viability, hepatocytes were also incubated with 10 % NL/HL rat serum and in presence of 5 µM Atv or 5 µM FF for 24 h; then the viability was measured with the MTT assay. In rat serum-containing media there was no loss of cell viability [ranged from 100 to 106 % compared to the control group (serum-free media)] with no significant difference being noted with cells in the presence of drugs or LPs (Figure 14, Table 9).

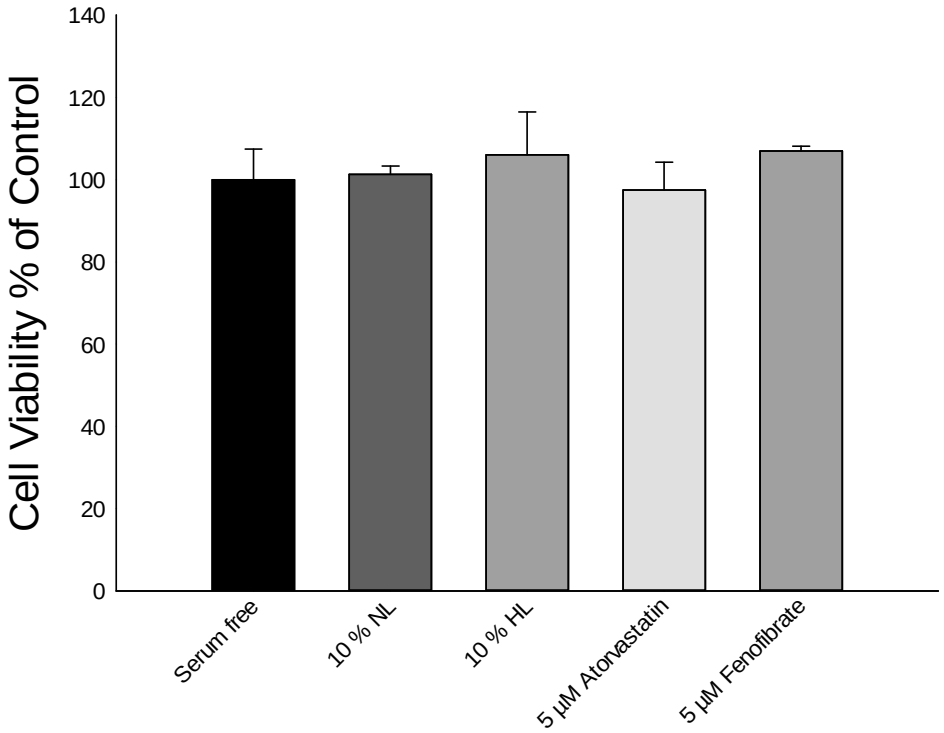


Figure 14. Effect of lipoproteins and drug treatment on cell viability. Freshly isolated rat hepatocytes were treated for 24 h with 10 % NL rat serum or 10 % HL rat serum or with 5µM antihyperlipidemic drugs (atorvastatin or fenofibrate). Triplicate reactions were performed for each experiment. The data are presented as mean ± standard deviation of the mean of 3 independent experiments (3 rats and n of 8 for each group).

Table 9: The percentage of cell viability using the MTT assay after 24h incubation (3 rats and n of 8 for each group).

% of cell viability mean±SD	
Serum free	100 ± 7.47
10 % NL serum	101 ± 1.96
10 % HL serum	106 ± 10.48
5 µM Atv in 10 % NL serum	97.5 ± 6.74
5 µM FF in 10 % NL serum	107 ± 1.1

To ensure also that poloxamer 407 by itself did not influence hepatocyte viability, some hepatocytes were spiked with NL serum and others with NL serum plus poloxamer 407 at concentrations (5 mg/mL) known to be present in serum *in vivo* after ip doses of lg/kg, 36 h after dosing. The viability of cells was determined by MTT assay. The results showed no significant difference in cell viability between NL and NL plus Poloxamer groups or between serum-free media and serum-free media plus Poloxamer 407 (Figure 15, Table 10).

Table 10: The percentage of cell viability using the MTT assay after 24h incubation with poloxamer 407 (5mg/mL) (3 rats and n of 8 for each group).

% of cell viability mean±SD	
Serum free	100 ± 3.1
10 % NL serum	100 ± 1.4
Poloxamer (5mg/mL) in serum free	98 ± 4.7
Poloxamer (5mg/mL) in 10 % NL serum	105 ± 2.7

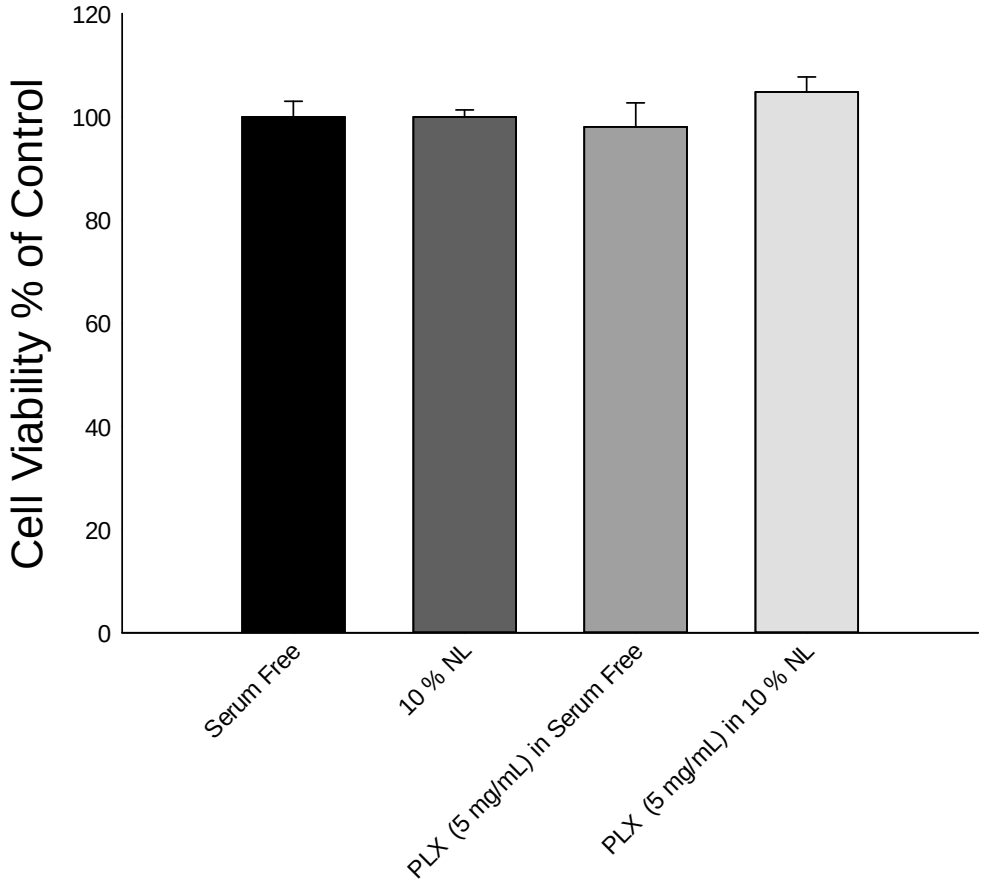


Figure 15. Effect of Poloxamer 407 on cell viability of primary rat hepatocytes. Cells were treated for 24 h with poloxamer (5 mg/mL). No significant change was observed in the cell viability in the presence of poloxamer in serum-free media or poloxamer in 10 % NL rat serum. Cell cytotoxicity was determined using the MTT assay. Triplicate reactions were performed for each experiment. The data are presented as mean ± standard deviation of 3 independent experiments (3 rats and n of 8 for each group).

3.5.2. Cyclosporine uptake study

A time dependent increase in CyA uptake was observed in all groups (Figure 16 to 17). Preincubation studies were conducted only using hepatocytes from three NL rats. Preincubation of NL hepatocytes with HL rat serum for 24 h led to an increase in the uptake of CyA nearly by 10 % compared to control the NL group, while when the cells were exposed to antihyperlipidemic drugs (Atv and FF), there was more increase in CyA uptake nearly up to 21 and 23 %, respectively. All comparisons were made to CyA coincubation with 2 % HL rat serum groups (Figure 16, Table 11).

Table 11: Time-dependent CyA uptake in primary rat hepatocytes. CyA coincubated with 2% NL rat serum, **(**3 independent experiments, n of 6 each group).

CyA (2.5 µg/mL) coincubated with 2 % NL serum				
Treatment groups	% CyA accumulation in primary rat hepatocytes Mean ±SD			
	5 min	15 min	30 min	60 min
A. 10 % NL	36.1±4.8	35.4±1.5	43.7±5.1	46.9±2.8
B. 10 % HL	32.2±7.3	30.4±2.6	46.3±4.8	66.8±5.4
C. 5µM Atorvastatin in 10% NL	36.3±5.2	42.9±1.3	56.3±2.9	56.4±5.09
D. 5µM Fenofibrate in 10% NL	31.9±3.6	43.4±2.8	57.6±5.1	58.2±6.2
Ranking*	[A=C] > [B=D]	[C=D] >A>B	[C=D] >[A=B]	B>[C=D] >A

Asterisk represents the statistics using one way ANOVA followed by Post-hoc Duncan's test. For each time point a comparison across groups was made using a one-way ANOVA followed by a Duncan post hoc comparison. Data analysis was performed using Sigma Plot 12. The result was considered statistically significant when $P < 0.05$.

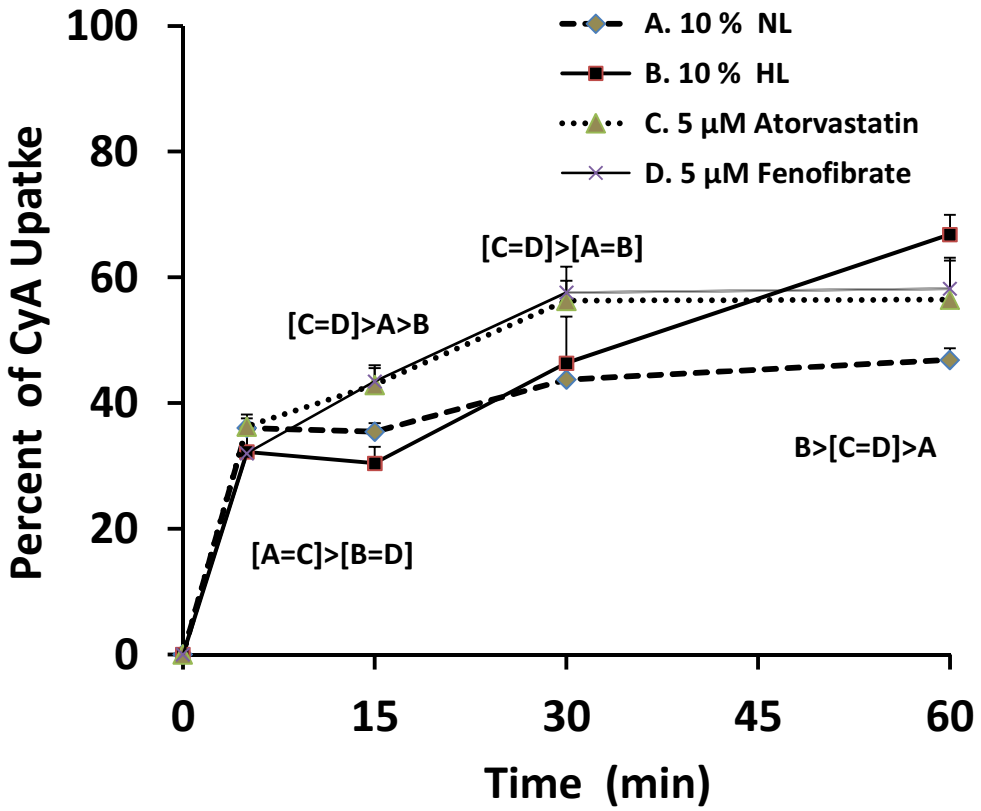


Figure 16. Percentage of CyA uptake (mean ± SD) in primary rat hepatocytes. Cells were obtained from normolipidemic rats and pre-incubated for 24 h with 10 % serum from normolipidemic (NL) or hyperlipidemic (HL) rats or 5 µM atorvastatin or fenofibrate. CyA was co-incubated with 2 % normolipidemic (NL) rat serum. Three rats were used for each group with 6 wells per time point for each rat. Statistically significant differences between amounts accumulated at any given time points are indicated.

Regarding preincubation results when CyA was coincubated with 2 % HL rat serum, HL caused uptake nearly 54 % increase in CyA uptake. Preincubation with 5 µM Atv caused an increase in uptake nearly up to 20 %. However, FF showed less increase, with only a 10 % increase. All comparisons were to NL (Figure 17, Table 12).

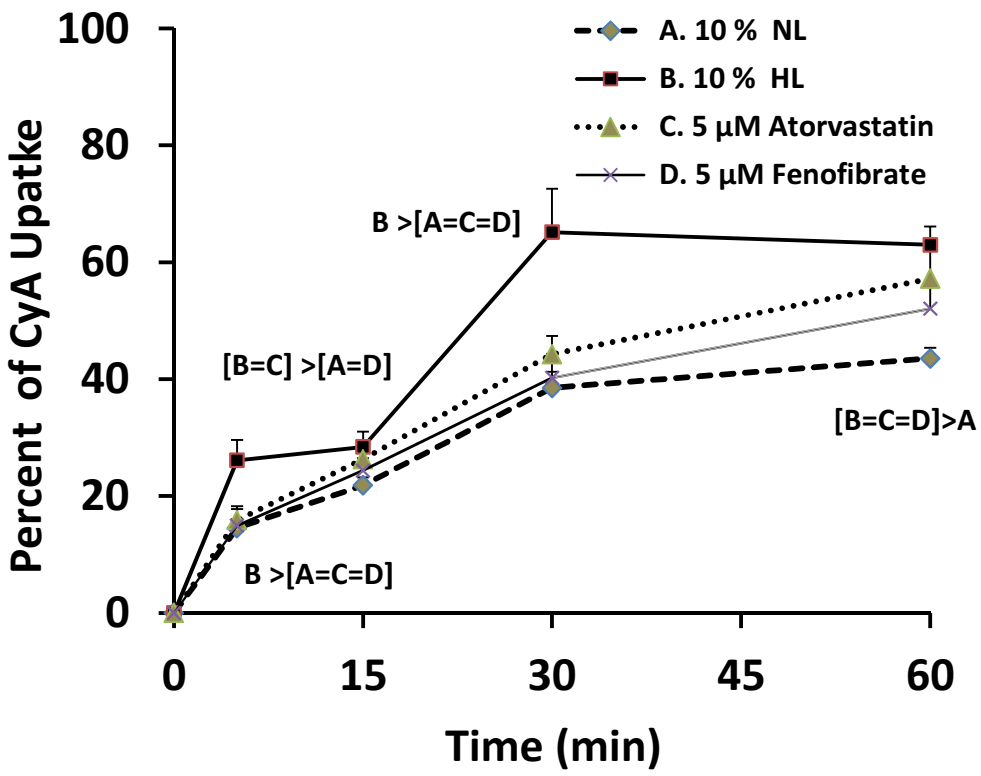


Figure 17. Percent of CyA uptake (mean ± SD) in primary rat hepatocytes. Cells were obtained from normolipidemic rats, pre-incubation for 24 h with 10 % serum normolipidemic (NL) or hyperlipidemic (HL) rats or 5 µM atorvastatin or fenofibrate. CyA was co-incubated with 2 % hyperlipidemic (HL) rat serum. Three rats were used for each group with 6 wells per time point. Statistically significant differences between amounts accumulated at any given time point are indicated.

CyA coincubated with HL rat serum caused additionally an increase of nearly 11 % in the uptake of CyA. In contrast, there was a clear and significant decrease of drug uptake by both NL and antihyperlipidemic drugs (Atv and FF), with CyA uptake being decreased by about 20, 21 and 28 %, respectively. All were compared to coincubation with NL rat serum (Table 13).

Table 12: Time-dependent CyA uptake in primary rat hepatocytes. CyA was coincubated with 2 % HL rat serum (3 independent experiments, n of 6 each group).

CyA (2.5 µg/mL) coincubated with 2 % HL serum				
Treatment groups	% of CyA accumulation in primary rat hepatocytes Mean ±SD			
	5 min	15 min	30 min	60 min
A. 10 % NL	14.5±1.5	21.8±1.4	38.5±2.8	43.5±1.8
B. 10 % HL	26.1±3.5	28.4±2.6	65.1±7.4	62.96±3.12
C. 5µM Atorvastatin in 10% NL	15.8±1.9	26.3±3.1	44.2±3.1	57.2±6.6
D. 5µM Fenofibrate in 10% NL	14.9±3.3	24.4±2.2	40.2±4.1	52.1±4.5
Ranking	B >[A=C=D]	[B=C] >[A=D]	B >[A=C=D]	[B=C=D]>A

For each time point comparisons across groups were made using a one-way AVOVA followed by Duncan post hoc comparison. Data analysis was performed using Sigma Plot 12. The results were considered statistically significant when $P < 0.05$.

The differences in AUC 0-1h among treatment groups were statistically significant between coincubation groups and also within pre-incubated groups (Table 13). The difference between pre-incubated treatment groups was compared with their respective NL control group and differences between coincubated hyperlipidemic groups and coincubated normolipidemic groups for each was also calculated. For statistical comparison, by using Bailer's method with Bonferroni correction , the AUC of percent of drug accumulated versus time was calculated using the linear trapezoidal rule from the time of adding the drug to the last measured time point up to 60 min, using the method to assess for significance of comparisons between AUC values.

Table 13: Summary of CyA uptake in primary rat hepatocytes. (3 independent experiments, n of 6 in each group).

Preincubation treatments	**CyA in 2 % NL serum**		**CyA in 2 % HL serum**		**% difference (AUC0-1h) µg·h/mL**
	(AUC0-1h) µg·h/mL	**% CyA increased**	**(AUC0-1h) µg.h/mL**	**% CyA increased**	**% increased or decreased**
A. 10 % NL	60±4.7	-	48±1.5	-	-20 ^
B. 10 % HL	66±4.9	10	74±4.6	54.2	10.8 ^
C. 5µM Atorvastatin in 10% NL	73±3.3	21.6	58±3.3	20.8	-20.5 ^
D. 5µM Fenofibrate in 10% NL	74±4.5	23.3	53±3.3	10.4	-28.4 ^
Ranking*	[C=D] >B>A	-	D>C> B>A	-	-

Asterisk represents the statistically significant difference between pre-incubated groups compared to control group in 10 % NL rat serum, ^ represents the statistically significant difference between coincubated hyperlipidemic groups and coincubated normolipidemic groups. The Bailer's method of assessing for significance of comparisons between AUC values was used with Bonferroni correction.

3.5.3. Effect of rat serum and antihyperlipidemic drugs on hepatic LDLr and MDR1A mRNA expression involved in CyA hepatic uptake by primary rat hepatocytes

Atv significantly up-regulated LDLr mRNA with an almost 5 fold increase compared to the 10 % NL rat serum control group. The HL rat serum increased LDLr mRNA 1.8 fold, but that was not significantly different from controls. In addition, LDLr mRNA levels did not change in the presence of NL rat serum or FF (Figure 18, table 14).

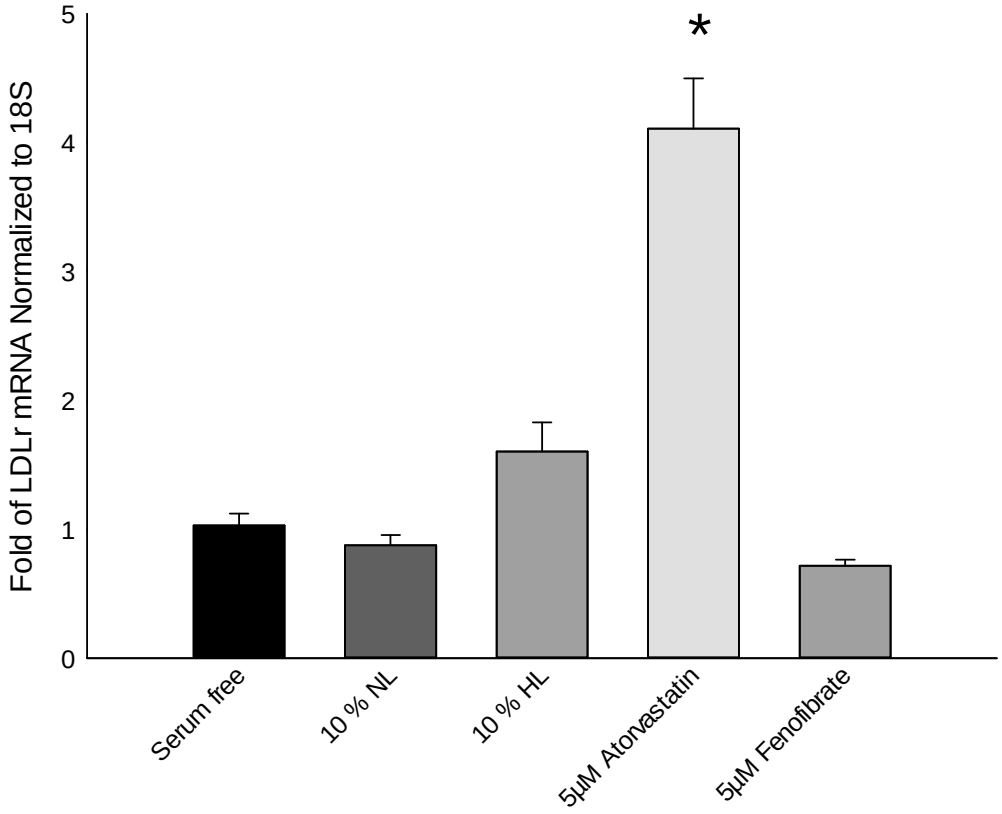


Figure 18. Freshly isolated rat hepatocytes were treated with 10 % NL rat serum or 10 % HL rat serum or 5 µM atorvastatin or fenofibrate for 24 h. LDLr mRNA levels were quantified using RT-PCR and normalized to 18S. Duplicate reactions were performed for each experiment, and data are presented as mean ± standard error of the mean of 3 independent experiments (3 rats and n of 6 in each group). (*) $P < 0.05$, compared to control (10 % NL).

Table 14: The effect of rat serum (10 % NL or HL) and antihperlipidemic drugs on transporting protein mRNA expression by isolated rat hepatocytes. Data shown as the mean of mRNA fold increase or decrease ± standard error of mean. (3 rats ,n of 6 for each treatment group).

Treatment groups	**LDLr (mean±SEM)**	**MDR1A (mean±SEM)**
A. Serum Free	1.03±0.09	0.99±0.10
B. 10 % NL	0.88±0.08	1.00±0.05
C. 10 % HL	1.60±0.22	0.69±0.06*
D. 5µM Atorvastatin	4.11±0.39*	0.67±0.06*
E. 5µM Fenofibrate	0.72±0.04	0.90±0.11
Ranking*	D>[A=B=C=E]	[C=D] > [A=B=E]

* ANOVA followed by post hoc ranking test (using Dunn's Method).

The MRD1 mRNA levels were down regulated in the presence of 5 µM Atv and 10% HL rat serum, with statistically significant reductions compared to the 10 % NL rat serum control group. MDR1A mRNA did not alter in presence of 5 µM FF or in the presence of serum-free media (Figure 19, table 14).

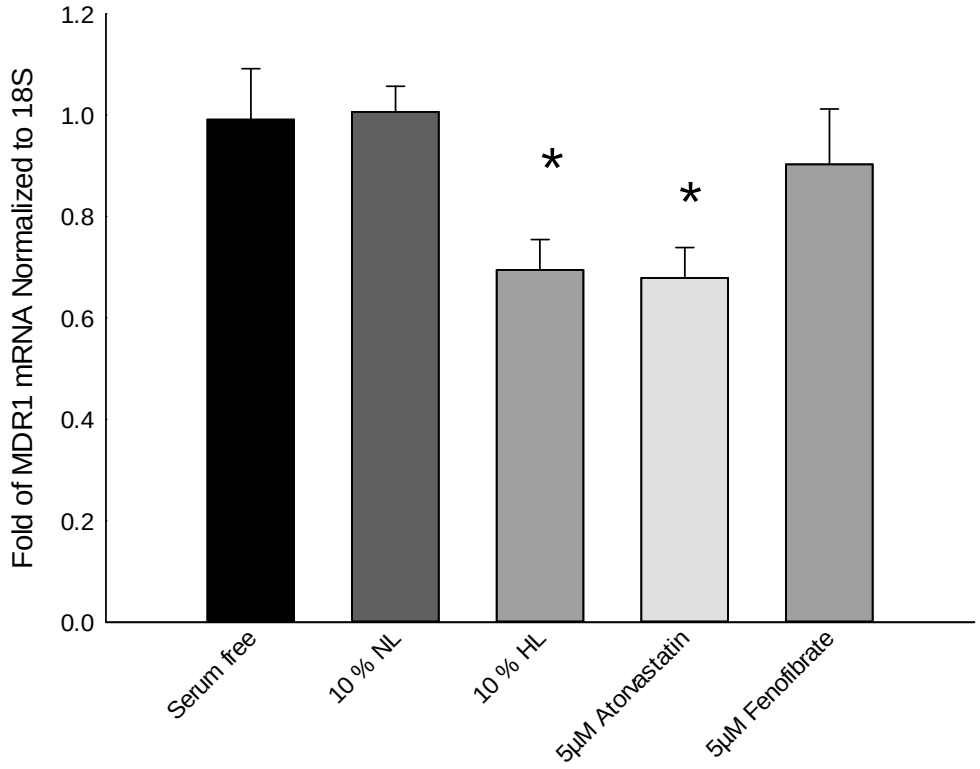


Figure 19. Freshly isolated rat hepatocytes were treated with 10 % NL rat Serum or 10 % HL rat serum or 5 µM atorvastatin or fenofibrate for 24 h. MDR1 mRNA levels were quantified using RT-PCR and normalized to 18S. Duplicate reactions were performed for each experiment, and data are presented as mean of mRNA fold increase or decrease ± standard error of the mean of 3 independent experiments (3 rats and n of 6 each group). (*) $P < 0.05$, compared to control (10 % NL).

4. Discussion

4.1. Development of an analytical method for azithromycin using Liquid Chromatography-Mass Spectrometry (LC-MS)

Several of the pre-existing methods cited for assay of AZ were performed in biological specimens, including plasma, serum, and tissues. These methods involve bioassays [176], which can have issues with inadequate sensitivity and cross-reactivity. Several high performance liquid chromatographic (HPLC) methods have also been described, using ultraviolet [177-181] and fluorescence detection [13, 14]. In order to increase sensitivity, pre-column derivatization followed by fluorescence detection has also been used, although this can introduce elements of time and complexity [182-184]. HPLC with electrochemical detection has also been used [185, 186]. Various extraction methods have also been reported for the extraction of AZ from plasma including protein precipitation [187], pressurized liquid extraction (PLE) [188], solid phase extraction [186, 189] and liquid–liquid extraction [131, 190]. Recently, the most sensitive techniques involve liquid chromatography coupled to mass spectrometry (LC-MS) [131, 187, 190, 191] or to tandem mass spectrometry (LC-MS/MS) [132, 192] .

For the present method workup, the extractability of AZ from plasma was tested in recovery experiments using diethyl ether, ethyl acetate, hexane and a TBME /hexane (50:50 % v/v) mixture. In contrast to what has been reported by some authors [131], we found diethyl ether to be the most effective in terms of removing endogenous substances and providing a higher recovery. Compared to

some other methods, our method is comparatively simple, sensitive, specific and reproducible. The terminal phase half-life of the drug is over 40 hours, and to characterize this phase, which begins approximately 24-48 hours after dosing, it is necessary to measure concentration ranges from 25 to 50 ng/mL. Thus, a reasonable target for the lowest quantifiable concentration of the drug for complete PK analysis after single doses is minimally achieved with a lower limit of quantitation of 10 ng/mL, which was achieved by the developed method.

The advantage of the current method is less complexity and shorter sample preparation time. To prepare 30 samples it took about 1.5 h to the time of injection, with an additional 300 minutes required for analytical run time. The matrix effect was negligible, and within the range of assay variability. Organic liquid-liquid extraction is a simple and effective method of affording sample cleanup for most analytes. Unlike the published complicated sample preparation, a one-step extraction procedure was used, which can reduce sample preparation times, which is important for large sample batches. Other assays required either a complicated extraction and/or derivatization procedure [182, 183]. The developed method has adequate sensitively to be used in PK studies. It can also be considered simple and cost effective. The disadvantage of the current method is the fact that instrumentation is quite expensive and is not as accessible as conventional HPLC in most laboratories. In addition, the LLQ was achieved by a using larger volume of matrix of 0.5 ml human plasma.

4.2. Azithromycin pharmacokinetics in an obese population

To our knowledge, we performed the first PK study that has examined AZ levels in obese patients. The assay was applied as a tool to investigate the effect of obesity on AZ PK. One might expect that the accelerated gastric emptying rate, higher cardiac output and increased gut perfusion could lead to an increase in drug bioavailability and rate of absorption [88]. However, from the relatively limited data that has been published it is difficult to make generalized conclusions regarding the effects of obesity on drug disposition [36, 94, 125, 193]. The rate and the extent of absorption of orally administered drugs do not significantly differ between obese and non-obese subjects in the case of the following drugs: cyclosporine [84], dexfenfluramine [85], midazolam [86] and propranolol [87]. In addition, the rate of absorption of a low-molecular-weight heparin, enoxaparin, after subcutaneous administration and after a single intravenous infusion appeared to be slower in the obese volunteers [44] while the extent of drug absorption did not change. Similarly, using ^{125}I-labeled rapid-acting insulin in patients with T2DM, there was no effect of obesity on absorption rate from subcutaneous injection sites.

The trend of our results was toward a decrease of both the AUC and the maximum serum concentrations of AZ in an obese population compared with healthy lean subjects. In general, the current findings appear to be in agreement with previous reports [115, 132, 175]. Each of the PK parameters (AUC_{0-24}, Cmax and Tmax) measured in our obese subjects was comparable with previous observations in healthy volunteers. In addition, oral clearance in our obese patients seems to be

higher than those reported in lean subjects. The mean partial half-lives ($t_{1/2}$) of obese patients were also calculated but we could not compare them to lean subjects since no data are available regarding elimination rate constants. The higher oral clearance might explain why the absorption of drug seems to be lower in obese patients. In addition, since we did also calculate the mean $t_{1/2}$, these values underestimate the true terminal phase $t_{1/2}$ of the drug and overestimate the true oral clearance, since AZ has long half-life over 40 h and longer sampling periods of at least up to 3 half-lives (~120h) would have been required to accurately measure half-life and calculate AUC from time zero to infinity $AUC_{(0-\alpha)}$ in order to calculate true oral clearance.

4.3. Effect of RYGB on azithromycin bioavailability

By using the assay we were also able to perform the first controlled study that has examined AZ levels after gastric bypass. A previous case report suggesting that amoxicillin and nitrofurantoin absorption may be compromised after gastric bypass appears to be the only other published report on this topic involving anti-infective agents [19]. The assay reported here was applied to analysis of AZ in a PK study following oral administration of a 500 mg AZ tablet to 14 obese patients who had undergone RYGB surgery. The 24 h AZ plasma AUC values in gastric bypass subjects were reduced by 32 % compared with matched control obese subjects. Gastric bypass circumvents the upper gut, which is the site of maximal absorption for AZ [47, 112, 194]. This might be an explanation for reduced AZ bioavailability post bypass. However, we found that the bypass group had a

higher oral clearance and that might explain why the absorption of drug seems to be lower in post RYGB. There was a lack of a difference in partial half-life between the two groups which argues against a difference in volume of distribution. These values were likely overestimating the true oral clearance since AZ has a long half-life over 40 h, and a longer sampling period at least up to 3 half-lives (~120 h) would have been ideally required to accurately measure half-life and calculate AUC from time zero to infinity Indeed our t½ estimates were much shorter.

RYGB surgery also leads to reduced levels of serum lipids, which causes the lipid/LDL levels to return to more normal values at ~ 2 years post-surgery. The improvement in HL is more prevalent with the malabsorptive procedures such as RYGB surgery and is sustained up to 1 year [195, 196]. After surgery there are significant beneficial changes in plasma LP and lipid profiles, with reductions in LDL, CHOL and TG, and increases in HDL [65, 66].

It has been reported that binding of AZ to plasma protein increases when its serum concentrations decrease, with 50 % binding at a lower serum concentration of 50 ng/mL and less than 12 % at higher serum concentration of 500 ng/mL [197]. As we observed in our patients that their plasma lipid levels returned to normal values post RYGB surgery. Subjects were matched for BMI; there is a possibility that the recovery from HL can change AZ disposition by altering AZ plasma or tissue unbound fractions and facilitating its tissue uptake by LP receptors and/or other drug transport proteins. It is possible that this procedure may alter drug PK by altering transporter expression and play a role in reducing

AZ absorption as it down regulates the transporters in the gut wall via its effect on lipids. Thus lower plasma concentrations in the surgical patients would imply corresponding decreases in tissue concentrations.

The efficacy of AZ is well correlated with the AUC to minimum inhibitory concentration (MIC) ratio [118]. Antibacterial action is optimized when this ratio is maximized, and increases with the initial dose ingested [118, 130]. This finding might have impact on clinical efficacy if tissue levels of the drug are not adequate for successfully clearing infection. The peak tissue concentrations achieved after ingestion of a 500 mg AZ dose range between 2–10 mg/L; therefore, tissue drug concentrations appear adequate for antibacterial activity since the AZ MIC for streptococcus pneumonia, the commonest cause of CAP, is ≤ 2 mg/L [115]. Thus, even with a reduced oral bioavailability, tissue levels are adequate for successfully clearing infection pathogens. However, there is a possibility of treatment failure with more resistant bacterial strains which need higher drug levels inside the tissue.

4.4. Cytokine levels in obese and post RYGB patients

A normalization of adipokine and inflammatory cytokine concentrations may occur after RYGB surgery [198]. For example, serum leptin, which is elevated in obesity, begins to drop after bariatric surgery toward those levels present in lean body weight subjects. The reduction in leptin was correlated with the magnitude of post-surgical decrease in body weight [199]. Another study examined the levels of leptin and adiponectin in 9 patients at 3 weeks post RYGB. The results showed

that leptin levels were significantly lower, with no difference in adiponectin levels observed. However, the mean reduction in body weight/BMI in these patients was very low, with only 7.4 % weight loss [200]. With the 30 % weight loss effect of the surgery, we have also observed this in our patients after bariatric surgery. There was an almost 50 % decrease in leptin levels at 19.24 ± 12.7 (mean ± SD), but significantly above that seen in lean subjects (Figure 8). All patients were still considered obese (mean BMI 36.8) at the time of the evaluation. However, leptin levels in the obese control group were 37.13 ± 16.7 ng/mL (mean ± SD) and within the normal range for obese subjects (values range between 3.9 and77.3 range ng/mL).

RYGB has been observed to cause a relatively quick (within 3 weeks) decrease in some other pro-inflammatory cytokines (TNF soluble receptor and C-reactive protein) and increase in adiponectin that stabilizes and does not continue to change with time as weight loss progresses. Other studies have shown that for other cytokines, (TNF-α, IL-6), however, there was no discernible decrease in cytokine levels [111, 201]. Our results also indicated that IL-6 levels were not different between patients' post-surgery (mean 25 months) compared to weight-matched obese controls. Despite this, however, the patients were still considered obese (BMI 36.8) at the time of the evaluation, although they had experienced substantial weight loss (30 %); the serum concentrations of IL-6 were still significantly above those seen in a lean healthy population. The data seem to imply that after the surgery there is a healing process associated with the surgery itself, changes in nutritional uptake, or longer term changes associated with the

weight loss and reduction in inflammation that begins to normalize these biomarkers. In an experimental setting, mild inflammation induced by the administration of bacterial lipopolysaccharide to healthy volunteers caused significant decreases in the CL of antipyrine, hexobarbital and theophylline [202], presumably due to a decrease in CYP expression. Increases in TNFα, IL6, CRP and α1 acid glycoprotein were demonstrated after administration of the endotoxin in the volunteers. Perhaps because of their relatively recent discovery, there has been no attention paid to the impact that adipokines, or their combination with cytokines, may have on altering other important aspects of drug disposition.

Inflammation is associated with changes in drug transporter expression. Inflammation in rats can lead to a suppression of P-glycoprotein (Pgp) in liver and various gastrointestinal segments [203, 204]. Human hepatocytes exposed to the cytokines TNFα and IL6 have likewise been seen to cause decreases in a number of the non-energy consuming transporters encoded by SLC genes, including OATP1B1/1B3/2B1, OCT1, OAT2 and sodium taurocholate-transporting polypeptide [205]. These decreases followed a dose response pattern, with larger decreases occurring in the presence of greater concentrations of each cytokine. The same group found that of a number of the energy consuming ABC proteins, only expression of bile salt export pump was diminished by TNFα; multiple drug resistance gene (MDR1; coding for Pgp), multidrug resistance gene-associated proteins (MRP) 2 to 4, and breast cancer resistance protein (BCRP) were unaffected. In contrast, IL6 caused decreases in MDR1, MRP2 and 4, and BCRP

[205]. In the liver of rats exposed to bacterial lipopolysaccharide findings similar to those in human hepatocytes were seen [206]. Some of the substrates for these transporters may represent therapies that are commonly administered to obese subjects. For example, OATP2B1 plays a role in the intestinal absorption of several statins, which are used to treat hyperlipidemia [207, 208].

AZ is a known substrate for organic anion transporting polypeptides (OATPs) [116, 117], and we observed in our patients that their plasma leptin and IL-6 levels were still higher post RYGB surgery compared to lean subjects. There is a possibility that the inflammatory biomarkers played a role in reducing AZ absorption as they down regulated these transporters in the gut wall. However, AZ is also substrate for P-glycoprotein and multidrug resistance-associated protein 2 (Mrp2) and these transporters play an important roles in AZ biliary excretion [116, 117].The down regulation of these transporters might lead to increases in AZ absorption which we did not see it in our study as AZ bioavailability had instead been reduced.

4.5. Effect of rat serum on hepatic mRNA expression by primary rat hepatocytes in vitro

Isolated primary hepatocytes are widely used as an *in vitro* tool to study hepatic drug uptake. They provide a full complement of intact plasma membrane (membrane permeability, active transport of drugs and active excretion) compared to *in vitro* systems such as cell lines. There is a possibility that HL may alter drug

PK by altering transporter expression. Transport proteins can be divided into two major groups. Uptake transporters mediate the uptake of drugs from the blood into hepatocytes, whereas export pumps actively secrete drugs or drug conjugates out of hepatocytes into bile [209]. Numerous influx (OATPs, MRPs, OCTs PEPT) and VLDL, LDL lipoprotein transporters and efflux (P-gp) transporter proteins can be involved in absorption; Vd and CL of many drugs and the distribution of these transporter proteins are vary throughout the body.

The receptors for LDL and VLDL particles were examined because they are potentially involved in cellular uptake of the LP-bound drug; they can function as drug transport proteins for drugs that are embedded in LPs. Our results had showed that HL rat serum downregualted lipoprotein receptors and could affect tissue uptake for wide range of highly lipophilic drugs. Indirectly they can also influence the rate or extent of drug CL by controlling the amount of drug that can access the drug metabolizing enzymes. During the drug development process the target goal for many new drug candidates is to be highly lipophilic. They become targets of P450 metabolism, leading to higher clearance and a stronger affinity for pharmacological receptors than more polar drugs [210]. The LDLr is extensively expressed in the liver and VLDLr extensively in heart and endothelial tissues [156, 158]. Our results showed that indeed LPs down regulate hepatic lipoprotein receptors such as LDLr and VLDLr. These findings could explain the changes in drug tissue concentrations in the HL state; however, depending on the type of tissue and degree of drug binding, tissue drug concentrations might change. Not

only will this impact the uptake of drugs into the liver but also into other tissues. CyA, AM, halofantrine, amphotericin B, midazolam, ketoconazole, nifedipine and nelfinavir are examples of lipophilic drugs known to bind with LPs, and their PK profile changes in the HL state in rat [135, 136, 142, 146, 150, 154, 160, 211, 212].

The OATPs are a family of influx transporters expressed in various tissues and are important determinants of anionic drug absorption. OATP1B1, OATP1B3 and OATP2B1 are expressed in the liver [207, 208, 213-216]. We measured the gene expression of OATP2B1, which has been reported to be a facilitator of AM influx [217]. What we observed was a significant down-regulating effect of HL on this gene that may impair transport, metabolism and biliary secretion of numerous drugs in the liver. OATP2B1 is expressed in several other tissues such as the intestines, kidney, and placenta [209]. Thus, this finding highlights not only that the AM uptake by liver might be changing but also that AM absorption and excretion could change in the HL state. Furthermore, several other drugs, e.g. benzyl penicillin, fexofenadine, ezetimibe glucuronide and montelukast, are known to be substrates for OATP2B1 [209]. The PK of these drugs might be affected by such changes in OATP protein expression in HL. OATP2B1 may play a significant role in the intestinal absorption of some statins (e.g., atorvastatin, fluvastatin and rosuvastatin) [207]. Atv is substrate of OATP2B1, OATP1B1 and OATP1B3 and they are major determinants of Atv hepatic uptake *in vivo* [216]; Atv is commonly used in treatment of HL.

The impact of HL itself on treatment with drugs such as Atv should also be considered when the drug is used in HL patients. This can have important effects on the disposition and potency of the drug. This is not an easy issue because patients with HL often suffer from other medical conditions such as obesity, diabetes and elevations in blood pressure which require additional medications for their treatment. An *in vitro* animal model of obesity has shown that OATP2 mRNA and protein levels were significantly diminished in obese rats with fatty livers [218]. However, here in our study, the poloxamer 407 HL rat serum model used is fairly restricted to increased plasma LP which does not result in full blown metabolic syndromes such as obesity and diabetes. Other studies also have shown that not only OTAB2B1 but also protein expression of OATP1A1 were significantly decreased in high-fat animals, while OATP1A1 and OATP1B2 expressions were significantly lower in HL rats when compared to controls [219]. Thus, we have evidence that hyperlipidemic serum (LPs) itself downregualted the expression of OATP mRNA in primary rat hepatocytes.

P-glycoprotein, also referred to as multidrug-resistance protein (MDR), is an example of an efflux transporter that is located on the blood-brain barrier, adrenal gland, kidney, intestine and liver with relatively different expression patterns [80, 220-222]. LPs caused a reduction in MDR mRNA in the liver; this suggests impairment of the biliary secretion of numerous of high molecular weight drugs in the liver. In addition, absorption and tissue distribution of P-gp substrates is expected to increase, whereas elimination might be reduced. AZ, sirolimus,

tacrolimus, digoxin, verapamil, diltiazem, and sotalol are known to be substrates for P-gp [65, 67]. They are more likely to be affected with the reduction of P-gp expression and/or function. In addition, some LP-bound medications such as CyA and AM have been found to have an inhibitory effect on Pgp; therefore they are expected to have drug interactions with their substrates adding on another factor beside the effect of HL itself on the transporter [151].

HL might also be associated with changes in CYP protein expression. Indeed, this in combination with increased LP-containing drug particles and down regulation of LP receptors, could lead to a decrease in drug metabolism. Drug clearance is affected by differences in CYP activity and the metabolic interactions of drugs and endogenous substances with these enzymes. Therefore, variability in CYP3A expression is expected to have substantial effects on both the efficacy and safety of narrow therapeutic window drugs like CyA and AM that are metabolized by CYP3As. CyA is metabolised by CYP3A enzymes [223, 224]. The two main metabolic reactions involved are hydroxylation and N-demethylation [224]. Several enzymes have been shown to be responsible for AM metabolite formation in the liver and intestines. CYP2D1, CYP2C11 and CYP3A2 are the major enzymes involved in AM metabolism in rats [225]. We had reason to believe that a change in CYP3A and/or other isoforms such as CYP2C11 and CYP2D1gene expressions by HL may have an impact on drug PK [149]. CYP1A1 and 1A2 facilitate the biotransformation of AM to the pharmacologically and toxicologically active metabolite, desethylamiodarone (DEA) [225]. Primary

cultured hepatocytes are useful tools for investigating drug metabolism, induction of drug metabolizing enzymes, and screening of cytotoxic and genotoxic compounds [167]. For this reason, using a subset of hepatocytes, we examined the gene expressions not only of transporter proteins but also other metabolizing enzymes for which CyA and AM are known to be substrates. We determined the effect of serum LPs on the hepatic CYP1A1, CYP2D1, CYP3A2 and CYP2C11. Our results were in line with the previous reports in which an experimental hyperlipidemia rat model (P407) had been shown to down regulate the expressions of CYP3A1/2 and CYP2C11 hepatic drug metabolizing enzymes [160, 226]. Additionally, in regards to CYP activity and/or expression, other models of HL resulted in comparable observations. As an example, researchers recently showed a decrease in the expression of a number of drug metabolizing enzymes and transport proteins was exhibited by the Zucker obese rat [227]. A down-regulation of hepatic expression of CYP3A after rats were fed a high fat diet has also been reported [228].

Our findings perhaps could help to explain the decrease in CyA and AM metabolism in hepatocytes. Since CYP2C11 and CYP3A1/2 are regulated by the transcription factors CAR and PXR, the inhibition of these nuclear transcriptional factors which might be responsible for down regulation [229]. These findings suggest that not only CyA and AM metabolism might be affected in an HL state. CYP450 enzymes catalyze the oxidative metabolism of a wide range of exogenous and endogenous lipophilic substrates [230]. CYP3As are the most abundant P450s in the liver.. In humans, CYP3A enzymes contribute to the

metabolism of approximately half the drugs that are currently in clinical use [114]. Therfore, these findings can impact a wide range of drugs for which CYP enzymes are responsible for their metabolism. They are expected to have drug interactions with their substrates, adding on another factor beside the effect of HL itself on the transporter.

4.6. Cyclosporine uptake study

In NL human plasma, 31 % of CyA is associated with combined LDL and VLDL classes and 44 % with HDL [163]. In human HL plasma, the CyA unbound fraction was found to be less than 10 % [142], and the drug shifted into the LDL/VLDL fractions from the HDL fraction [163]. Poloxamer 407 caused 32.9 and 42.3-fold increases in TG and CHOL in rat plasma, respectively. CHOL concentrations were greatly increased in the TRL and the LDL fractions; they were increased by 125 and 13 fold, respectively compared with NL rat plasma [161]. In HL rats, the CyA unbound fraction was found to be from 16 to 30 % [142], and the CyA AUC in plasma, blood, kidney, and liver was increased compared with NL rats. However, the AUC of CyA was decreased in heart and spleen [146]. Previous studies had suggested that the LDL may be playing a role in cellular drug uptake of CyA, aminoglycosides and anionic liposomes [231-233]. Recently, a study had shown that LDLr overexpression was associated with an increased uptake of radiolabelled CyA [234]. However, other studies have reported instead that LDL decreased CyA uptake into cells via the LDLr receptor [235].

The current study using an *in vitro* cellular system found that when CyA was co-incubated with NL rat serum, there was a significant increase in the CyA uptake when hepatocytes were preincubated with diluted HL rat serum nearly up to a 10% increase. In addition, the same trend was observed with a significant and even greater increase in CyA uptake when hepatocytes were preincubated with either 5 µM Atv or FF [nearly up to 21 % and 23 % increase, respectively]. This could be explained due to the effect of HL and drug treatments on the efflux transporter. The increase in CyA uptake when preincubated with HL rat serum and Atv could be attributed to a decrease in P-gp efflux transport. This ultimately results in more drugs being inside the cells. Therefore, we examined the expression of the MDR gene, which codes for P-gp for which CyA is known to be a substrate and inhibitor. Atv caused downregulation of P-gp mRNA nearly 33 % (Table 14). As a result, more CyA will be inside the cells. Our gene expression results are in line with another study, which showed that Atv treated with 1, 10 and 20 µM of Atv caused dose-dependent down regulation of P-gp mRNA of nearly 60 % in HepG2 cells, explained as a decrease in mRNA stability [236].

In addition, HL also caused down-regulation of P-gp mRNA by almost 31 % decreases. This possibly could explain the mechanism behind our findings with the preincubated HL groups. Unexpectedly, FF increased CyA uptake more than the two other treatments, up to 23 %. It has been reported that FF has an inhibitory potency against P-gp at concentrations of 25 µM, which are much

higher than in our study [237]. FF did not alter P-gp or LDLr mRNA levels, perhaps because the inhibitory drug concentration is much lower here, at only 5 µM.

Interestingly, the addition of HL sera led to significant increases up to 54 % compared to the NL preincubation group. Atv led to a significant increase in CyA uptake (nearly 20 %), while FF caused only a 10% increase of CyA uptake compared with NL preincubation group. These findings could be explained by the effect of treatments on LDLr-mediated uptake and was confirmed by RT-PCR results as Atv caused a 5 fold increase in LDLr mRNA compared with the NL control group. Our results were in line with other studies. In humans, 20 mg Atv daily for 4 weeks increased LDLR mRNA expression by approximately 30–40 % in mononuclear blood cells of normal subjects [1]. Atv also up-regulated LDLR mRNA expression in circulating mononuclear cells in humans [238].

In rat, Atv (0.5 to 10 µM) concentrations also increased LDLr expression 5 and 2 fold in HepG2 and Hep3B cells respectively, and almost 3 fold in the liver of Sprague-Dawley rats [239]. However, HL and FF did not change the LDLr mRNA. FF is known to act on an enzyme in the blood called LP lipase. The structure of the LDL substances already present is altered when this enzyme breaks down fats, resulting in a decrease in the triglyceride levels. This results in an increased particle size, which the body can more easily break down. However, this is not the case in our results since FF preincubated had washed out before CyA was added.

It has been reported that CyA binds more with TRL following with LDL in HL rat plasma. However, CyA binding shifts into the HDL fraction in NL rat plasma [161]. Unexpectedly, the observations of CyA co-incubated with diluted HL rat serum were against our hypothesis. With HL, the addition of HL rat serum led to restricted accessibility of CyA to the cells and was associated with less CyA available inside the cells (Table 9). HL caused a decrease of CyA uptake ranging from 20 to 28 %. These results suggest that the LDLr plays a minor role in CyA hepatic uptake. This was clear with Atv in which CyA uptake increased by the same trend up to 20 % in both NL and HL coincubated groups. This was in line with our findings regarding the LDLr gene expression in which the HL preincubated group did not change LDLr mRNA expression. This raised the possibility of other factors besides LP receptors being involved. The mechanisms are not clear but it perhaps could suggest that the CyA-bound fraction transported by other ways could be affected by preincubation with LPs rather than the LDLr; further studies are needed to clarify these findings.

Many patients develop HL after transplantation, and the use of CyA in combination with steroids has caused an increased incidence of post-transplant dyslipidemia [240, 241]. Statins are commonly used for treatment of dyslipidemia. Our findings are highly clinically significant and relevant as renal transplant recipients typically receive multidrug therapy. Therefore, there is the possibility of CyA and antihyperlipidemic drugs (ATV and FF) interacting when they are coadministered to these patients. CyA is highly bound to LPs in plasma,

and the lipid-lowering effect of statins will possibly alter its free fraction. In humans, Atv has been shown to increase the CyA AUC by approximately a 10 % - 13 % in stable liver transplant recipients [242]. However, other studies have shown instead a 9.5 – 13.5 % decrease in CyA AUC and maximum concentrations (Cmax) with coadministration of statin therapy [240, 243, 244]. The current literature conflicts in regard to the impact of Atv on CyA PK in renal transplant recipients. In our study, Atv caused an increase in hepatic CyA uptake in rats by nearly 20 %, and led to more CyA accessible to the liver. Our findings indicate that the combination of an inhibition of CYP450-mediated metabolism, an up regulation of LDLr mRNA and a downregulation of P-gp mRNA could be a possible contributing mechanism for this interaction. In addition, FF might also increase CyA hepatic uptake when coadministered with CyA to hyperlipidemic transplant patients. In general, these findings highlight that not only can CyA hepatic concentrations be affected by antihyperlipidemic drugs but also there is the possibility of an increase in CyA uptake in other tissues such as heart or kidney possessing LP receptors.

5. Conclusion

A selective, simple, sensitive and rapid liquid chromatographic–electrospray ionization mass spectrometric method for determination of AZ in human plasma was first developed and validated. This assay offers a simple and relatively rapid alternative tool for quantifying AZ levels that exhibits selectivity and sensitivity, with validated lower limits of quantification of 10 ng/mL using volumes 0.5 mL of plasma which are suitable for PK studies.

The present method was successfully applied to measure AZ concentrations in the samples of 14 obese women and demonstrated that the method is reproducible. To our knowledge, we have performed the first PK study that has examined AZ levels in obese patients. Each of the PK parameters measured in our obese subjects were comparable with previous observations in healthy volunteers. Until more studies on large numbers of patients can be done, dosage adjustment of AZ does not seem to be needed in obese populations.

By using the assay we were also able to perform the first controlled study that has examined AZ levels after gastric bypass. After RYGB, AZ concentrations were substantially reduced in gastric bypass subjects compared with controls. Although the clinical relevance is currently uncertain, dose modification and/or closer clinical monitoring for treatment failure may be required. In addition, the remission effects of post-surgery diseases like obesity and hyperlipidemia that are usually associated with the effect of the surgery itself on the distribution and

clearance of drugs must be considered before attributing abnormal serum concentrations of drugs to malabsorption and making final conclusions after RYGB surgery. In general, until more PK studies are available, practitioners should apply the principles of pKa and log P of individual drugs in an attempt to predict the potential impact of the RYGB on a drug's absorption, which requires a more in-depth look at mechanisms of drug absorption. More studies should be done on the changes in disease states (hyperlipidemia and obesity) associated with the surgery.

Regarding cytokine levels, the present analysis has added to previously published data by analyzing the serial changes in leptin and IL-6, 25 months after RYGB. Our results were in line with previous studies; however, the levels of both leptin and IL-6 were higher compared to published literature data from lean subjects. This suggests that the surgery might initiate an inflammatory state, with reductions in biomarkers to normal levels not seen until longer after. Complications associated with the surgery could, at least in part, underlie some of these responses in the inflammatory markers. An explanation for the potential delay in the reduction of the inflammatory biomarkers is that the severe energy restriction after this type of surgery leads to persistent stress that can elevate certain markers.

From our hepatocyte work, the results showed that serum LP downregualted all hepatic mRNA of the genes that were studied. The extent of decrease in

expression was most noticeable for OATP2B1 (81 % reduction), and for CYP2D1 (84 % decrease). Our results confirmed our hypothesis that HL indeed downregualted mRNA expression of LP receptors such as LDLr and VLDLr and OATP2B1 transporter proteins, as well as a number of metabolizing enzymes such as CYP1A1,CYP2D1, CYP2C11 and CYP3A2 and the P-gp efflux transporter. All of these effects could lead to change in the PK of protein-bound drugs and drugs which are known to be substrates for these proteins. These changes are expected not only in the liver but also in other tissues in which these transporters and enzymes are known to be expressed. More studies are needed to determine the possible influence of LPs on the expression of other influx transporters like OCT and OTP in liver and other tissues. Furthermore, studies on the effect of LPs on other common CYP enzymes that known to be involved in drug disposition are needed.

Our uptake study results seem contrary to expectations based on hepatic CyA uptake increases via LDL receptors in HL serum as LPs restricted CyA access to the cells. However, our uptake studies showed that HL preincubated caused increased CyA uptake by almost 10% with an additional effect of LPs on CyA uptake when CyA was coincubated with HL rat serum. These findings suggest that HL increased CyA uptake via other complex mechanisms besides inhibiting P-gp transporters, and the possibility of involvement of LDL receptors in increasing CyA uptake cannot be completely ruled out. In addition, in the HL state, antihyperlipidemic drugs (Atv and FF) led to an increase in CyA hepatic

uptake that could potentially cause a clinically significant interaction and increase CyA PD in other tissues via LDLr, P-gp and other unknown mechanisms.

In conclusion, a clear understanding of the essential components of CMS is needed together with data to support the relative importance of each component on drug PK. Our findings could explain some variability in drug PK and unexpected dose versus effect outcomes in cardiometabolic patients that could contribute to both HL and the obesity state.

6. Future Directions

In our LC-MS analysis we reported that the LLQ was 10 ng/mL. Future work will be needed to measure such drug concentrations using 100 µL or less matrix volume. This would be helpful for collecting human samples at more frequent and longer times in clinical experiments.

Regarding PK studies, additional studies comparing AZ intracellular and tissue concentrations between gastric bypass patients and controls is needed to rule out the possibility of treatment failure, especially with more resistant bacterial strains. Further studies could be done using a suitable study design that allows for longer sampling periods to accurately measure half-life and calculate oral clearance and exclude any possibility of changes in clearance and/or volume of distribution as a result of the surgery. In addition, the effects of obesity on changes in levels of proinflammatory cytokines and on drug transporters and metabolizing enzymes could be studied further using an obese rat model.

HL was found to affect not only hepatic expression of LP receptors but also other influx and efflux transporters besides metabolizing enzymes. Further studies are required to explore the mechanisms behind these findings in other tissues such heart, kidney and intestine. Specific experiments using different LP concentrations should be performed.

We demonstrated that further studies will be required to accept the significant influence of LDLr on CyA uptake. Using hepatocytes from HL rats might result in a better explanation of our findings with the HL preincubation group, since HL increased hepatic uptake significantly despite the lower fu with HL rat serum. This pointed to the possibility of the effect of Poloxamer itself on hepatocytes in vivo. In addition, the effect of Poloxamer itself on the hepatic protein expression is worthy of future study.

We used only one drug concentration to study the pattern of drug uptake. In future a wide range of drug concentrations might be useful. Shorter experimental times with more time points should be considered to better understand the influence of LDLr on CyA uptake.

Finally, since HL was shown to potentiate the CyA-antihyperlipidemic drug interactions, further studies could be explored to determine the effect of HL on CyA toxicity in the presence of these drugs; such studies could be done in vivo in rats to see whether or not it affects drug response or toxicity in other tissues like the kidney.

7. References

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