Clinical Pharmacokinetics and Pharmacodynamics of Statins and Metformin in Obese Patients Pre- and Post-Gastric Bypass Surgery

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Clinical Pharmacokinetics and Pharmacodynamics of Statins and Metformin in Obese Patients Pre- and Post-Gastric Bypass Surgery

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Abstract

Obesity is a major health concern, with 39.8% of adults in the US being obese in 2015-2016. Bariatric surgery is the recommended treatment strategy for obese patients with a body mass index (BMI) of ≥ 40 kg/m^2 or ≥ 35 kg/m^2 with comorbidities. Obese individuals are at a higher risk of diseases of diabetes, coronary heart diseases, hyperlipidemia, respiratory problems, sleep apnea, and others. As a result, obese individuals who undergo bariatric surgery are usually on multiple medications, and most of the medications may still be needed after the surgery. Roux-en-Y gastric bypass surgery (RYGB) is one type of bariatric surgery, that results in a reduction in the stomach pouch size and the bypass of the first section of small intestine. There is scarce research regarding to the effects of RYGB on the pharmacokinetics (PK) and pharmacodynamics (PD) of medications that are widely prescribed to severely obese patients. With the popularity of RYGB in the US, an understanding of its effects on the PK, PD and PK/PD correlations of the commonly prescribed medications in this patient population is timely and crucial for rational and proper dosing modification of the medications post-RYGB. Hyperlipidemia and type 2 diabetes are two of the most prevalent comorbidities in obese patients, with statins and metformin being the first medication choices for hyperlipidemia and type 2 diabetes, respectively. In this project, the PK and PD of 3 statins and metformin were studied longitudinally in each subject through the course of 12 months post-surgery.

The objective of our study was to investigate the impacts of RYGB on the PK/PD of three of the most widely used statins which are simvastatin, atorvastatin, and their active metabolites as well as rosuvastatin, and metformin. Our study is innovative because it is the first longitudinal study for individual subjects with the same patient's pre-surgery conditions as their own controls. In addition, our study monitored the active metabolites and parent drug of statins and investigated the effect of RYGB on statin metabolisms. We hypothesize that RYGB will decrease the absorption of simvastatin, atorvastatin, and rosuvastatin, as well as metformin and affect the PK/PD of these medications and their active metabolites, if any, in patients post-surgery. To achieve the objective of our study, three specific aims were formulated:

1. a) To develop and validate a simultaneous LC-MS/MS assay for statins, simvastatin, atorvastatin, and their active metabolites, as well as rosuvastatin (no metabolites) in lipemic plasma samples from obese patients to assess the concentrations of the drugs and metabolites before and after RYGB.

   b) To develop and validate an LC-MS/MS assay for metformin in lipemic plasma samples from obese patients to assess the concentrations of metformin before and after RYGB.

2. To monitor and characterize the effects of RYGB on the PK/PD of simvastatin, atorvastatin, and their active metabolites, as well as rosuvastatin for the time course of 12 months post RYGB.

3. To monitor and characterize the effects of RYGB on the PK/PD of metformin for the time course of 12 months post RYGB.

First, we have successfully accomplished the objective of aim 1, where we have developed and validated two LC-MS/MS methods. The first method simultaneously quantifies simvastatin, atorvastatin, along with their active metabolites, and rosuvastatin. To our knowledge, this is the first report of simultaneous quantification of simvastatin, its active metabolite (simvastatin acid), atorvastatin, its two active metabolites (2-hydroxy atorvastatin and 4-hydroxy atorvastatin), and rosuvastatin in human plasma samples. The second LC-MS/MS method was utilized to quantify metformin concentrations in human plasma samples. Both methods have been validated in plasma with low (<300 mg/dl) and high (>300 mg/dl) triglyceride levels. The methods showed no interference with the quantification of all analytes, nor matrix effect from the triglycerides, and suitable for drug monitoring for statins and metformin, respectively, in lipemic plasma samples. Overall, two specific, accurate, robust and reliable methods were developed and validated for the quantification of three statins along with their

active metabolites and metformin at the LLOQ of 0.25 ng/ml in plasma with either low or high triglyceride levels.

Second, we have successively investigated the impacts of RYGB on statins and their active metabolites. Our study is the first report on the longitudinal effects of RYGB on the PK of simvastatin (n=9), atorvastatin (n=5), along with their active metabolites, and rosuvastatin (n=12) in the same subject pre- and post-surgery. The study showed a trend of significant decrease in the individual and mean plasma concentrations on the same dose per unit body weight [(nM)/(mg/kg)] of atorvastatin from 38.81 ± 2.36 at baseline to 16.30 ± 3.41 and 9.75 ± 2.52 (nM)/(mg/kg) at 3- and 6-months follow -up visits, respectively. The same trend of significant decrease was observed for the two hydroxy active metabolites of atorvastatin, 2-hydroxy and 4-hydroxy atorvastatin, with mean concentrations of 44.82 ± 6.11 and 18.75 ± 4.18 at baseline, respectively. The mean concentrations of 2-hydroxy and 4-hydroxy atorvastatin decreased to 14.62 ± 2.40 and 5.12 ± 1.20 (nM)/(mg/kg), respectively, by 3 months post-RYGB and stabilize at 10.26 ± 2.95 and 2.94 ± 0.80 (nM)/(mg/kg), respectively, at 6 months post-RYGB. Rosuvastatin individual and mean plasma concentrations on the same dose per unit body weight [(nM)/(mg/kg)] also showed the trend of decrease from 213.07 ± 22.87 at baseline to 122.56 ± 9.67 and 83.28 ± 6.39 (nM)/(mg/kg) at 3 and 6 months post-RYGB, respectively.

For simvastatin and its active metabolite, simvastatin acid, the trend was opposite with an increase in their plasma concentrations on the same dose per unit body weight [(nM)/(mg/kg)] post-RYGB. The mean concentrations for simvastatin and simvastatin acid increased from 8.52 ± 3.04 and 9.96 ± 3.99 (nM)/(mg/kg) at baseline, respectively, to 11.29 ± 1.96 and 24.89 ± 6.90 (nM)/(mg/kg) at 3-month and stabilized at 28.45 ± 3.54 and 39.32 ± 7.95 (nM)/(mg/kg) at 6-month visits post-RYGB, respectively. The effect of RYGB on simvastatin and simvastatin acid concentrations seem to be normalized to the baseline levels at 12 months post-surgery with mean plasma concentrations of 7.09 ± 0.42 and 10.45 ± 2.39 (nM)/(mg/kg), respectively.

These differential impacts on statin PK were consistent with the PD observations on LDL rebound with patients on atorvastatin and rosuvastatin, but the rebound was not apparent with patients on simvastatin when doses were reduced post-RYGB. A preliminary PK/PD correlation between the summation of the molar concentrations of atorvastatin, 2-hydroxy-atorvastatin and 4-hydroxy-atorvastatin and LDL values showed that the threshold of effective atorvastatin with active metabolites decreased from 40 nM pre-surgery to 20 nM at 3 and 6 months post-RYGB. The LDL concentrations were correlated with patients' BMI and total atorvastatin (with metabolites) concentrations post-RYGB in a linear model. A preliminary PK/PD correlation between simvastatin acid molar concentrations and LDL values showed that a trend of decrease in LDL levels with increase in SMV-A

concentrations at 3 and 6 months post-surgery. However, LDL reduction seems to level off at SMV-A concentrations higher than 5-10 nM. In addition, the LDL levels were correlated with the ratio of simvastatin acid/simvastatin concentrations and BMI in a linear model with no interaction between BMI and the simvastatin acid/simvastatin ratio post-RYGB. Moreover, both atorvastatin and simvastatin showed decreases in their metabolisms to the active metabolites post-RYGB. The ratios of 2-hydroxy atorvastatin/atorvastatin and 4-hydroxy atorvastatin/atorvastatin varied significantly at baseline among subjects, with 0.39-2.06 and 0.14-0.88, respectively. Among the subjects who completed the follow-up visits, the ratios decreased by 3 months post-RYGB by about 60%, then remained relatively constant at the lower levels. The ratio of simvastatin acid/simvastatin also varied significantly at baseline among subjects, with a range of 0.10-7.85. Among the 9 subjects on simvastatin, 5 had the PK data at the follow-up visits and showed a wide range of 3-60% decrease in simvastatin metabolism at 3 months or 6 months post-RYGB, as expressed by the ratios of simvastatin acid/simvastatin, then remained relatively constant at levels of lower ratios afterwards.

The surgery itself influenced the lipid panel in patients by decreasing LDL in the non-statin group from 110 mg/dl at baseline (higher than the optimal level of <100 mg/dl) to 91 mg/dl at 1-year post-RYGB. Combined statin group had optimal LDL levels of 90, 77, 82, and 96 mg/dl at baseline, 3, 6, and 12 months post-RYGB,

respectively. The significantly higher LDL in non-medicated patients was corrected by RYGB. The mean TG value pre-surgery in atorvastatin group (101 mg/dl) resembled those of non-statin group (118 mg/dl), but lower than those in simvastatin and rosuvastatin groups (165-183 mg/dl). At 1-year post-RYGB, TG levels decreased in all groups with 87 mg/dl in atorvastatin group, 103-116 mg/dl in simvastatin and rosuvastatin groups, and 91 mg/dl in non-statin group. The high pre-operative values of TG in simvastatin and rosuvastatin groups were corrected post-RYGB. Mean HDL levels in statin groups was 42-50 mg/dl at baseline and increased significantly at 12-month (58-64 mg/dl) follow-up visits. A similar time profile was observed in non-statin group with baseline mean HDL level of 47 mg/dl and increased to 58 mg/dl at 12-month visit. The weight loss was merely RYGB related, and statin treatments did not affect the weight loss outcomes post-RYGB comparing to non-statin group.

Finally, we characterized the effect of RYGB on metformin PK/PD with 31 subjects in the metformin group. There was a trend of continuous decrease in metformin concentrations on the same dose per unit body weight [(ng/ml)/(mg/kg)] basis after surgery, with a range of 2.1-240.2 (ng/ml)/(mg/kg) at baseline that continuously reduced to 1.8-146.7, 0.6-110.6, and 0.1-12.9 (ng/ml)/(mg/kg) at 3, 6, and 12 months post-RYGB, respectively. The surgery itself proved to have a lowering effect on HbA1c. However, only 26.3-47.3% of the patients in our study reached complete remission state at any time point post-RYGB. Our results disagreed with

the general notion that RYGB yields complete remission or resolution of type 2 diabetes in patients. The treatment failure in diabetic patients post-RYGB in our study could be correlated to the decreasing metformin concentrations post-surgery.

Overall, we have achieved the goals of our specific aims. The complexity of the anatomical and physiological changes after RYGB makes the deduction of a conclusion challenging, especially with the small subject number as in our study. However, the observed longitudinal changes in the PK and PD of statins and metformin in the same individual subjects after the surgery suggest that the concentrations of statins and metformin should be monitored and the dosing regimen can be rationally adjusted after RYGB to ensure the therapeutic efficacy of the treatment with minimal adverse effects. In addition, patients who stop statins and/or metformin treatment post-RYGB should be followed-up closely to ensure they do not have recurrence of hyperlipidemia or diabetes.

The merits of the study lie in the longitudinal monitoring and characterization of the impacts of RYGB on the PK and PD of three statins, atorvastatin, simvastatin, and rosuvastatin, along with their active metabolites, as well as the first line oral antidiabetic medication, metformin. The study utilizes the individual subjects as their own controls from pre-RYGB, to monitoring at 1 week, and at 1, 3, 6, and 12 months post-RYGB. In addition, the uniqueness of this study is being the first study

to monitor the active metabolites of simvastatin and atorvastatin post-RYGB and provide PK/PD correlations of LDL with BMI and total active atorvastatin concentrations, as well as with BMI and simvastatin acid/simvastatin concentration ratios post-RYGB. The models with future validations offer the potential to rationally adjust the dose regimen of atorvastatin and simvastatin post-RYGB.

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%EWL	Percentage Excess Weight Loss
2-OH-ATV	2-Hydroxy Atorvastatin
4-OH-ATV	4-Hydroxy Atorvastatin
AMPK	AMP-Activated Protein Kinase
ATV	Atorvastatin
BMI	Body mass index
CDC	Centers for Disease Control and Prevention
FDA	Food and Drug Administration
FLU	Fluvastatin
HbA1c	Glycated Hemoglobin
HDL	High-density Lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HQC	High (Concentration) Quality Control
IS	Internal Standard
LDL	Low-density Lipoprotein
LLOQ	Lower Limit of Quantification
LOV	Lovastatin
LQC	Low (Concentration) Quality Control
MATE	Multidrug and Toxin Extrusion
MI	Multiple Imputation

MQC	Medium (Concentration) Quality Control
NIH	National Institute of Health
OATP	Organic Anion Transporter Polypeptides
OCT	Organic Cation Transporter
PD	Pharmacodynamics
PK	Pharmacokinetics
PMAT	Plasma Membrane Monoamine Transporter
PRV	Pravastatin
QC	Quality Control
RSV	Rosuvastatin
RYGB	Roux-en-Y gastric bypass surgery
SMV	Simvastatin
SMV-A	Simvastatin Acid
SRM	Selected Reaction Monitoring
TC	Total Cholesterol
TG	Triglyceride

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Chapter 1. Introduction

1.1. Obesity

1.1.1. Definition, prevalence and comorbidities

Obesity is a complex condition of excessive fat accumulation that has mostly a negative impact on the individual's overall health. Body mass index (BMI, kg/m^2) is used as a descriptor for the assessment of overweight and obesity, and it is calculated by dividing body weight (kg) by squared height (m^2). Obesity is defined as having a BMI of ≥ 30.0 kg/m^2, and is categorized into classes based on BMI as described in **Table 1** (Jensen et al., 2014). Obesity can develop from a factor or a combination of factors including, but not limited to, diet, lifestyle, genetics, metabolism and environment (Motycka et al., 2011).

Table 1. Classification of BMI

Weight Category	**Adult BMI (kg/m^2)**
Underweight	< 18.5
Normal Weight	18.5 – 24.9
Overweight	25.0 – 29.9
Obesity (Class I)	30.0 – 34.9
Obesity (Class II)	35.0 – 39.9
Extreme Obesity (Class III)	≥ 40

* Based on the 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society(Jensen et al., 2014).

The percentage of adults in the United States with overweight and obesity (BMI of ≥ 25.0 kg/m2) has been reported by the Centers for Disease Control and Prevention (CDC) as 71.6% of adults in 2015-2016. Obesity prevalence (BMI ≥ 30 kg/m2) has been on the rise for the last two decades, with an increase in obesity prevalence from 30.5% of adults in the United States in 1999-2000 to 39.8% in 2015-2016 (Hales et al., 2017). According to the CDC latest report, all states had an obesity rate in adults of > 20% in 2017, with seven states (Alabama, Arkansas, Iowa, Louisiana, Mississippi, Oklahoma, and West Virginia) having an obesity rate in adults of ≥ 35%. The trend of increase in obesity prevalence is alarming, since only five years before that (in 2012) all states had an obesity rate of < 35% (Centers for Disease Control and Prevention, 2018).

The association of obesity with an increased risk of many disease conditions have been established (Li et al., 2015). The increased risk of type 2 diabetes is 64% higher in obese patients compared to normal weight individuals (Must and McKeown, 2000). The risk of abnormal lipid metabolism (dyslipidemia) is also higher with obesity, with increases in total cholesterol (TC), low-density lipoprotein (LDL), and triglyceride (TG), as well as a decrease in high-density lipoprotein (HDL) (Jarolimova et al., 2013). Comorbidities of obesity also include cardiovascular diseases, hypertension, depression, sleep apnea, and certain cancers. Obesity is also associated with a higher mortality rate, especially from cardiovascular diseases (Sampsel and May, 2007).

1.1.2. Treatment strategies to deal with obesity

1.1.2.1. Diet, physical activity, and behavioral therapy

Low-calorie diet, physical activity, and behavioral therapy are recommended for overweight individuals with BMI ≥ 25 kg/m^2, even individuals with no comorbidities. The recommendation from the 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults is to follow a diet that decreases the calories intake to a level that will cause weight loss. Low calorie diet can cause an average of 8% reduction in total weight in a period of six months. Along with diet, exercise and physical activity is a crucial part of any weight loss and maintenance plan. In addition to causing a decrease in weight, exercise reduces the risk for cardiovascular diseases. Behavioral therapy is another important aspect in the treatment plan for obesity to help patients change their life style and incorporate low calorie diet and physical activity into their daily routine. A combination therapy of all three is usually the most successful approach to reach the goal of weight loss and maintenance (Jensen et al., 2014).

1.1.2.2. Pharmacotherapy

If combined therapy of low-calorie diet, physical activity, and behavioral therapy did not produce the desired weight loss results, pharmacotherapy can be included in the treatment plan. Pharmacotherapy is recommended for patients with BMI ≥

30 kg/m^2 with no comorbidities, or BMI ≥ 27 kg/m^2 with comorbidities (Jensen et al., 2014). Phentermine is the most commonly used anti-obesity medication for short-term use. For long-term use, five anti-obesity medications are approved in the US (orlistat, lorcaserin, liraglutide, phentermine/topiramate, naltrexone/bupropion) (Gadde et al., 2018). Pharmacotherapy should never be used alone to cause weight loss but should be incorporated as a part of a treatment plan that include lifestyle interventions such as a low-calorie diet and an increase in physical activity (Jensen et al., 2014). The medication can be continued as long as it causes a satisfactory weight loss or help the patients maintain the loss of weight. However, continuous monitoring of patients is recommended because adverse side effects from anti-obesity medications have been observed in patients on these medications such as nausea, vomiting, dry mouth, diarrhea, fecal incontinence, and insomnia (Gadde et al., 2018). If any adverse effects are observed, the medication should be discontinued, and a different treatment plan should be attempted (Jensen et al., 2014).

1.1.2.3. Bariatric surgery (weight loss surgery)

Bariatric surgery is recommended for patients with clinically severe obesity according to the 2013 AHA/ACC/TOS guideline (Jensen et al., 2014). Surgery is recommended for extremely obese patients with BMI ≥ 40 kg/m^2 or BMI ≥ 35 kg/m^2 with comorbidities. The surgery should be reserved for patients with whom other

weight loss strategies with less complexity have not been successful and patients with comorbid conditions that may pose a life threat. Obese individuals who undergo bariatric surgery should be monitored lifelong by a multidisciplinary team of healthcare providers (Jensen et al., 2014).

1.2. Bariatric surgery procedures

1.2.1. Background

Bariatric surgery procedures have become increasingly popular for the treatment of obesity. Bariatric surgery procedures use either restrictive, malabsorptive or combined both restrictive/malabsorptive techniques to cause weight loss (American Society for Metabolic and Bariatric Surgery). Restrictive procedures limit the amount of food intake by an individual, usually by decreasing the gastric pouch size. Adjustable gastric band and sleeve gastrectomy are types of restrictive bariatric procedures for weight loss. Malabsorptive procedures, such as bilio-pancreatic diversion with or without duodenal switch, that work by shortening the length of the small intestine which comes in contact with food and diverting the biliopancreatic secretions to limit the absorption of food. Combined restrictive/malabsorptive procedures use both restriction and malabsorption techniques to cause weight loss. The combined restrictive/malabsorptive technique is used in Roux-en-Y gastric bypass surgery (RYGB) (Nguyen and

Varela, 2017). **Figure 1** shows a depiction of the different types of bariatric surgery (Stephen et al., 2012).

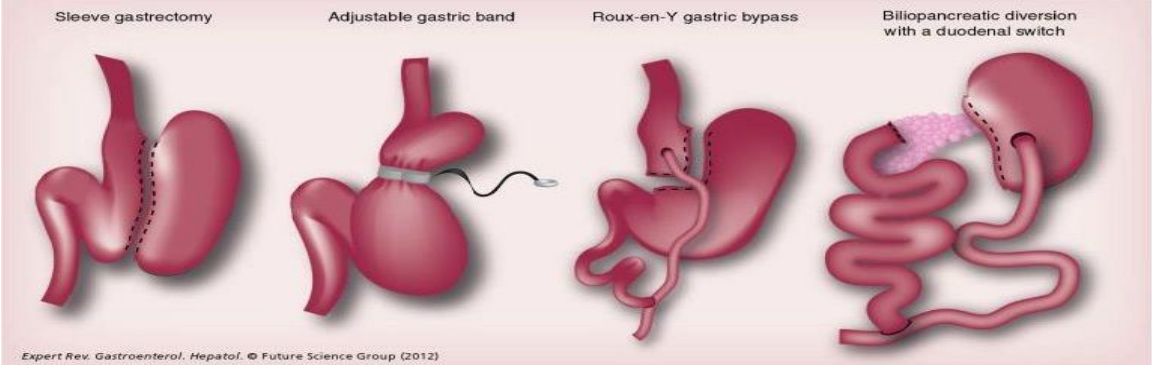


Figure 1. Types of bariatric surgery (Stephen et al., 2012)

The estimated numbers of bariatric surgery procedures performed in the United States from 2011 to 2017 showed an increase by 44.3% from 158,000 in 2011 to 228,000 in 2017. The number of RYGB accounted for more than one-third of the performed bariatric surgery procedures in 2011-2013, and almost one-quarter of the performed bariatric surgery procedures in 2014-2015. In 2016-2017, RYGB accounted for about one-fifth of the performed bariatric surgery procedures, next to sleeve gastrectomy, gastric band, and biliopancreatic diversion with duodenal switch procedures (with 59.39, 2.77, and 0.70%, respectively), in the United States as shown in **Table 2** (American Society for Metabolic and Bariatric Surgery, 2018). This decrease of RYGB percentage is due to the increased popularity of sleeve gastrectomy procedure in the United States, which is considered less invasive than

RYGB. However, RYGB is still considered the gold standard of bariatric surgery because it has a more sustained and durable weight loss results (Maciejewski et al., 2016, Golzarand et al., 2017, Lager et al., 2017, Melissas et al., 2017). The statistics worldwide shows a different trend, with RYGB accounting for 46.3% of the performed bariatric surgery procedures in the period of 2013-2017 (Higa et al., 2017).

Table 2. Estimates of bariatric surgery numbers in the United States, 2011-2017

	2011	2012	2013	2014	2015	2016	2017
Total	158,000	173,000	179,000	193,000	196,000	216,000	228,000
Sleeve gastrectomy	17.80%	33.00%	42.10%	51.70%	53.61%	58.11%	59.39%
RYGB	36.70%	37.50%	34.20%	26.80%	23.02%	18.69%	17.80%
Gastric Band	35.40%	20.20%	14.00%	9.50%	5.68%	3.39%	2.77%
BPD-DS	0.90%	1.00%	1.0%	0.40%	0.60%	0.57%	0.70%

* Based on the American Society for Metabolic and Bariatric Surgery: Estimates of bariatric surgery numbers, 2011-2017 (American Society for Metabolic and Bariatric Surgery, 2018). BPD-DS, Biliopancreatic diversion with duodenal switch.

1.2.2. Roux-en-Y gastric bypass surgery (RYGB)

RYGB is one kind of bariatric surgery procedures that employ both malabsorptive and restrictive techniques to cause weight loss. The stomach is made smaller by

creating a small pouch (15-30 ml) by stapling at the proximal end of the stomach, limiting the individual's food intake at one time as shown in **Figure 2**. A Roux limb is created by transecting 30 cm distal to the ligament of Treitz that suspends the distal duodenum, and this limb is connected to the small gastric pouch that was created. When food is ingested, it will bypass the whole duodenum and part of the jejunum (bypassed limb), which represents the malabsorptive approach of the surgery. Bile salts and pancreatic enzymes will mix with food at the common limb.

The surgery can be performed using either a proximal or a distal approach, which is usually decided by the surgeon's preference. Most surgeons use the proximal approach where the Roux and bypassed limbs have a fixed length (Quan et al., 2017). The length of the Roux limb in the proximal approach depends on the BMI of patients (75 cm for BMI ≤ 50 kg/m^2 and 150 cm for BMI > 50 kg/m^2), while the bypassed limb is usually 50-100 cm in length. The common limb length in the proximal approach varies (50-850 cm), depending on the anatomy of patient since the small intestine length varies drastically (range 300-1000 cm). In the distal approach, the common limb length is fixed to 100-150 cm, and the Roux and bypassed limbs vary in length (Nguyen et al., 1999, Quan et al., 2017). The distal bypass theoretically can lead to superior weight loss outcomes because of the shorter common limb for absorption (100-150 cm and 50-850 cm in the distal and proximal bypass, respectively) (Quan et al., 2017). The anatomical changes that

are introduced into the gastrointestinal tract by the surgery are expected to affect the absorption and/or disposition of orally administered medications.

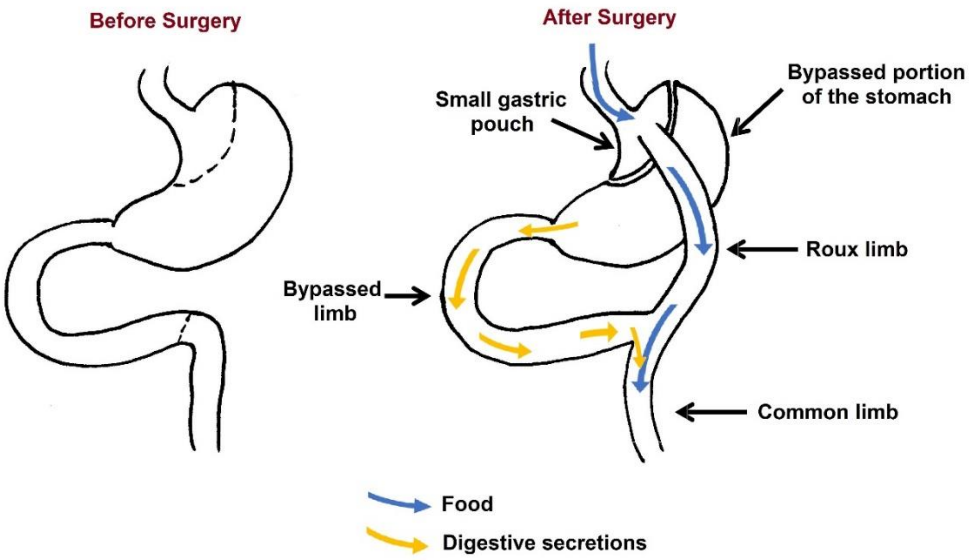


Figure 2. Roux-en-Y gastric bypass surgery

1.2.3. RYGB and possible effects on pharmacokinetics and pharmacodynamics of medications

RYGB introduces anatomical and physiological changes in the body that are expected to affect the pharmacokinetics (PK) and possibly the pharmacodynamics (PD) of orally administered medications. The smaller gastric pouch is expected to cause a reduction in gastric mixing, which decreases disintegration/dissolution of orally administered medications (Padwal et al., 2010). The surgery causes an

increase in the gastric pH of the small stomach pouch from 2 to > 4, due to the decrease of available parietal cells (HCl producing cells) (Smith et al., 2011). This change may affect the absorption of medications in different ways. Basic medications need acidic environment for dissolution, acidic medications need acidic pH to have more unionized form of the medications for passive absorption process, and some transport mediated medications are transported by a pH dependent transporter across the intestinal wall. The increase in pH may lessen the absorption of these medications (Milne, 1965, Mitra and Kesisoglou, 2013). On the other hand, medications that are degraded by the acidic environment in the stomach will have an increase in their bioavailability due to the increased pH (Smith et al., 2011).

Another important anatomical change in the gastrointestinal tract after RYGB is the bypass of the proximal small intestine (duodenum and part of the jejunum). This may affect the PK of medications in several ways. The surface available for absorption in the small intestine will be smaller due to the bypass of the proximal small intestine where the concentration of villi and microvilli is the highest (Pang, 2003). The location of transporters (influx or efflux) that are involved in the drug absorption will be critical when the proximal small intestine is bypassed (Smith et al., 2011). If the transporters expression is the highest in the proximal small intestine, the absorption and sequential bioavailability will be decreased (involving

influx transporter) or increased (involving efflux transporter). Transporters are also a key component of enterohepatic circulation (Roberts et al., 2002). Enterohepatic circulation is a process by which the drug circulates by biliary excretion after reaching the liver, and back to the intestine, where it is reabsorbed. Disruption in the enterohepatic circulation process may occur after RYGB due to the dramatic anatomical changes in the intestinal tract and exposure to bile and pancreatic enzymes in addition to the reduced surface area, which may cause a decrease in the initial and repeated absorption (Giuliano et al., 2012, Bhutta et al., 2015).

Another important component of the gastrointestinal tract is metabolizing enzymes (mainly CYP3A4 and CYP3A5). These enzymes are expressed highly in the proximal small intestine and their expression decreases from the proximal to the distal segments of the small intestine (Srinivas, 2016). Medications which are substrates to these metabolizing enzymes are expected to have less intestinal first pass metabolism (Hachon et al., 2017). The intestinal transit time will also decrease after RYGB, which may affect the absorption rate of medications, especially from extended release formulation (Smith et al., 2011).

The available research is scarce regarding to the effects of RYGB on the PK/PD of medications that are most commonly used in obese patients (Yska et al., 2013, Srinivas, 2016). With the high number of patients who have undergone RYGB in the United States and worldwide, it is crucial and timely to have a better

understanding than the current knowledge of the impacts of RYGB on the PK/PD of most commonly prescribed medications in this obese patient population. In this project, the studies were focused on statins and metformin, as they are the common medications, respectively, for hyperlipidemia and type 2 diabetes, two most prevalent comorbidities in obese patients. We hypothesize that RYGB will decrease the absorption of simvastatin, atorvastatin, and rosuvastatin, as well as metformin and affect the PK and PD of these medications and their active metabolites.

1.3. Statins

1.3.1. Statin history and mechanism of action

Statins, also known as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, were first discovered in the 1970s, starting with compactin and lovastatin which are natural products derived from fungi (Endo, 2010). They were valued for their cholesterol lowering properties in an era when high cholesterol levels were first appreciated as a risk factor for heart diseases in the 1960s. However, clinical trials with compactin were terminated in 1980, due to its long-term toxicity in animals (Tobert, 2003, Endo, 2010). Clinical trials with lovastatin were also suspended until further toxicity studies are performed, due to the significant structural similarities between compactin and lovastatin. Lovastatin clinical studies were resumed after the suspension lift and received the approval

by the Food and Drug Administration (FDA) in 1987 (Tobert, 2003). Many statins were introduced into the worldwide market following the lovastatin approval, including simvastatin (1988 in Sweden to be approved by FDA in 1998), pravastatin (1991 in Japan to be approved by FDA in 2000), fluvastatin (approved by FDA in 1994), atorvastatin (approved by FDA in 1997), cerivastatin (approved by FDA in 1998 and withdrawn in 2001), rosuvastatin (approved by FDA in 2003), and pitavastatin (2003 in Japan to be approved by FDA in 2009).

Simvastatin is a semisynthetic product that differs from the structure of lovastatin by having an additional methyl group on the side chain. Pravastatin is a biotransformation product of compactin. Fluvastatin, atorvastatin, cerivastatin, rosuvastatin, and pitavastatin are compounds from entire chemical synthesis. However, all statins have structural similarities to HMG-CoA as shown in **Figure 3** (Tobert, 2003, Endo, 2010, Sasaki, 2010). Compactin, lovastatin and simvastatin have a lactone ring in their structures, and the lactone form are inactive. They convert to the active open acid form by enzymatic hydrolysis *in vivo* to resemble the HMG-CoA structure. All other statins are administered in the active open acid form (Fong, 2014).

Statins exert their action by competing with HMG-CoA for HMG-CoA reductase enzyme active site in the hepatocytes. Statins prevent the conversion of HMG-CoA into mevalonic acid, which is the rate limiting step in cholesterol biosynthesis. The

inhibition on HMG-CoA leads to a reduction in LDL levels in plasma and hepatocytes, and an upregulation of LDL-receptors to keep cholesterol intracellular hemostasis in the hepatocytes as shown in **Figure 4** (Stancu and Sima, 2001).

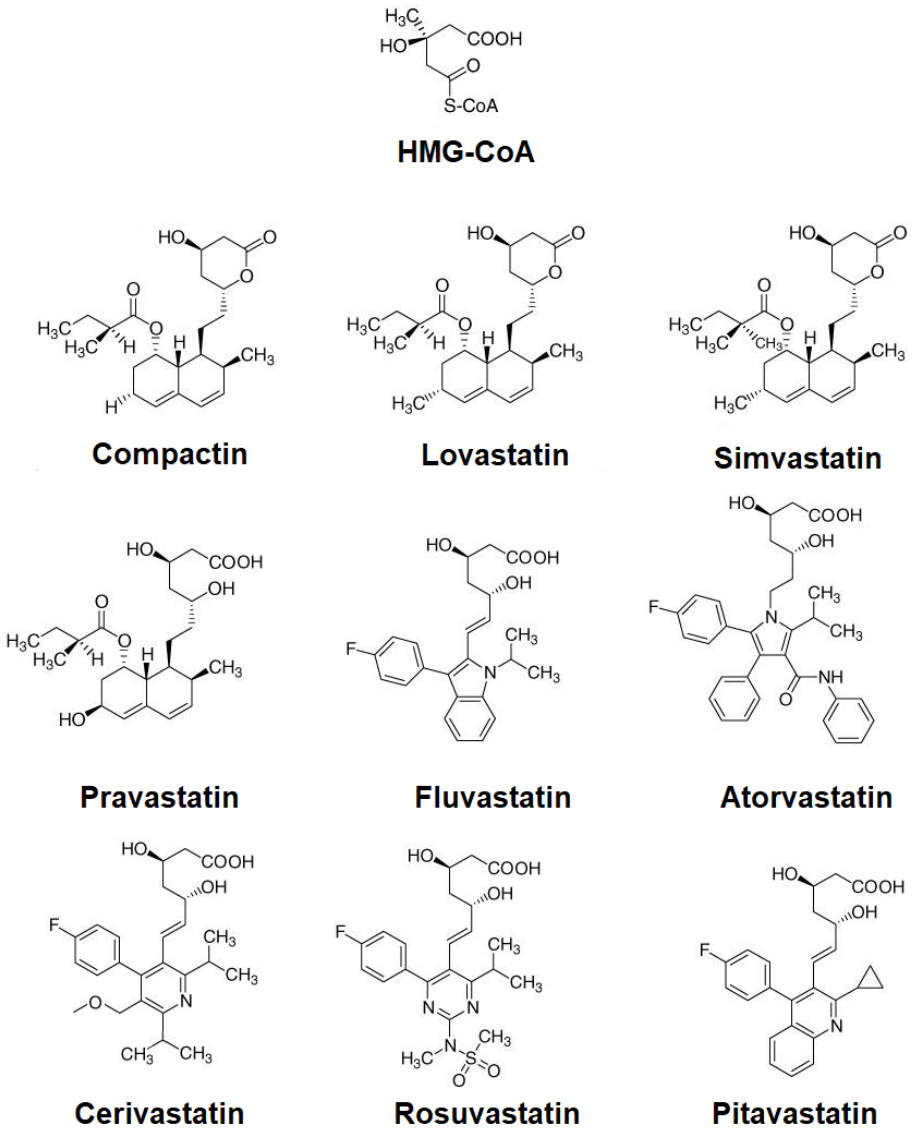


Figure 3. Structures of statins

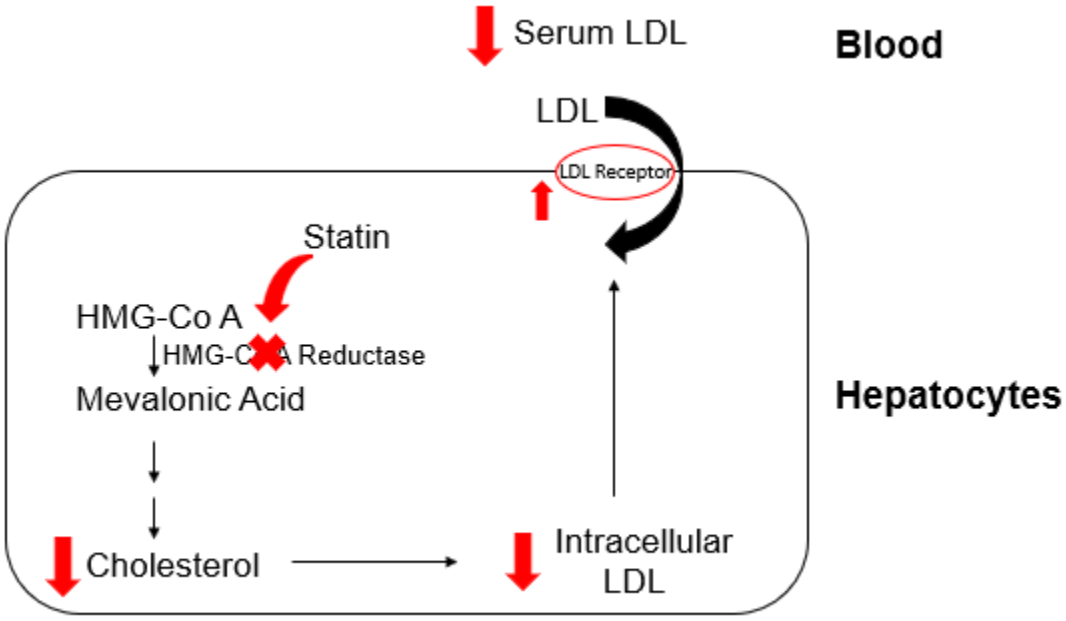


Figure 4. Mechanism of action of statins

1.3.2. Clinical pharmacokinetics of simvastatin

Simvastatin (SMV) is a relatively lipophilic statin administered as a prodrug in the inactive lactone form. It is rapidly absorbed in the gastrointestinal tract by passive diffusion (approximately 60-80 % of the dose), but only 5 % of the administered dose reaches the systematic circulation (Klotz, 2003, Schachter, 2005). The low bioavailability of SMV is attributed to its low solubility in the gastrointestinal fluids and the extensive first pass effect of metabolism to 6′-hydroxy simvastatin, 3″-hydroxy simvastatin, 6′-exomethylene simvastatin, 3′-hydroxy simvastatin, 6′-hydroxymethyl simvastatin, and 6′-hydroxycarboxyl simvastatin (Mauro, 1993, Garcia et al., 2003). SMV is a substrate for CYP3A4 enzyme and P-glycoprotein efflux transporter in the enterocytes, which also contributes to its low bioavailability (Klotz, 2003). SMV and its hydroxy acid metabolite simvastatin acid (SMV-A) extensively bind to plasma protein (95-98%). The high protein binding, the low plasma concentrations and the hydrophilic nature of the hydroxy acid form of SMV-A limit the distribution of SMV and SMV-A to tissues other than the liver (Mauro, 1993, Garcia et al., 2003).

Structures of SMV and SMV-A are represented in **Figure 5**. SMV converts to SMV-A by hydrolysis, and the reaction is reversible. The ratio of SMV/SMV-A in the intestine has been reported as 4:1 from simulated *in vitro* studies (Garcia et al., 2003). SMV activation occurs mainly in the liver by carboxylesterases which form

the active form, SMV-A (Vree et al., 2003). In the liver, both SMV and SMV-A undergo extensive metabolism by CYP3A4 enzyme to their respective hydroxyl metabolites. In addition, SMV-A and SMV-A hydroxyl metabolites undergo glucuronidation, which in turn undergo spontaneous elimination of the glucuronide portion to form the lactone form (Prueksaritanont et al., 2002).

Figure 6 shows the general metabolic pathways of statins. SMV and its active form, SMV-A, have short half-lives of 2 and 1.9 hours, respectively (Lennernas and Fager, 1997, Schachter, 2005). Elimination of SMV occurs mainly by biliary excretion. Renal excretion of SMV is not considered a major route of elimination with only 13% of radioactive simvastatin dose excreted in urine (Mauro, 1993, Schachter, 2005).

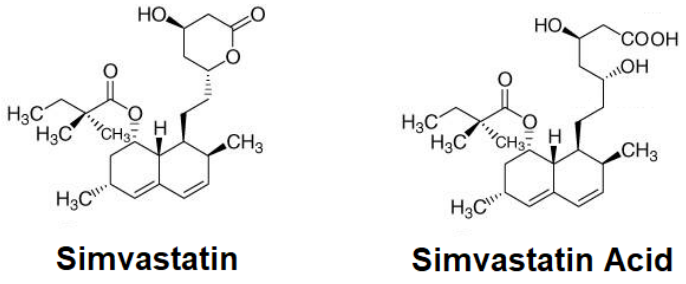


Figure 5. Structures of SMV and SMV-A

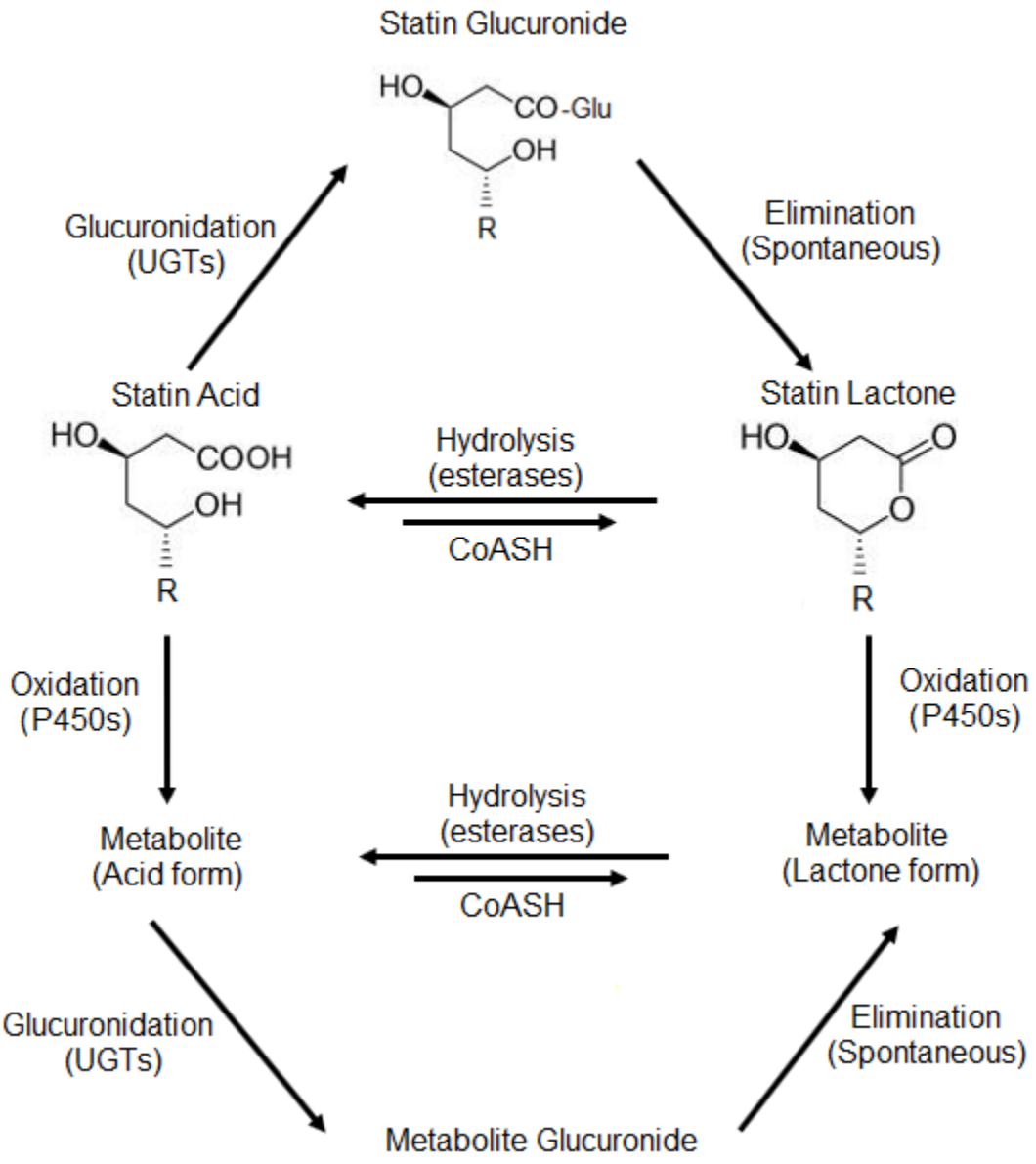


Figure 6. General metabolic pathways of statins, adapted from (Prueksaritanont et al., 2002, Fujino and Kojima, 2006)

1.3.3. Clinical pharmacokinetics of atorvastatin

Atorvastatin (ATV) is a synthetic statin that is administered in the active open acid form. ATV hydroxy acid form is relatively lipophilic, with a lipophilicity comparable to the lactone form of SMV (Garcia et al., 2003). The hydroxy acid form of ATV is in equilibrium with its lactone form *in vivo*. Clinical studies have shown that the ratio of the AUC of ATV acid: lactone was approximately 1 (ranged 1.1-1.3) (Lennernas, 2003). ATV is absorbed by passive diffusion in the gastrointestinal tract with 30% absorption and 12% bioavailability (Klotz, 2003, Schachter, 2005).

ATV is a substrate of the intestinal CYP3A4 enzyme and P-glycoprotein efflux transporter. It extensively binds to plasma protein (98%), which limits the systemic exposure to the free form of the drug. In addition, the extensive first pass metabolism by intestinal and hepatic CYP3A4 metabolic enzyme converts the drug into the more hydrophilic 2- and 4-hydroxylated metabolites (2-OH-ATV and 4-OH-ATV) **(Figure 7)**. These metabolites are active and tend to remain in the liver (the target organ) (Garcia et al., 2003, Lennernas, 2003).

ATV acid form as well as its two active metabolites undergo glucuronidation, which is believed to be the mechanism by which the acid form converts to the lactone form as shown in **Figure 6** (Prueksaritanont et al., 2002). Biliary excretion is the major route of elimination of ATV and its metabolites. The half-life of atorvastatin

is 14 h, but its enzyme inhibition half-life is extended to 20-30 hr by the active metabolites (Schachter, 2005).

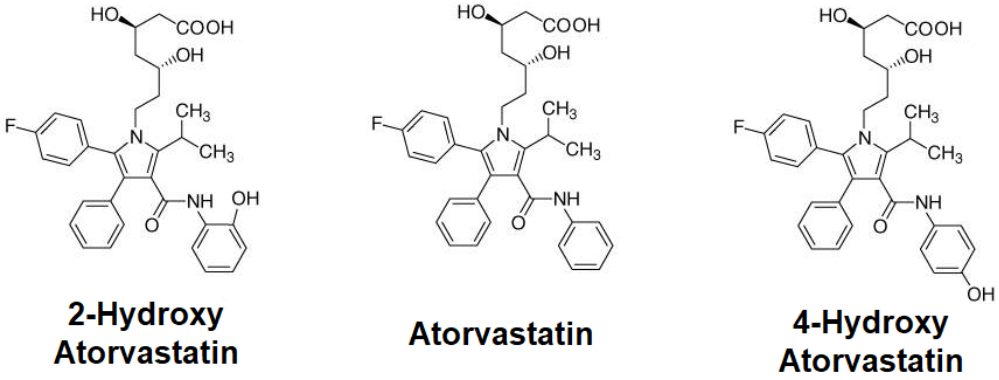


Figure 7. Structures of ATV and its two metabolites (2-hydroxy and 4-hydroxy ATV)

1.3.4. Clinical pharmacokinetics of rosuvastatin

Rosuvastatin (RSV) is another synthetic statin which, in contrast to SMV and ATV, is relatively hydrophilic. Due to its hydrophilicity, RSV does not cross the cell membrane by passive diffusion; RSV absorption and uptake into the hepatocytes is facilitated by the organic anion transporter polypeptides (OATP) (White, 2002, Johnson et al., 2017). RSV has a bioavailability of 20%, and is 90% plasma protein bound (Schachter, 2005). RSV shows a selective distribution to the liver and undergoes minimal metabolism by CYP2C9 metabolic enzyme. Approximately 90% of the HMG-Co A reductase inhibitory activity is attributed to the parent

compound. However, glucuronidation is believed to be the major route for RSV metabolism with secondary peaks being observed in individual plasma concentration-time profiles which are indicative of enterohepatic circulation (Keating and Robinson, 2008). RSV has a relatively long elimination half-life of 19 hr. Liver is the major organ responsible for RSV elimination (by biliary secretion), with renal excretion contributing by only 10% of RSV elimination (Schachter, 2005).

1.3.5. Statins and RYGB

Literature that discusses the impacts of RYGB on the PK of statins are lacking. There were no studies on SMV or RSV in patients who underwent RYGB. Only two publications were available in the literature about ATV systemic exposure following RYGB in a short (3-8 weeks) and a long (21-45 months) follow-up study in the same cohort of patients (Skottheim et al., 2009, Jakobsen et al., 2013), respectively. The study reports that ATV systemic exposure represented by AUC_{0-8} (ng*hr/ml) significantly changed over time post-RYGB with large inter- and intra-individual variability. The study concludes that patients on ATV following RYGB should be observed regularly to ensure therapeutic effects with minimum adverse events. However, the study did not monitor the two active metabolites of ATV (2-OH-ATV and 4-OH-ATV) which are responsible for approximately 70% of the

HMG-CoA reductase circulating inhibitory activity of ATV *in vivo* (Pfizer Ireland Pharmaceuticals, 2009).

1.4. Metformin

1.4.1. Metformin history and mechanism of action

Guanidine is the main active component of *Galega officinalis*, an herb that was used for its antihyperglycemic activities since the medieval period (Bailey, 2017). Guanidine is the parent compound that was used to synthesize three biguanides (metformin, phenformin, and buformin) in the late 1950s (Bailey, 2017). The uses of phenformin and buformin were discontinued in the late 1970s, due to the increased incidences of lactic acidosis, a known side effect of biguanides (Bailey, 2017). Metformin structure shows methyl substitutions on two merged guanidine molecules (1,1-dimethyl biguanide) as shown in **Figure 8**. Compared to the other two biguanides, metformin is favorable, because it is less likely to cause lactic acidosis at the therapeutic dose.

Metformin was first approved by the FDA in 1994 for the treatment of type 2 diabetes, and has become the first line oral treatment of type 2 diabetes (Song, 2016, Bailey, 2017). Although metformin has been in use for a very long time, its mechanism of action is still not fully elucidated. In the liver, metformin decreases glucose production by targeting the mitochondria. Metformin inhibits the

respiratory chain complex 1, which leads to the suppression of ATP production and consequently increases ADP: ATP and AMP: ATP ratios. As a result of this energy imbalance, AMP-activated protein kinase (AMPK) will be activated to restore energy homeostasis (Rena et al., 2017). Activation of AMPK switches on all the catabolic pathways that lead to ATP generation and switches off the cellular pathways that consume ATP. This will lead to the inhibition of glucose production in the hepatocytes which is the main effect of metformin. Other molecular mechanisms of metformin have been proposed by acting on the lysosome instead of the mitochondria to activate AMPK and acting by AMPK independent pathway to decrease hepatic gluconeogenesis. It has been also proposed that metformin act in the intestine by increasing glucose utilization and metabolism in the enterocytes (Stumvoll et al., 2007, Viollet et al., 2012, Rena et al., 2017).

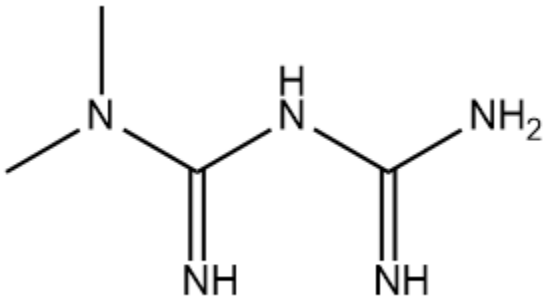


Figure 8. Metformin structure

1.4.2. Clinical pharmacokinetics of metformin

Metformin is considered a weak base with most of the drug in the ionized state at physiological pH (about 99.9% is ionized at physiological pH). Metformin is not significantly absorbed via passive diffusion. The absorption of metformin happens predominantly from the proximal small intestine (Gong et al., 2012). Transporters in the intestine involved in metformin absorption are plasma membrane monoamine transporter (PMAT) and organic cation transporters (OCT3 and OCT1), of which metformin is transported by PMAT and OCT3 from the intestinal lumen to the enterocytes, and by OCT1 from the enterocytes to the blood (Graham et al., 2011). The oral bioavailability of metformin is about 55%. Metformin does not undergo significant metabolism and is mainly eliminated by the renal excretion. In the kidney, OCT2 is responsible for the transport of metformin from blood to the renal tubular cells, while OCT1 and multidrug and toxin extrusion (MATE1 and MATE2-K) transport metformin from the renal tubular cells to the urine (Graham et al., 2011).

1.4.3. Metformin and RYGB

There is a scares information in literature regarding the impact of RYGB on the PK of metformin. One study was found in the literature that investigate the impact of RYGB on metformin absorption and bioavailability. The control group for the study

were BMI-matched non-surgical individuals. The systemic exposure of metformin represented by $AUC_{0-\infty}$ increases by 21% and bioavailability increased by 50% in RYGB subjects compared to the control group. In addition, AUC_{0-8} of glucose levels is significantly lower (14%) in RYGB subjects. However, the study design poses a concern of neglecting inter-individual variability by comparing with a separate, BMI-matched non-surgical group. In addition, the study was performed by administering a single dose of metformin to non-diabetic individuals post-surgery (Padwal et al., 2011).

Chapter 2. Objectives and Specific Aims

2.1. Hypotheses

2.1.1. Hypothesis A: RYGB will decrease the absorption of SMV, ATV and RSV and affect the PK/PD of these drugs and their active metabolites in patients post-surgery

Atorvastatin and simvastatin and are relatively lipophilic, and mainly absorbed by passive diffusion (Garcia et al., 2003). Since there is a decrease in the intestinal surface area available for passive absorption post-RYGB, a decrease in atorvastatin and simvastatin absorption is expected. In addition, atorvastatin and simvastatin have low bioavailability, because they are substrates of CYP450 metabolic enzyme system and P-glycoprotein (P-gp) efflux transporter in the enterocytes (Garcia et al., 2003, Schachter, 2005). Published studies have shown a decrease in the absorption and bioavailability after RYGB for tacrolimus and cyclosporin A, drugs that are substrates for both CYP450 and P-gp (Marterre et al., 1996, Rogers et al., 2008).

Rosuvastatin is relatively hydrophilic and its absorption is facilitated by organic anion transporting polypeptide (OATP2B1, a pH-dependent transporter) with enhanced transporter activity at acidic pH (Varma et al., 2011). Since OATP2B1 is expressed at similar levels along the intestine (Meier et al., 2007), a decrease in the transporter availability is expected post-RYGB due to the bypass of the

proximal part of the small intestine. In addition, there are reports of increase in the gastrointestinal pH after RYGB due to a decrease in the production of hydrochloric acid in the gastric pouch (Smith et al., 2011). The decrease in gastrointestinal acidity after RYGB is likely to reduce the absorption of rosuvastatin via OATP2B1.

2.1.2. Hypothesis B: RYGB will decrease the absorption of metformin and affect its PK/PD in patients post-surgery.

Metformin has a relatively low bioavailability and its absorption occurs primarily in the proximal small intestine by a saturable process through plasma membrane monoamine transporter (PMAT) (Gong et al., 2012). Since the proximal small intestine is bypassed in RYGB patients, the absorption of metformin is expected to decrease.

2.2. Objectives

a) To investigate and characterize the impacts of RYGB on the PK and PD of three most widely used statins (SMV, ATV, and their active metabolites, as well as RSV).

b) To investigate and characterize the impacts of RYGB on the PK and PD of metformin.

c) To correlate PK/PD of SMV, ATV, RSV, and metformin post-RYGB for potential rational guideline for dosing these medications for patients post RYGB.

2.3. Specific Aims

2.3.1. Specific Aim 1

a) To develop and validate a simultaneous LC-MS/MS assay for statins (SMV, ATV and RSV) and their active metabolites in plasma samples from obese patients to monitor the concentrations of the drugs and their active metabolites before and after the surgery.

b) To develop and validate an LC-MS/MS assay for metformin in plasma samples from obese patients to monitor the concentrations of metformin before and after the surgery.

The assay methods will be validated in plasma samples with both low (< 300 mg/dl) and high (> 300 mg/dl) TG levels to ensure the reliability of the assay methods in the quantification of statins and their active metabolites, and metformin in plasma sample from obese patients pre- and post-surgery.

2.3.2. Specific Aim 2

To characterize the longitudinal effects of RYGB on the PK, PD and PK/PD correlations of SMV, ATV and RSV and their active metabolites in the same subjects through the course on 12 months post-RYGB. Concentrations of statins and their active metabolites will be monitored and normalized by unit dose per

body weight [(nM)/(mg/kg)] at baseline (pre-surgery) and at 3, 6, and 12 months post-surgery. The lipid panel analysis of TC, TG, LDL, and HDL will be performed at the same time points pre- and post-surgery. PK/PD correlations will be performed using the molar concentrations of statins and their active metabolites with LDL. The time profiles of weight loss outcomes will also be constructed.

2.3.3. Specific Aim 3

To characterize the longitudinal effects of RYGB on the PK, PD and PK/PD correlation of metformin in the same subjects through the course on 12 months post-RYGB. Concentrations of metformin will be monitored and normalized by unit dose per body weight [(ng/ml)/(mg/kg)] at baseline and at 3, 6, and 12 months post-surgery. Glycated hemoglobin (HbA1c) and glucose concentrations will be monitored at the same time points pre- and post-surgery. PK/PD correlations will be performed using the concentrations of metformin with HbA1c.

Chapter 3. Materials and Methods

3.1. Materials

3.1.1. Chemicals and Materials

- 2-hydroxy and 4-hydroxy ATV (Toronto Research Chemicals Inc., Toronto, Ontario, Canada) were used as standard metabolites of ATV for the LC-MS/MS analysis.
- Acetonitrile of LC-MS grade (Sigma-Aldrich Corp., St. Louis, MO, USA) was used for the preparation of mobile phases, stock solutions, washing solvents for columns, and samples processing for LC-MS/MS assays.
- Ammonium formate for mass spectrometry (Sigma-Aldrich Corp., St. Louis, MO, USA) was used to prepare the mobile phases for LC-MS/MS assays.
- ATV (Sigma-Aldrich Corp., St. Louis, MO, USA) was one of the statins used in this clinical study to prepare standard solutions for the LC-MS/MS analysis.
- Blank human plasma (Equitech-Bio, Inc., Kerrville, TX, USA) was used to validate the LC-MS/MS method and prepare the standard calibration curve for samples quantification.
- Ethyl acetate of LC-MS grade (Alfa Aesar, Ward Hill, MA, USA) was used as the extraction solvent for statins and metabolites.
- Fluvastatin (Sigma-Aldrich Corp., St. Louis, MO, USA) was used as the internal standard in the LC-MS/MS assay of statins and metabolites.

- Formic acid for mass spectrometry (Sigma-Aldrich Corp., St. Louis, MO, USA) was used to prepare the mobile phases for LC-MS/MS assays.
- Methanol of LC-MS grade (Sigma-Aldrich Corp., St. Louis, MO, USA) was used for preparation of stock solutions, washing solvents for columns, and samples processing for LC-MS/MS assays.
- Metformin-d6 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used as the internal standard in the LC-MS/MS assay of metformin.
- Metformin standard (Sigma-Aldrich Corp., St. Louis, MO, USA) was the anti-diabetic medication used in this clinical study to prepare standard solutions for the LC-MS/MS analysis.
- RSV (Toronto Research Chemicals Inc., Toronto, Ontario, Canada) was one of the statins used in this clinical study to prepare standard solutions for the LC-MS/MS analysis.
- SMV-A (Toronto Research Chemicals Inc., Toronto, Ontario, Canada) was used as a standard metabolite of SMV for the LC-MS/MS analysis.
- SMV (Sigma-Aldrich Corp., St. Louis, MO, USA) was one of the statins used in this clinical study to prepare standard solutions for the LC-MS/MS analysis.
- Triglyceride mix (Sigma-Aldrich Corp., St. Louis, MO, USA) was used to spike the plasma to create lipemic plasma for the LC-MS/MS assays.

- Water of LC-MS grade (Sigma-Aldrich Corp., St. Louis, MO, USA) was used for the preparation of mobile phases, washing solvents for columns, and samples processing for LC-MS/MS assays.

3.1.2. Supplies

- ACQUITY UPLC BEH C18 column (2.1×100 mm I.D., 1.7 µm, Waters, Milford, MA, USA) was used for the separation of statins and their active metabolites.
- Centrifuge tubes with printed graduations and flat caps of 15 and 50 ml (VWR international, Radnor, PA, USA) for solvent mixing and storage.
- Glass scintillation vials (VWR international, Radnor, PA, USA) for storing stock solutions in -20°C.
- Microcentrifuge tubes of 1.7 ml (VWR international, Radnor, PA, USA) were used during sample preparation.
- Pipette tips (VWR international, Radnor, PA, USA) with various ranges (1-200, 100-1250 µl) were attached to the pipettes to deliver plasma and solvents quantities accurately.
- Polypropylene vials of 0.1 ml (Chrom Tech, Inc., Apple Valley, MN, USA) with pre-assembled black screw caps with slit were used as sample holders during LC-MS/MS analysis.
- Spherisorb SCX column (4.6×50 mm I.D., 5.0 µm, Waters, Milford, MA, USA) was used for the separation of metformin.

3.1.3. Equipment

- Air dryer/Nitrogen evaporator (N-EVAP 116, Organomation Associates, Inc., Berlin, MA, USA) was used for drying the samples during the extraction procedures.
- API 3200-Qtrap and 5500-Qtrap triple quadrupole mass spectrometer (SCIEX, Framingham, MA, USA) equipped with a TurboIonSpray™ ion source was used as the mass detector in LC-MS/MS analysis.
- Balance (NewClassic MF, Mettler Toledo International Inc., Columbus, OH, USA) was used to weigh the standards for making the stock solutions.
- Centrifuge, Microfuge 20R (Beckman Coulter, Inc., Brea, CA, USA) was used to centrifuge the samples during the extraction procedures.
- Pipettes (VWR international, Radnor, PA, USA) of the ranges 2-20, 20-200, 100-1000 µl were used to deliver plasma and solvents quantities accurately.
- Ultrahigh performance liquid chromatography systems (Waters, Milford, MA, USA) were used for the chromatographic separation in LC-MS/MS analysis.
- Vortex-2 Genie (Scientific Industries, Inc., Bohemia, NY, USA) was used for agitation and mixing the samples during samples preparation.

3.1.4. Software

- Analyst 1.6.3 LC-MS/MS acquisition software (SCIEX, Framingham, MA, USA) was used to control the mass spectrometer instruments and to perform peak integration and quantification of samples.
- ChemDraw Prime 16.0 (Perkin Elmer, Waltham, MA, USA) was used to draw all the chemical structures of medications.
- Design-Expert 11 (State-Ease, Minneapolis, MN, USA) was used to attempt modeling PK/PD correlations.
- Empower 3 (Waters, Milford, MA, USA) was used to control the liquid chromatography system attached to the 3200-Qtrap mass spectrometer.
- MassLynx 4.1 (Waters, Milford, MA, USA) was used to control the liquid chromatography system attached to the 5500-Qtrap mass spectrometer.
- SPSS Statistics Grad Pack 25 Premium (IBM, Armonk, NY, USA) was used to perform all the statistical analyses.

3.2. Methods

3.2.1. Simultaneous LC-MS/MS assay method for the quantification of statins and their active metabolites

3.2.1.1. Chromatographic conditions

Chromatographic analysis was performed using an ultrahigh performance liquid chromatography system (Waters, Milford, MA, USA). SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, RSV and fluvastatin (FLU; used as the internal standard, IS) were separated on ACQUITY UPLC BEH C18 column (2.1 × 100 mm I.D., 1.7 µm). The mobile phases consisted of 10 mM ammonium formate and 0.04% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). A gradient elution was used for the separation as follows: 0-1 min, 30% B; 1-1.5 min, 30-60% B; 1.5-2 min, 60-80 % B; 2-2.5 min, 80-95% B; 2.5-3 min, 95% B; 3-3.5 min 95-80% B; 3.5-4 min, 80% B; 4-4.5 min, 80-60% B; 4.5-5 min, 60-30% B. The elution was performed at a flow rate of 0.4 ml/min, with injection volume of 5 µl, sample temperature of 20°C, and column temperature of 40°C.

3.2.1.2. Mass spectrometry conditions

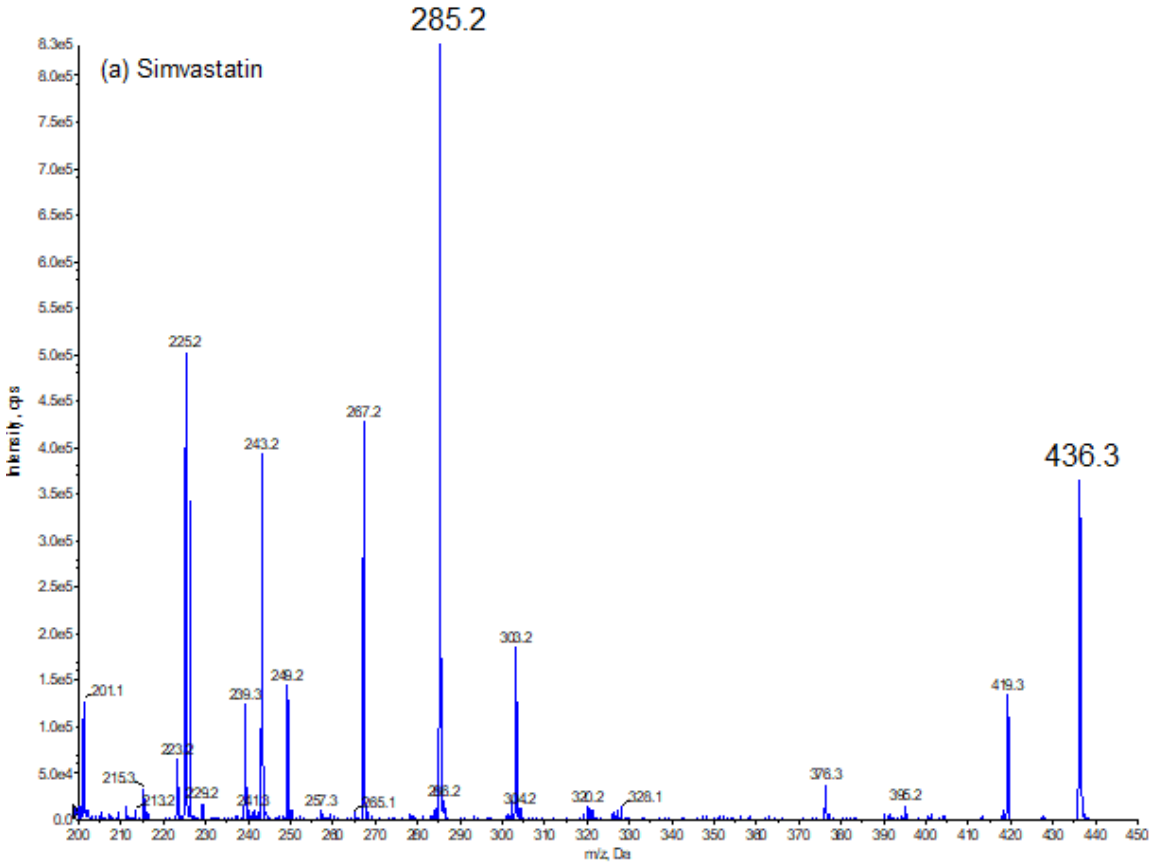
LC-MS/MS analysis was performed using an API 5500-Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Framingham, MA, USA) equipped with a TurboIonSpray™ source. The concentrations of SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, RSV and FLU in plasma samples were determined by Selected Reaction Monitoring (SRM) method to detect transitions ions in the positive ion mode. The quantifications were performed with the transitions of m/z 436.3 → 285.2 for SMV, m/z 437.2 → 303.2 for SMV-A, m/z 559.2 → 440.3 for ATV, m/z 575.4 → 440.3 for 2-OH-ATV and 4-OH-ATV, m/z 482.3 → 258.1 for

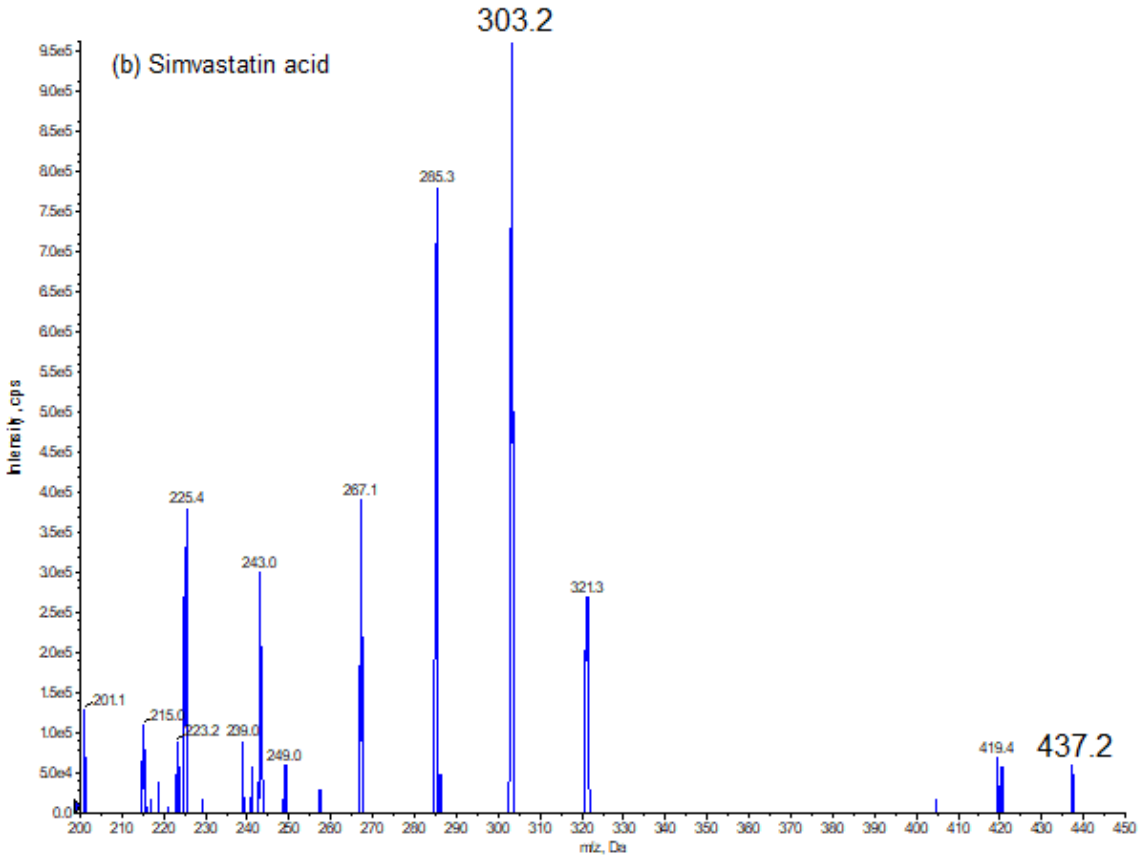
RSV, and m/z 412.3 → 224.2 for FLU. The main working parameters for mass spectrometer were set as follows: ionspray voltage, 5.5 kV; ion source temperature, 500°C; gas1, 20 psi; gas2, 20 psi; curtain gas, 20 psi; and collision gas, high. Compound-dependent parameters for the analytes and the IS were set as summarized in **Table 3**. By using these parameters, the positive ion SRM product ion spectra for all the analytes and the IS were established as shown in **Figure 9**.

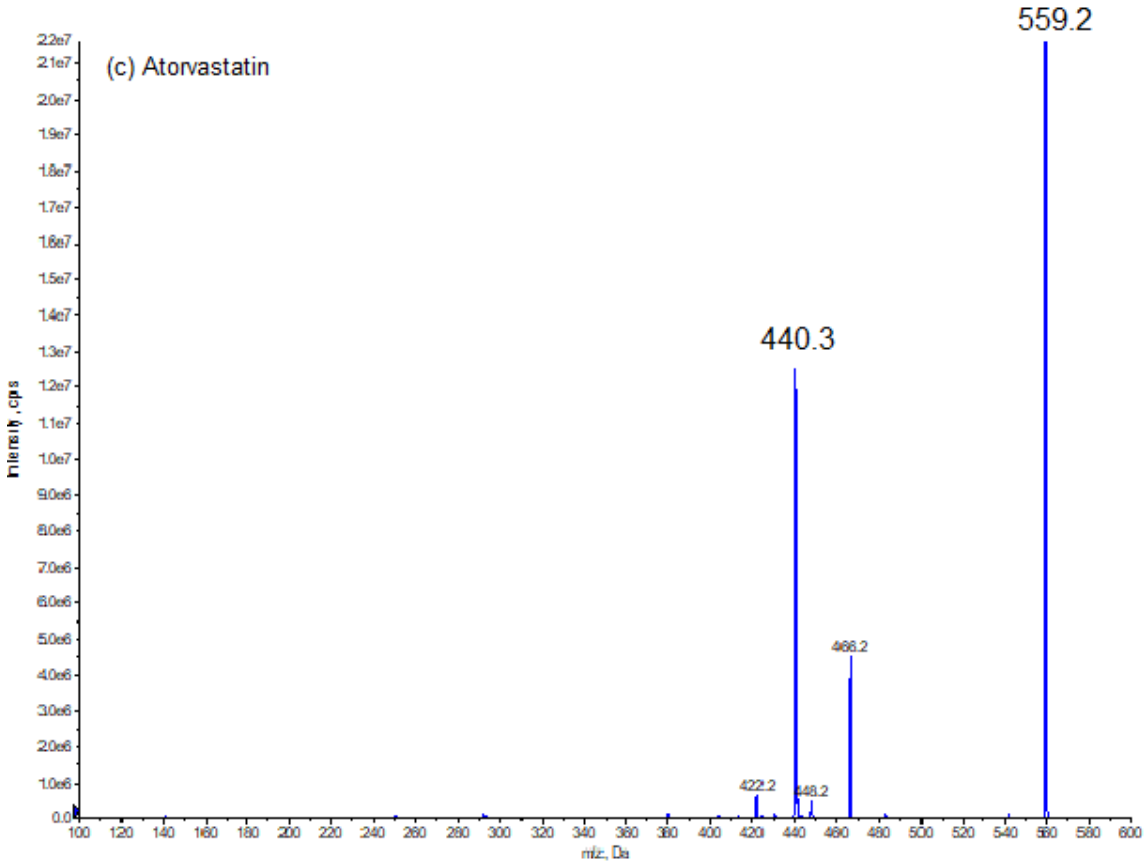
Table 3. Compound-dependent parameters for SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, RSV, and FLU in SRM mode for LC-MS/MS analysis

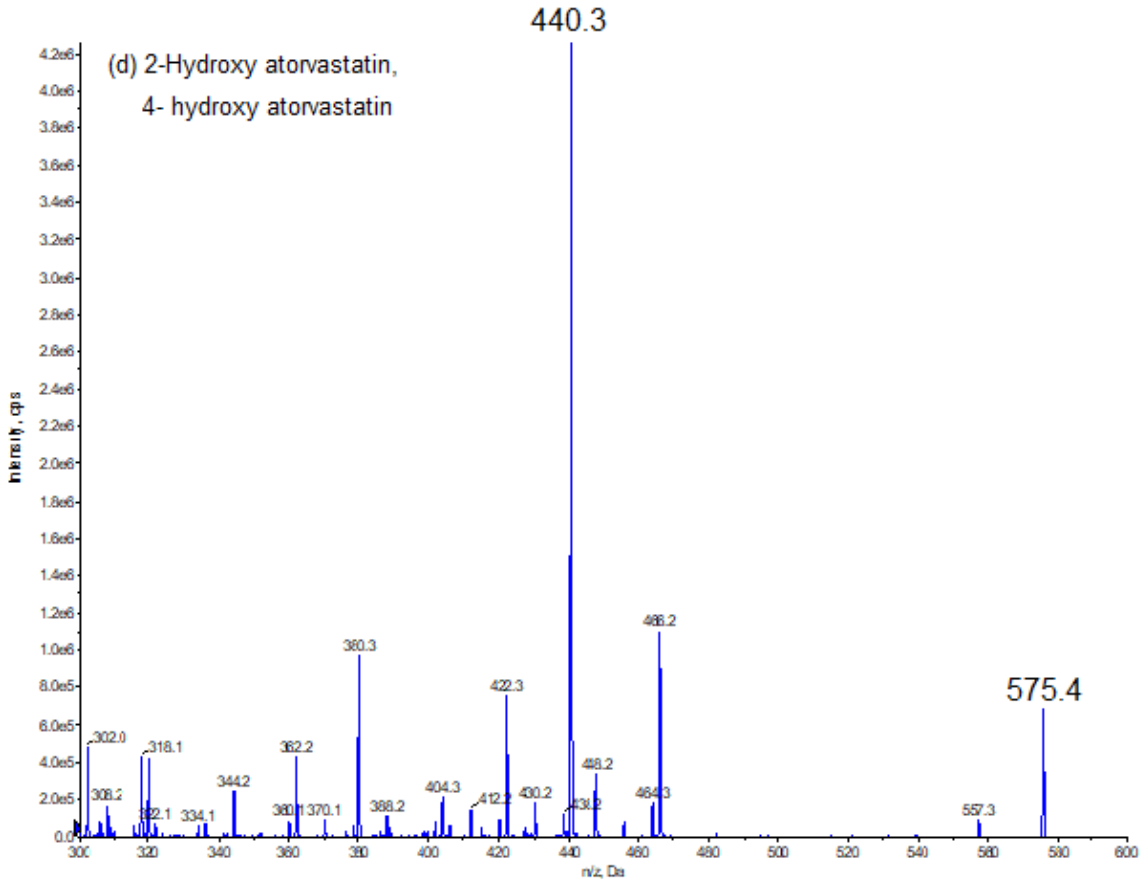
Analyte	Dwell Time (ms)	DP* (V)	EP* (V)	CE* (V)	CXP* (V)
SMV	100	100	8	13	19
SMV-A	100	100	7	11	16
ATV	100	195	9	29	11
2-OH-ATV 4-OH-ATV	100	210	4	28	17
RSV	100	100	9	41	12
FLU	100	130	7	40	35

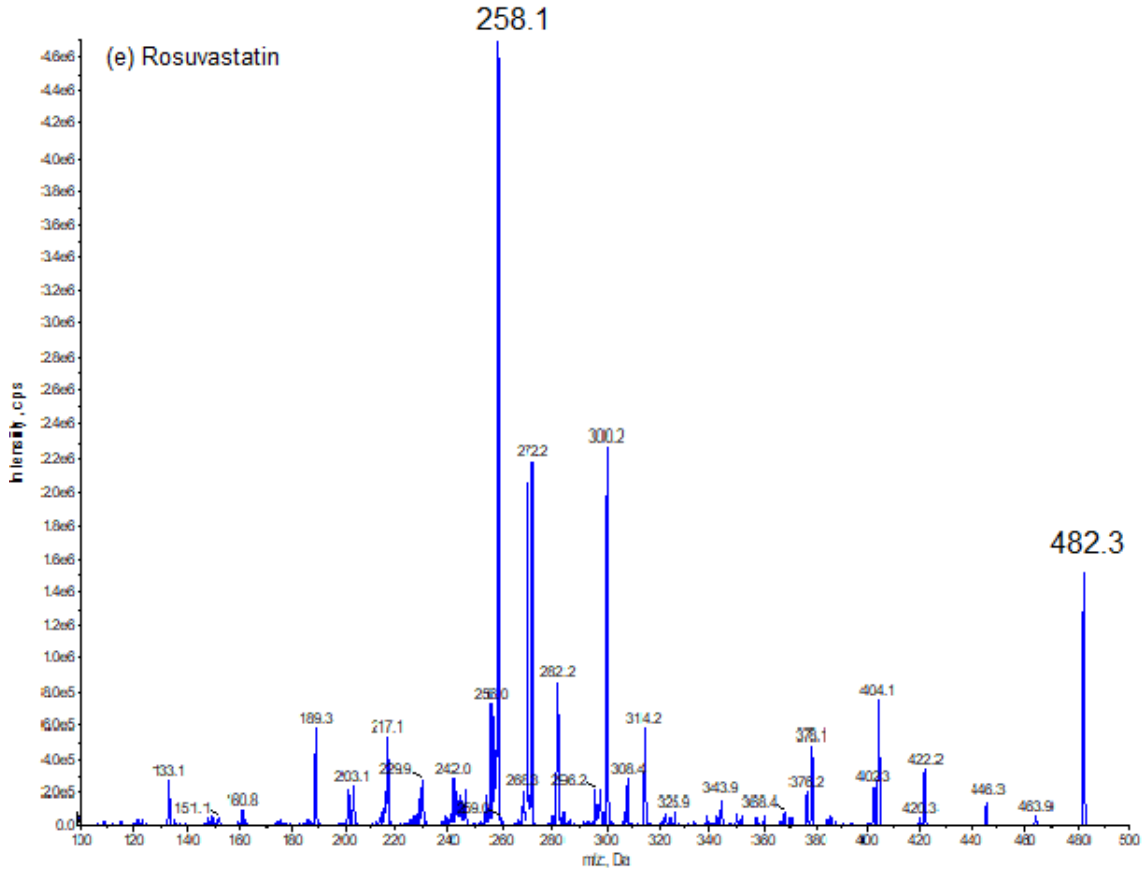
*DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.


285.2
(a) Simvastatin
436.3
225.2
287.2
243.2
303.2
249.2
201.1
239.3
419.3
223.2
215.3
213.2
229.2
257.3
265.1
286.2
304.2
320.2
328.1
376.3
396.2
Intensity, cps
m/z, Da


(b) Simvastatin acid
303.2
285.3
267.1
225.4
243.0
321.3
201.1
215.0
223.2
239.0
249.0
419.4
437.2
Intensity, cps
m/z, Da


(c) Atorvastatin
559.2
440.3
466.2
422.2
448.2
Intensity, cps
m/z, Da


440.3
(d) 2-Hydroxy atorvastatin,
4- hydroxy atorvastatin
Intensity, cps
m/z, Da
575.4
486.2
380.3
422.3
362.2
318.1
302.0
344.2
404.3
468.2
430.2
386.2
370.1
334.1
308.2
322.1
557.3


(e) Rosuvastatin
258.1
482.3
300.2
272.2
282.2
314.2
404.1
189.3
217.1
242.0
133.1
151.1
160.8
203.1
308.4
343.9
422.2
446.3
Intensity, cps
m/z, Da

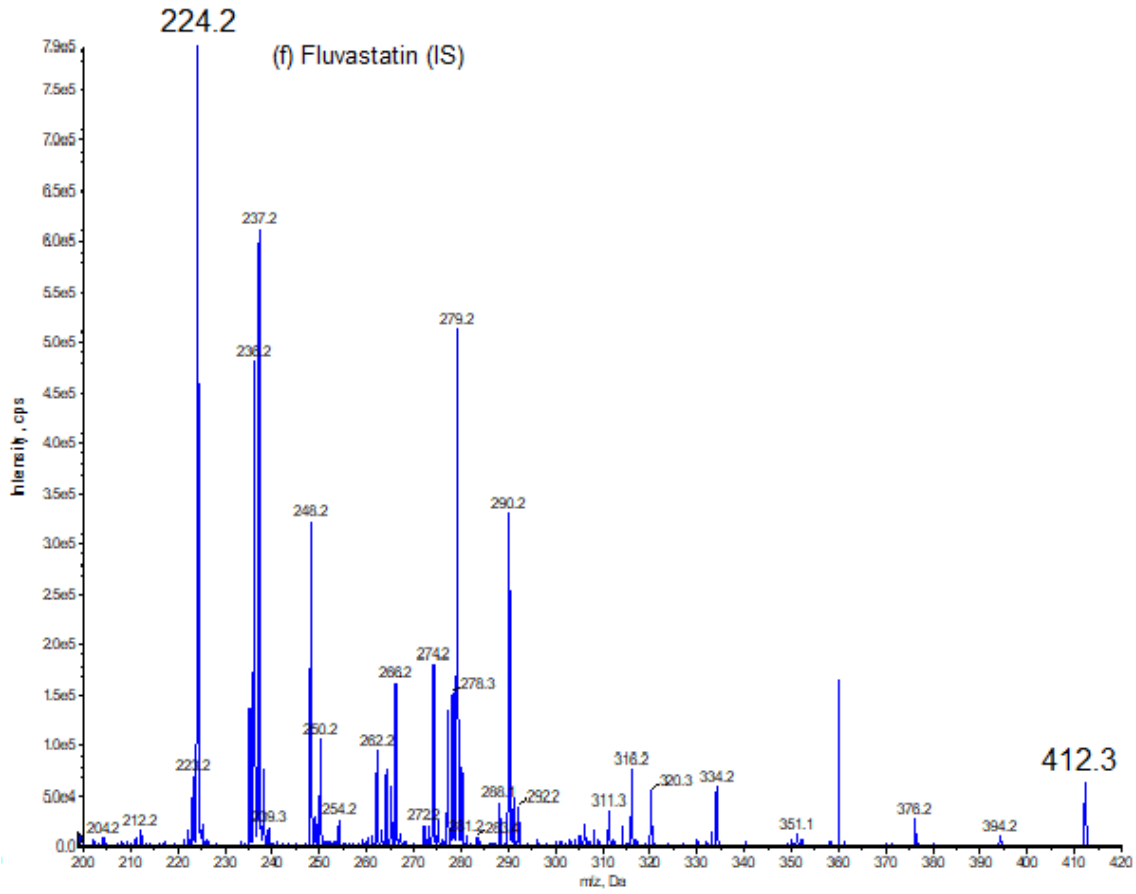


Figure 9. Representative SRM positive product ion mass spectra for (a) SMV, (b) SMV-A, (c) ATV, (d) 2-OH-ATV and 4-OH-ATV, (e) RSV, and (f) FLU. The transitions were m/z 436.3 → 285.2 for SMV, m/z 437.2 → 303.2 for SMV-A, m/z 559.2 → 440.3 for ATV, m/z 575.4 → 440.3 for 2-OH-ATV and 4-OH-ATV, m/z 482.3 → 258.1 for RSV, and m/z 412.3 → 224.2 for FLU

3.2.1.3. Preparation of calibration standards and quality control samples

The standard stock solutions of SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, RSV and FLU were prepared separately at a concentration of 50 µg/ml each in methanol. Stock solutions were stored at -20°C until used for the preparation of working solutions. A mixture of 10 µg/ml each (SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, RSV) was prepared from the stock solutions by dilution with acetonitrile. Standard working solutions were prepared by serial dilutions of the 10 µg/ml standard working solution mixture with acetonitrile to obtain concentrations of 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 0.8, and 1 µg/ml. These standard working solutions were used to spike the plasma samples to yield the calibration standards of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 80, and 100 ng/ml, respectively. Low quality control (LQC), medium quality control (MQC), and high quality control (HQC) standards were selected at concentrations of 0.5, 5, 80 ng/ml, respectively.

3.2.1.4. Plasma sample preparation

Liquid-liquid extraction was used for plasma samples preparation. A plasma sample (200 µl) was spiked with 10 µl of triglyceride mixture (as needed for lipemic group) and 20 µl of IS (500 ng/ml in acetonitrile). Blank plasma samples were also spiked with 20 µl of the required analyte concentrations to prepare the samples for calibration curve of individual analytes and the QC samples. Ethyl acetate (1 ml)

was then added, and the mixture was vortexed for 3 minutes. After centrifugation with 17,968 x g for 20 minutes at 4ºC, the upper phase was transferred to another vial and evaporated to dryness in a stream of air at room temperature (25 ºC). The residue was reconstituted in 100 µl of water/acetonitrile (70:30 v/v) for LC-MS/MS analysis.

3.2.2. LC-MS/MS assay method for metformin quantification

3.2.2.1. Chromatographic conditions

Chromatographic analysis was performed using an ultrahigh performance liquid chromatography system (Waters, Milford, MA, USA). Spherisorb SCX column (4.6 ×50 mm I.D., 5.0 µm, Waters, Milford, MA, USA) was used to analyze plasma samples containing metformin using metformin-d6 as the IS. The mobile phase consisted of water/acetonitrile/formic acid/ammonium formate (100:100:0.1:1.2). An isocratic elution was used for the elution at a flow rate of 1.0 ml/min, with injection volume of 10 µl, sample temperature of 20°C, and column temperature of 25°C.

3.2.2.2. Mass spectrometry conditions

LC-MS/MS analysis was performed using an API 3200-Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Framingham, MA, USA) equipped with a TurboIonSpray™ source. The concentrations of metformin in

plasma samples were determined by SRM method to detect transitions ions in the positive ion mode using the respective $[M+H]^+$ ions m/z 130 → 71 for metformin and m/z 136 → 77 for metformin-d6. The main working parameters for mass spectrometer were set as follows: ionspray voltage, 5.5 kV; ion source temperature, 550°C; gas1, 25 psi; gas2, 30 psi; curtain gas, 25 psi; and collision gas, high. Compound-dependent parameters for metformin and metformin-d6 were set as follows: DP, 65 V; EP, 7 V; CE, 31 V; CXP 7 V. By using these parameters, the positive ion SRM product ion spectra for metformin and metformin-d6 were established as shown in **Figure 10**.

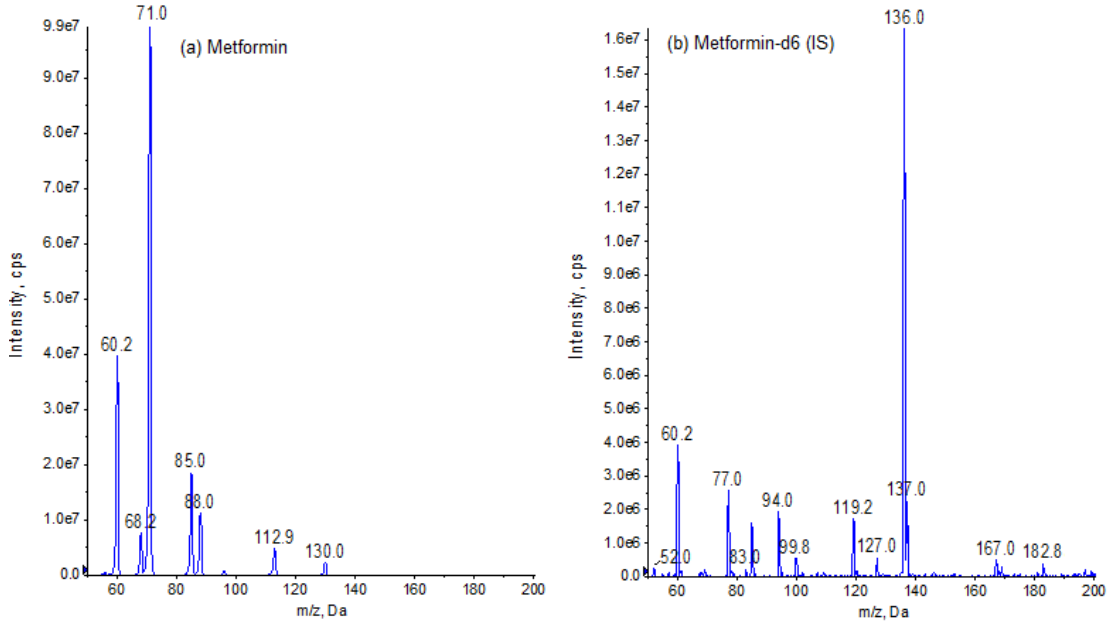


Figure 10. Representative SRM positive product ion mass spectra for (a) metformin and (b) metformin-d6 (IS) with the transitions of m/z 130 → 71 and m/z 136 → 77, respectively

3.2.2.3. Preparation of calibration standards and quality control samples

The standard stock solutions of metformin and metformin-d6 were prepared separately at a concentration of 1 mg/ml each in acetonitrile/water (9:1). Stock solutions were stored at -20°C until used for the preparation of working solutions. Metformin standard working solutions were prepared by serial dilutions of the stock solution with acetonitrile/water (1:1) to obtain metformin concentrations of 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2.5, 5, 8, and 10 µg/ml. These standard working solutions were used to spike the plasma samples to yield the calibration standards of 0.25, 0.5, 1, 2.5, 5, 10, 20, 50, 100, 250, 500, 800, and 1,000 ng/ml, respectively. LQC, MQC and HQC standards were selected at concentrations of 0.5, 20, 800 ng/ml, respectively.

3.2.2.4. Plasma sample preparation

Protein precipitation method was used for plasma samples preparation. A plasma sample (100 µl) was spiked with 10 µl of triglyceride mixture (as needed for lipemic group) and 10 µl of metformin-d6 solution (1,000 ng/ml in acetonitrile/water; 1:1). Blank plasma samples were also spiked with 10 µl of the required analyte concentration to prepare the samples for calibration curve of metformin and the QC samples. Mixture of acetonitrile/methanol (4:1; 500 µl) was then added, and the mixture was vortexed for 2 minutes. After centrifugation with 17,968 x g for 15

minutes at 4ºC, the upper phase was transferred to another vial and evaporated to dryness in a stream of air at room temperature (25 ºC). The residue was reconstituted in 100 µl of water/acetonitrile (1:1) for LC-MS/MS analysis.

3.2.3. Method validation of LC-MS/MS assays

Method validation was performed according to the US FDA Guidelines of "Bioanalytical Method Validation: Guidance for Industry" (Food and Drug Adminisration, 2018) for (1) selectivity and specificity, (2) sensitivity and carryover, (3) linearity, (4) accuracy and precision, (5) extraction recovery (6) matrix effect, and (7) stability. Each analytical run included samples of double blank plasma (no analytes nor IS), blank (no analytes, with IS), and calibration standards. Replicate sets (n=6) of QC samples were included in the run.

3.2.3.1. Selectivity and specificity

Selectivity and specificity were evaluated by analyzing six different lots of un-pooled blank human plasma matrix samples with triglyceride levels of 52-103 mg/dL. The same six lots were spiked with triglyceride mixture (to reach 352 to 403 mg/dL), and the selectivity and specificity were assessed in the presence of high triglyceride levels. The double blank samples should not have any interference at the retention time of each analyte and the internal standards.

3.2.3.2. Sensitivity and carryover

Sensitivity was assessed by analyzing six replicates of samples spiked with the analytes to reach the lower limit of quantification (LLOQ) concentration, with accuracy and precision within 20% of the nominal concentration of the LLOQ. The LLOQ was determined as the concentration producing a peak response of at least 5:1 of the response of blank plasma at the same retention time. Carryover was assessed by injecting 5 samples at the highest standard concentration in the calibration curve followed by three blank injections. Carryover acceptance criteria is less than 20% of the LLOQ response.

3.2.3.3. Linearity

Linear calibration curves were constructed by plotting the peak area ratios of individual analyte/IS versus the analyte concentrations over the range of 0.25-100 ng/ml for statins and their metabolites, and the range of 0.25-1000 ng/ml for metformin. The linearity was assessed using linear regression analysis. Calibration curves were constructed in plasma with low and high triglyceride levels.

3.2.3.4. Accuracy and precision

Quality control samples, containing LLOQ, LQC, MQC, and HQC concentrations of 0.25, 0.5, 5, and 80 ng/ml for statins and their metabolites, and 0.25, 0.5, 20,

and 800 for metformin, respectively, were used to establish the accuracy and precision of the assays. QC samples used for the determination of accuracy and precision were prepared from a stock solution different from the one used to prepare the calibration curve samples. Both intra-day and inter-day accuracy and precision were evaluated by analyzing six replicates of the four different concentrations on three different days. The accuracy was expressed as a percentage of the nominal concentration, and the precision was expressed by the % of coefficient of variation. The acceptable criteria are ± 15% for accuracy, and ≤ 15% for precision, except at LLOQ, where the acceptable deviation is up to 20% for accuracy and precision. The accuracy and precision of the methods were assessed in plasma with low and high triglyceride levels.

3.2.3.5. Extraction recovery

Recovery of the analytes and the internal standards from the extraction methods was evaluated by comparing the peak areas of each analyte and IS between two samples, where the plasma was spiked with the analytes after and before the extraction (six each of LQC, MQC and HQC concentrations), respectively. Recovery does not need to be 100 %, according to FDA guidelines, but should be consistent, precise, and reproducible. Extraction recovery was performed with low and high triglyceride levels for each analyte. For the internal standards, extraction recovery from high triglyceride plasma was performed.

3.2.3.6. Matrix effect

Matrix effect was evaluated by comparing the peak areas of individual analytes in plasma samples that were extracted before spiking the analyte with that in neat solution. Matrix effect was determined using six different sources of plasma at the three different QC concentrations. At first, matrix effect was performed with plasma from six different individuals with triglyceride levels of 52-103 mg/dL. The test was repeated with plasma that was spiked with a known concentration of triglycerides to reach concentrations more than 300 mg/dl (352-403 mg/dl). Matrix effect on the internal standards was assessed using the plasma with high triglyceride levels.

3.2.3.7. Stability

Tests of stability on freeze and thaw, bench-top, long-term, processed sample, and stock solution were performed using six replicates at each of the QC concentration levels. Freeze and thaw stability was determined after three cycles of freeze and thaw from -80ºC to room temperature. Bench-top stability was assessed after the QC samples were kept at room temperature for 4 hours. Long-term stability was assessed after the QC samples were kept at -80ºC for one month. Processed sample stability was determined by the quantification of QC samples prior to and after storage in the autosampler (20ºC) for 24 hours. Stock solution stability was

determined by comparing the analysis results of samples freshly prepared and after storage at -20°C for 1 month.

3.2.4. Inclusion criteria and clinical study design

3.2.4.1. Statins study

Adult patients who met the guidelines for bariatric surgery defined by NIH (National Institutes of Health, 1998) and decided to undergo RYGB after discussion with the participating bariatric surgeon at Houston Methodist Hospital and agreed to participate in the study were recruited (IRB approved protocol ID Pro00007036). Patients were divided into two groups based on their statins' intake (statin and non-statin groups). The statin group was subdivided into groups based on which statin they were prescribed as part of their routine care pre-surgery. The five statins that were used by patients were SMV, ATV, RSV, pravastatin, and lovastatin. The group of patients who were not on any statin (non-statin group) was used as a control group to take into consideration of the effects from the surgery itself. Demographic data, weight and height were recorded at baseline (pre-surgery), and weights were continuously monitored at follow-ups of 1 week, 1, 3, 6, and 12 months. Weight loss outcomes were reported as mean initial BMI, BMI reduction from baseline, and percentage excess weight loss (%EWL). The % of patients in follow-ups from the original number of patients in each group at baseline was calculated at each time point. Concentrations of SMV and its active metabolite

SMV-A, ATV and its two active metabolites, 2-OH-ATV and 4-OH-ATV, and RSV were quantified by our validated LC-MS/MS method (El-Zailik et al., 2019). Since pravastatin and lovastatin groups have very small sample sizes (n=3 and n=1, respectively), and most of their plasma samples were not available, they were excluded from the statin study. Lipid panel analysis of TC, TG, LDL, and HDL was performed at Houston Methodist Hospital on the blood samples at baseline, and at 3, 6, and 12 months post-surgery (El-Zailik et al., 2019).

3.2.4.2. Metformin study

The same patients that were recruited for the statin study were also divided into groups based on their diabetes status and the use of antidiabetic medication as part of their routine care. The first group was for subjects who had been taking metformin prior to the surgery (metformin group). The second group was for subjects who were diabetics, but on other antidiabetic medications, such as insulin and sulfonylurea (other antidiabetic group). The last group was for non-diabetics who are not on any antidiabetic medications (non-diabetic group). The data from the non-diabetic group were used as a control to account for any effect of the surgery on the weight loss outcomes and PD parameters. Demographic data, weight and height were recorded at baseline, and weights were continuously monitored at follow-ups of 1 week, 1, 3, 6, and 12 months. Weight loss outcomes were reported as mean initial BMI, BMI reduction from baseline, and %EWL. The

% follow-up from the original number of patients in each group at baseline was calculated at each time point. Blood samples for the determination of metformin concentrations were collected immediately before the surgery as the individual's own control, and at 3, 6, and 12 months of follow-up visits. The LC-MS/MS assay that was developed and validated was used to quantify metformin concentrations in patients' blood samples. Glucose concentrations and glycated hemoglobin (HbA1c) levels were determined at baseline and at 3, 6, and 12 months of follow-up visits at Houston Methodist Hospital. Glycemic outcomes were reported as complete remission, partial remission, improvement, unchanged, or recurrence. Complete and partial remission were defined as having HbA1c < 6% and 6-6.4%, respectively, with no medication. Improvement was defined as having a decrease in HbA1c to < 6.5% from higher than 6.5%, or the decrease in medication requirement. Recurrence was defined as having HbA1c levels ≥ 6.5% or returning to medication after remission (Brethauer et al., 2015).

3.2.5. Statistical analysis

Comparison of the demographic data among groups was performed using ANOVA followed by Tukey test (3 or more groups) or Student's t-test (2 groups) for age, mean BMI, and excess weight at baseline. Fisher's Exact test was utilized for sex and race comparison. To compare weight loss outcomes and PD parameters among the groups (3 or more groups), either ANOVA followed by Tukey test or

Kruskal-Wallis followed by Dunn's test were performed, depending on the distribution of data and the fulfillment of parametric test assumptions. To compare weight loss outcomes and PD parameters between 2 groups, either Student's t-test or Mann Whitney test were used depending on the distribution of data and the fulfillment of parametric test assumptions. Within each group, the comparison was performed among different time points before and after the surgery with Friedman's test (non-parametric test), mostly because the data failed the assumption of sphericity and hence repeated measure ANOVA could not be performed (Nayak and Hazra, 2011, Laerd Statistics, 2018). All the statistical analysis was performed using SPSS® Version 25. The significant level of all statistical analysis was set at $p < 0.05$.

Missing data percentage was 33.7% in PD data and 13.4% in weight loss outcomes. Missing data of more than 10% is most likely to cause bias in the statistical analysis. Missing data can be handled using two approaches, either ignoring the missing values by listwise or pairwise deletion or by imputation. Deletion of the cases that has missing data is plausible for large data sets but not for limited size data where there could be a loss of power for the statistical analysis (Bennett, 2001). Imputation is preferred for small data sets, where the missing values are imputed either by single value (single imputation) or multiple values (multiple imputation). Multiple imputation (MI) has the advantage over single imputation of minimizing bias by accounting for the uncertainty that accompany

each imputed value (Kang, 2013). Multiple imputation of 5 imputations was used to deal with the missing data in the data set (Graham et al., 2007). The statistical analysis of the 5 imputations was performed on each data set, and the results were pooled together from all the 5 imputed data sets (Dong and Peng, 2013, Biering et al., 2015).

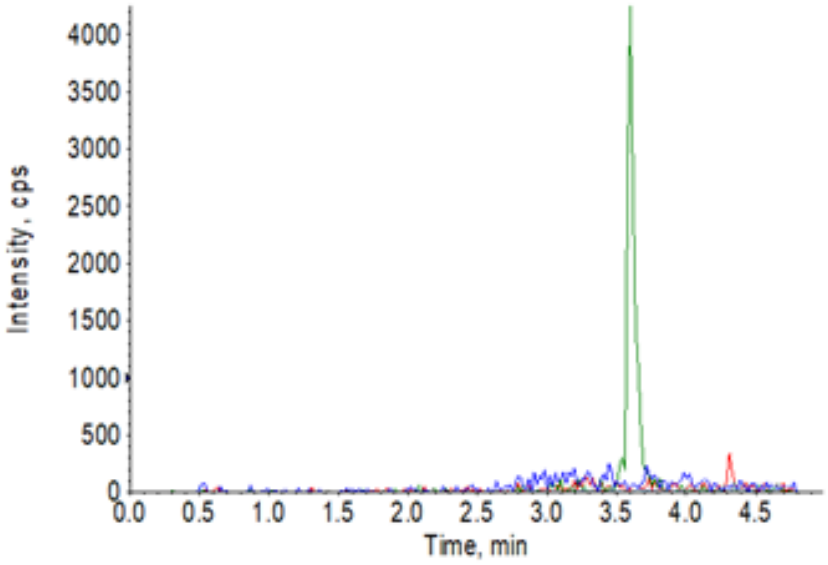
Chapter 4. Results and Discussion

4.1. Validation of LC-MS/MS assay of statins and their active metabolites

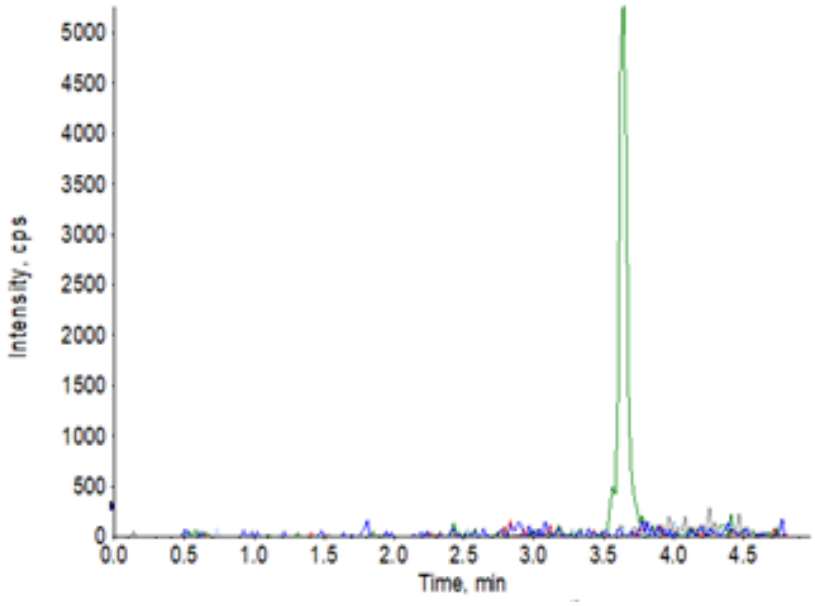
4.1.1. Selectivity and specificity

The interference from endogenous plasma components was evaluated for all six analytes and the internal standard by inspecting the chromatograms of processed blank plasma that contains low or high triglyceride concentrations. The blank plasma chromatograms from plasma with low and high triglyceride levels did not show any significant interference at the retention times of 3.8 minutes for SMV, 3.6 minutes for SMV-A, 3.2 minutes for ATV, 3.1 minutes for 2-OH-ATV, 2.8 minutes for 4-OH-ATV, 2.9 minutes for RSV, and 3.3 minutes for the IS, as shown in **Figure 11.** The peak showing in the upper most chromatogram at 3.6 minutes was observed at the transition for RSV in blank plasma with both low and high triglyceride levels. This indicates that this peak is from an endogenous substance from the plasma, which did not interfere with RSV quantification which elutes at 2.9 minutes.

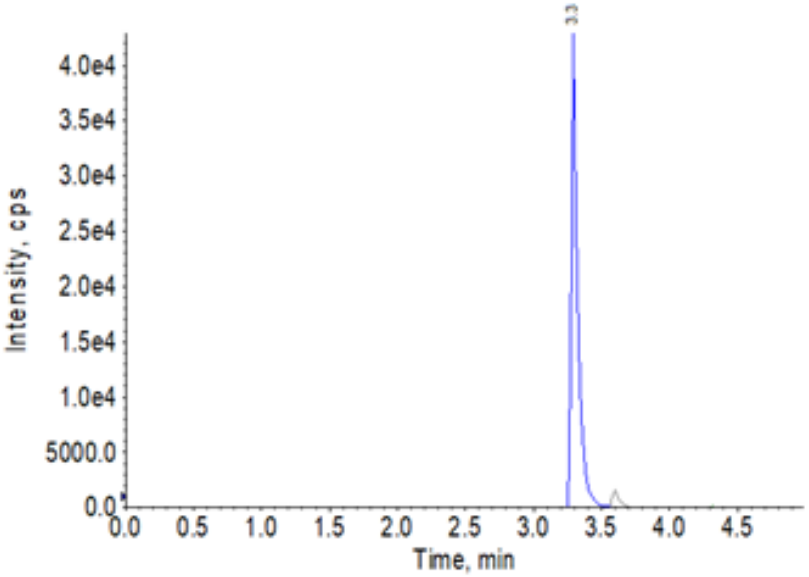
(a) Blank Plasma, Low TG Levels



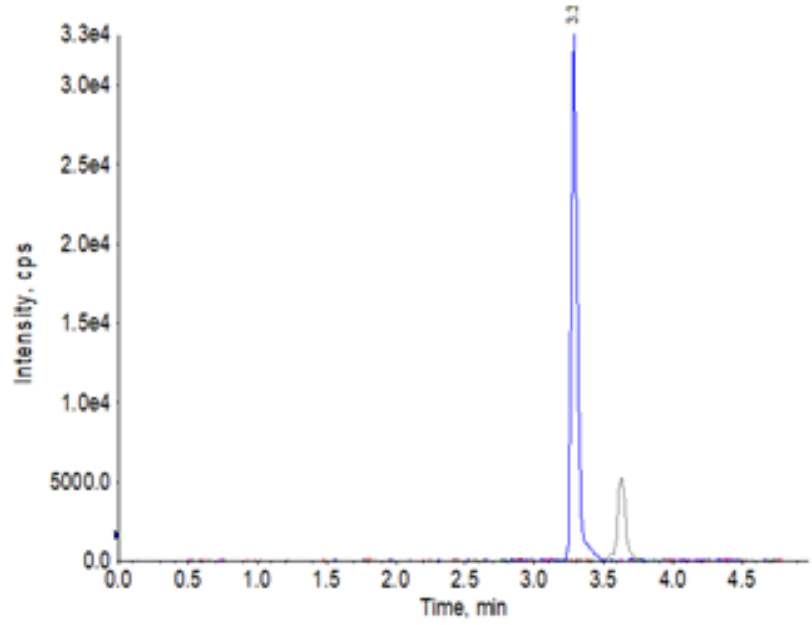
(b) Blank Plasma, High TG Levels



(c) IS, Low TG Levels



(d) IS, High TG Levels



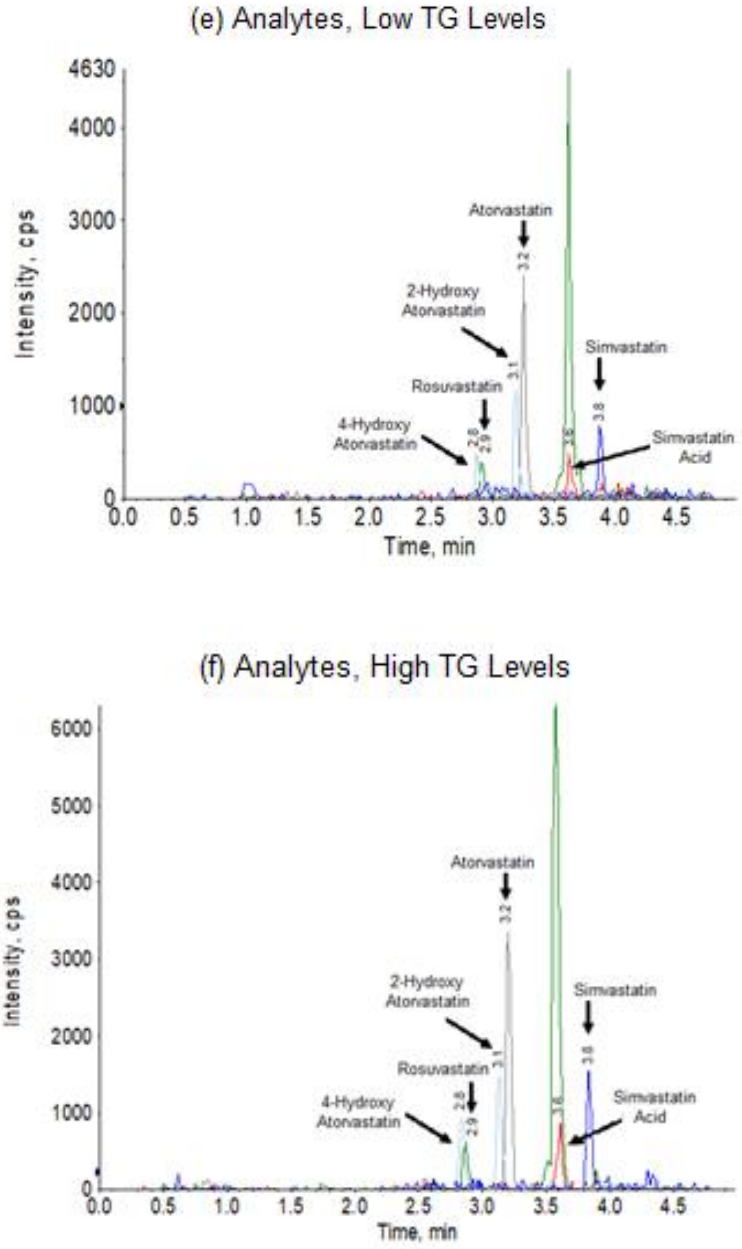


Figure 11. Representative chromatograms of (a, b) blank plasma, (c, d) IS at 50 ng/ml, and (e, f) the six analytes at LLOQ of 0.25 ng/ml in plasma with low (a, c, e) and high (b, d, f) triglyceride levels. The retention times for SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, RSV, and IS are 3.8, 3.6, 3.2, 3.1, 2.8, 2.9, and 3.3 min, respectively

4.1.2. Sensitivity and carryover

The lower limit of quantification (LLOQ) was determined for all analytes to be 0.25 ng/ml based on a response > 5 times of the baseline in blank plasma for each analyte. The lower panel in **Figure 11** shows the simultaneous chromatograms at LLOQ for all six analytes. Carryover was < 10% of LLOQ signal for all analytes in the 3 blank samples injected following 5 injections of samples at the highest standard concentration. Carryover acceptance criterion of < 20% of LLOQ signal was fulfilled for all the six analytes.

4.1.3. Linearity

The linearity over the concentration range of 0.25-100 ng/ml for all analytes was confirmed by constructing calibration curves for each analyte in plasma with both low and high triglyceride levels. Weighting factor of 1/*x* of the analytes to the IS signals vs. analytes' concentrations produced the best fit linear regression equations for the concentration-detector response relationship. **Table 4** present the mean ± standard deviation for the coefficients of determination (r^2) and slopes of the calibration curves from inter-day batches for all analytes.

Table 4. Linearity of the calibration curves for SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, and RSV in plasma with low and high triglyceride levels [data are presented as mean ± standard deviation (% CV)]

	Low TG Levels (n=6)		High TG Levels (n=4)	
Analyte	Slope	r^2	Slope	r^2
SMV	0.045 ± 0.004 (7.94)	0.987 ± 0.009 (0.92)	0.051 ± 0.002 (4.07)	0.990 ± 0.008 (0.75)
SMV-A	0.014 ± 0.001 (8.85)	0.991 ± 0.007 (0.72)	0.013 ± 0.001 (7.97)	0.990 ± 0.004 (0.42)
ATV	0.086 ± 0.009 (9.94)	0.991 ± 0.005 (0.51)	0.085 ± 0.004 (4.43)	0.994 ± 0.003 (0.28)
2-OH-ATV	0.053 ± 0.005 (8.68)	0.990 ± 0.004 (0.43)	0.049 ± 0.002 (3.39)	0.994 ± 0.001 (0.12)
4-OH-ATV	0.019 ± 0.002 (9.69)	0.990 ± 0.006 (0.56)	0.020 ± 0.001 (6.04)	0.991 ± 0.004 (0.24)
RSV	0.018 ± 0.001 (7.53)	0.991 ± 0.006 (0.64)	0.017 ± 0.001 (6.83)	0.995 ±0.001 (0.15)

4.1.4. Accuracy and precision

Tables 5 and 6 represent the intra- and inter-day accuracy and precision values of SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, RSV from plasma samples with low and high triglyceride levels, respectively. The intra-day accuracy and precision values for plasma with low triglycerides were 96-104% and 4-8% for SMV, 95-103% and 5-12% for SMV-A, 95-109% and 5-12% for ATV, 98-104% and 4-11% for 2-OH-ATV, 95-106% and 4-10% for 4-OH-ATV, 96-105% and 3-13% for RSV. The inter-day accuracy and precision values for the assay in plasma with low triglycerides ranged from 99-101% and 6-8% for SMV, 98-100% and 7-9% for SMV-A, 96-106% and 6-10% for ATV, 100-103% and 5-9% for 2-OH-ATV, 97-

102% and 7-9% for 4-OH-ATV, 97-101% and 6-9% for RSV **(Table 5).** The values for the intra-day accuracy and precision for plasma with high triglycerides ranged from 94-104% and 4-13% for SMV, 93-103% and 4-11% for SMV-A, 87-104% and 3-13% for ATV, 90-114% and 5-11% for 2-OH-ATV, 89-100% and 5-12% for 4-OH-ATV, 93-102% and 6-14% for RSV. The assay values for inter-day accuracy and precision for plasma with high triglycerides ranged from 98-99% and 6-10% for SMV, 95-100% and 7-10% for SMV-A, 98-99% and 8-11% for ATV, 96-103% and 8-12% for 2-OH-ATV, 93-98% and 6-10% for 4-OH-ATV, 94-99% and 7-11% for RSV **(Table 6).** The inter- and intra-day accuracy and precision values of the method were not affected by high triglyceride levels in the plasma.

Table 5. Intra-day (3 different days) and inter-day accuracy and precision of SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, and RSV at LLOQ and three QC concentration levels for plasma with low triglyceride levels

Concentration (ng/ml)	Day 1 (n=6)		Day 2 (n=6)		Day 3 (n=6)		Inter-day (n=18)	
	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)
SMV								
0.25	101.30 ± 7.52	7.42	96.80 ± 3.56	3.68	99.70 ± 5.40	5.41	99.27 ± 5.71	5.75
0.5	97.53 ± 4.88	5.00	103.95 ± 5.42	5.21	99.00 ± 6.82	6.89	100.16 ± 6.11	6.10
5	104.38 ± 7.92	7.59	95.53 ± 7.36	7.71	95.72 ± 4.88	5.10	98.54 ± 7.71	7.82
80	103.50 ± 6.44	6.22	98.07 ± 6.09	6.21	100.22 ± 8.12	8.10	100.59 ± 6.91	6.87
SMV-A								
0.25	97.58 ± 8.53	8.75	103.13 ± 12.81	12.42	99.32 ± 4.64	4.67	100.01 ± 9.04	9.04
0.5	102.22 ± 6.13	5.99	94.72 ± 5.72	6.04	97.65 ± 6.96	7.13	98.19 ± 6.71	6.83
5	101.05 ± 8.80	8.71	97.15 ± 4.93	5.08	97.98 ± 7.33	7.48	98.73 ± 6.98	7.07

80	96.30 ± 8.49	8.81	101.70 ± 4.85	4.77	98.67 ± 6.65	6.74	98.89 ± 6.80	6.88
ATV								
0.25	104.02 ± 6.69	6.43	109.43 ± 9.17	8.38	103.50 ± 6.47	6.25	105.65 ± 7.61	7.20
0.5	95.95 ± 8.79	9.16	97.48 ± 7.07	7.25	95.05 ± 9.07	9.54	96.16 ± 7.92	8.23
5	102.63 ± 12.43	12.11	100.75 ± 9.48	9.41	98.57 ± 8.28	8.40	100.65 ± 9.74	9.68
80	100.22 ± 5.32	5.31	97.68 ± 7.00	7.17	100.67 ± 4.89	4.85	99.52 ± 5.62	5.65
2-OH-ATV								
0.25	102.93 ± 8.41	8.17	104.05 ± 6.63	6.37	101.25 ± 6.23	6.15	102.74 ± 6.82	6.64
0.5	103.35 ± 7.74	7.49	97.95 ± 9.41	9.61	99.17 ± 10.70	10.79	100.16 ± 9.11	9.10
5	100.27 ± 7.63	7.61	101.63 ± 10.81	10.63	98.55 ± 10.47	10.62	100.15 ± 9.24	9.23
80	98.28 ± 5.22	5.31	99.68 ± 5.71	5.73	102.17 ± 4.31	4.22	100.04 ± 5.08	5.08
4-OH-ATV								
0.25	97.18 ± 3.55	3.65	105.67 ± 7.34	6.95	104.15 ± 9.25	8.89	102.33 ± 7.69	7.52
0.5	98.18 ± 7.23	7.37	98.58 ± 9.09	9.22	95.60 ± 9.67	10.12	97.46 ± 8.31	8.53

5	102.52 ± 3.76	3.67	101.27 ± 9.09	8.97	98.32 ± 8.72	8.87	99.81 ± 7.40	7.41
80	95.90 ± 9.57	9.98	95.02 ± 7.80	8.21	100.58 ± 4.02	4.00	97.17 ± 7.48	7.70
RSV								
0.25	99.00 ± 12.37	12.50	104.75 ± 4.60	4.39	96.20 ± 8.37	8.70	99.98 ± 9.23	9.24
0.5	101.85 ± 7.71	7.57	100.28 ± 10.04	10.02	102.30 ± 10.16	9.93	101.48 ± 8.85	8.72
5	98.65 ± 10.96	11.11	96.30 ± 9.85	10.23	96.58 ± 3.23	3.34	97.18 ± 8.25	8.49
80	99.68 ± 7.03	7.05	97.03 ± 4.96	5.12	102.15 ± 4.89	4.79	99.62 ± 5.78	5.81

Table 6. Intra-day (3 different days) and inter-day accuracy and precision of SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, and RSV at LLOQ and three QC concentration levels for plasma with high triglyceride levels

Concentration (ng/ml)	Day 1 (n=6)		Day 2 (n=6)		Day 3 (n=6)		Inter-day (n=18)	
	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)
SMV								
0.25	98.53 ± 8.73	8.86	98.00 ± 7.10	7.25	97.61 ± 8.72	8.93	98.05 ± 7.73	7.88
0.5	101.20 ± 12.69	12.54	95.20 ± 6.08	6.39	96.23 ± 10.69	11.11	97.54 ± 9.96	10.21
5	97.67 ± 5.16	5.28	93.63 ± 7.90	8.44	104.28 ± 3.90	3.74	98.53 ± 7.14	7.25
80	99.79 ± 7.56	7.57	95.02 ± 5.04	5.30	98.70 ± 5.60	5.68	97.84 ± 6.16	6.29
SMV-A								
0.25	102.33 ± 8.73	8.53	101.00 ± 6.48	6.42	98.08 ± 7.84	7.99	100.47 ± 7.50	7.46
0.5	100.80 ± 10.15	10.07	95.07 ± 8.43	8.86	102.50 ± 11.55	11.27	99.46 ± 10.06	10.11
5	96.20 ± 7.67	7.98	99.32 ± 7.91	7.97	93.68 ± 7.75	8.28	96.40 ± 7.68	7.97

80	96.47 ± 7.77	8.06	93.37 ± 3.45	3.69	96.28 ± 8.16	8.47	95.37 ± 6.55	6.87
ATV								
0.25	101.64 ± 3.03	2.98	87.42 ± 4.70	5.37	104.04 ± 12.75	12.25	97.70 ± 10.68	10.93
0.5	95.87 ± 8.17	8.52	100.30 ± 12.96	12.92	100.17 ± 9.75	9.73	98.78 ± 10.07	10.20
5	101.97 ± 9.05	8.87	101.49 ± 9.03	8.90	93.70 ± 8.25	8.80	99.05 ± 9.13	9.21
80	95.52 ± 7.75	8.11	99.18 ± 8.75	8.82	101.82 ± 6.10	5.99	98.84 ± 7.63	7.72
2-OH-ATV								
0.25	100.83 ± 6.94	6.88	111.00 ± 6.72	6.06	89.93 ± 6.08	6.77	100.59 ± 10.80	10.74
0.5	90.72 ± 9.71	10.71	103.37 ± 8.28	8.01	113.67 ± 7.03	6.19	102.86 ± 12.82	12.47
5	97.70 ± 5.01	5.13	100.76 ± 9.00	8.94	93.32 ± 10.21	10.94	97.26 ± 8.47	8.71
80	93.85 ± 7.75	8.26	93.42± 6.53	6.99	100.18 ± 6.34	6.33	95.82 ± 7.22	7.54
4-OH-ATV								
0.25	98.00 ± 7.21	7.36	88.67 ± 10.66	12.02	92.71 ± 8.20	8.84	93.12 ± 9.16	9.84
0.5	94.15 ± 8.15	8.65	98.48 ± 5.52	5.61	99.12 ± 7.51	7.58	97.25 ± 7.09	7.29

5	98.48 ± 5.20	5.28	91.83 ± 5.82	6.34	97.20 ± 5.55	5.71	95.84 ± 5.98	6.24
80	97.08 ± 8.65	8.91	98.00 ± 7.46	7.61	100.26 ± 10.24	10.21	98.45 ± 8.43	8.56
RSV								
0.25	96.88 ± 9.88	10.19	98.20 ± 13.62	13.87	100.42 ± 11.60	11.55	98.50 ± 11.18	11.35
0.5	93.25 ± 8.91	9.56	92.72 ± 11.01	11.87	97.00 ± 10.66	10.99	94.32 ± 9.81	10.40
5	101.67 ± 6.39	6.29	98.55 ± 7.53	7.64	95.77 ± 7.43	7.76	98.66 ± 7.15	7.25
80	96.33 ± 7.23	7.50	95.45 ± 8.96	9.39	100.38 ± 7.41	7.39	97.39 ± 7.75	7.96

4.1.5. Extraction recovery

The extraction recovery of all analytes from plasma with low and high triglyceride levels was reproducible at the three QC concentration levels and above 88% and 91 %, respectively **(Table 7).** The extraction recovery was in the range of 98-100% for SMV, 88-96% for SMV-A, 97-100% for ATV, 92-98 for 2-OH-ATV, 97-99% for 4-OH-ATV, and 88-95 for RSV in plasma with low triglycerides levels. The range was similar for all analytes in plasma with high triglycerides levels with recovery range of 95-97% for SMV, 91-98% for SMV-A, 93-97% for ATV, 95-97 for 2-OH-ATV, 95-97% for 4-OH-ATV, and 94-98 for RSV. The extraction recovery for the IS was tested only in plasma with high triglyceride levels and the recovery was 88.12 ± 7.84%. Since the extraction recovery of the IS from high TG plasma was above 85%, we did not test for the extraction recovery using low TG plasma.

Table 7. Extraction recovery (mean ± SD) of SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, and RSV at three levels of QC samples from human plasma with either low or high triglyceride levels using liquid-liquid extraction method

	Low TG Levels			High TG Levels		
Concentration (ng/ml), n = 6	0.5	5	80	0.5	5	80
SMV	99.69 ± 5.28	98.37 ± 4.42	99.82 ± 7.31	95.21 ± 10.05	97.26 ± 8.83	97.40 ± 6.80
SMV-A	88.49 ± 6.48	96.04 ± 6.01	94.32 ± 4.80	91.29 ± 3.21	96.46 ± 3.80	97.75 ± 5.89
ATV	98.84 ± 6.77	96.94 ± 7.86	100.37 ± 8.18	93.06 ± 8.21	96.41 ± 4.99	97.36 ± 3.03
2-OH-ATV	92.15 ± 6.20	97.71 ± 4.52	96.31 ± 4.17	94.93 ± 12.20	95.29 ± 2.52	96.97 ± 5.03
4-OH-ATV	99.17 ± 6.33	97.97 ± 8.92	96.97 ± 9.60	94.86 ± 12.82	96.67 ± 9.51	96.47 ± 8.75
RSV	87.99 ± 6.28	94.62 ± 6.67	95.36 ± 4.75	97.77 ± 10.42	94.18 ± 4.41	95.28 ± 10.02

4.1.6. Matrix effect

The matrix effect was not significant for all analytes in plasma with either low or high triglyceride levels. The mean ± SD of the matrix effect for each analyte in low and high triglyceride plasma are represented in **Table 8.** Matrix effect was in the range of 93-110% in plasma with low triglyceride levels and in the range of 92-103% in plasma with high triglyceride levels. Matrix effect was tested for the IS in plasma with high triglyceride levels and was 93.51 ± 6.67%.

Table 8. Matrix effect (mean ± SD) of SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, and RSV at three levels of QC samples from human plasma samples with low (n=6 at each QC level) and high (n=6 at each QC level) triglyceride levels

Concentration (ng/ml)	SMV	SMV-A	ATV	2-OH-ATV	4-OH-ATV	RSV
Low TG Levels						
0.5	97.76 ± 4.14	92.63 ± 7.41	110.42 ± 6.39	102.93 ± 8.35	101.84 ± 9.10	93.53 ± 2.64
5	96.61 ± 9.08	99.98 ± 8.29	102.78 ± 10.23	96.50 ± 11.48	96.06 ± 5.96	94.64 ± 10.12
80	96.23 ± 5.93	103.01 ± 8.48	97.45 ± 4.40	99.08 ± 7.15	97.84 ± 10.47	93.83 ± 9.04
High TG Levels *						
0.5	99.05 ± 6.04	97.01 ± 7.53	102.76 ± 9.19	103.23 ± 13.68	100.68 ± 8.20	98.02 ± 8.65
5	93.62 ± 5.94	97.85 ± 6.75	97.05 ± 7.98	91.59 ± 6.08	102.52 ± 5.56	98.20 ± 5.71
80	97.02 ± 7.49	101.12 ± 7.99	94.17 ± 7.42	101.62 ± 5.74	97.97 ± 7.99	97.72 ± 7.40

* High TG levels in plasma samples were achieved by spiking with triglycerides to reach ≥ 300 mg/dl (352-403 mg/dl) triglyceride concentrations, t-test showed no statistically significant difference between the matrix effect at all concentration levels between plasma with low and high TG levels

4.1.7. Stability

The conditions under which the stability experiments were performed reflect the expected conditions during sample preparation, storage, and analysis. **Table 9** presents the results of the stability experiments performed under different conditions. Results of freeze and thaw stability test showed that all analytes were stable (92-99%) for three cycles when stored at -80ºC and thawed to room temperature. Bench-top stability test showed that the samples were stable (90-100%) for 4 hr at room temperature (the time required for sample preparation). Long-term stability test results showed that the analytes were adequately stable (92-98%) for up to 1 month in plasma when stored at -80ºC. Processed samples can be analyzed overnight (24 hr) in the autosampler at 20ºC without any concern about the stability of all analytes (92-100%). Lastly, stock solutions of the analytes (50 µg/ml, each) were stable (94-100%) up to one month when stored at -20ºC.

Table 9. Stability of SMV, SMV-A, ATV, 2-OH-ATV, 4-OH-ATV, and RSV in human plasma under various conditions (n=6); RT, Room temperature; all data are presented as mean ± SD

	% of Initial Concentration				
Concentration (ng/ml)	Freeze and Thaw, three cycles, -80ºC to RT	Bench-top, RT for 4 hr	Long-term, -80ºC for 1 month	Processed sample, autosampler, 20ºC for 24 hr	Stock solution, -20°C for 1 month
SMV					
0.5	96.92 ± 8.88	96.75 ± 6.12	95.62 ± 3.86	92.30 ± 3.22	99.98 ± 2.82
5	94.83 ± 6.84	97.05 ± 8.45	94.48 ± 10.69	94.46 ± 3.00	94.59 ± 3.18
80	91.58 ± 5.77	99.23 ± 8.51	96.32 ± 5.07	99.74 ± 2.43	94.50 ± 4.71
SMV-A					
0.5	97.22 ± 5.62	98.97 ± 3.50	92.75 ± 5.46	98.68 ± 3.67	95.53 ± 5.36
5	98.83 ± 7.36	99.82 ± 6.69	98.45 ± 3.37	95.39 ± 7.87	94.69 ± 5.79
80	98.74 ± 2.33	92.42 ± 5.75	94.55 ± 7.77	94.77 ± 5.40	94.61 ± 5.06
ATV					
0.5	94.43 ± 3.92	90.35 ± 2.28	91.67 ± 5.12	93.21 ± 6.02	95.17 ± 7.43
5	95.67 ± 7.45	93.27 ± 3.81	98.05 ± 5.95	94.87 ± 4.40	95.58 ± 5.97
80	96.82 ± 3.14	91.67 ± 1.77	96.88 ± 8.50	91.97 ± 3.53	98.68 ± 11.38
2-OH-ATV					
0.5	98.38 ± 4.74	96.75 ± 6.20	98.00 ± 8.84	98.12 ± 2.49	96.82 ± 5.66
5	93.83 ± 8.69	98.27 ± 10.90	92.63 ± 5.67	96.70 ± 6.84	94.95 ± 8.75
80	96.28 ± 4.03	94.10 ± 6.69	94.05 ± 3.22	97.00 ± 6.12	97.15 ± 7.02
4-OH-ATV					
0.5	93.55 ± 3.63	93.63 ± 8.91	92.95 ± 5.36	94.62 ± 9.28	99.78 ± 9.96
5	96.55 ± 9.10	96.83 ± 11.15	95.35 ± 6.25	98.13 ± 7.10	98.28 ± 8.96
80	98.12 ± 9.21	97.02 ± 7.95	96.33 ± 7.23	97.10 ± 5.75	94.45 ± 4.42
RSV					
0.5	98.8 ± 9.08	92.85 ± 5.44	93.78 ± 5.34	97.12 ± 4.27	94.87 ± 6.93
5	96.52 ± 10.42	97.60 ± 9.34	97.82 ± 5.47	96.30 ± 9.38	95.28 ± 6.30
80	98.65 ± 6.56	92.83 ± 5.08	96.47 ± 7.58	97.42 ± 8.45	97.28 ± 5.66

4.1.8. Conclusion

A simultaneous assay method for the quantification of SMV ATV, along with their active metabolites, and RSV was developed and validated. This is the first report of an analytical method for the simultaneous quantification of these three statins and their active metabolites. The method was validated for plasma with low (< 300 mg/dl) and high triglyceride (> 300 mg/dl) levels and proved to be reliable, accurate, selective, and sensitive. The method was used successfully for the quantification of SMV ATV, and RSV along with their active metabolites in the clinical samples from Aim 2 of this dissertation work.

4.2. Validation of LC-MS/MS assay of metformin

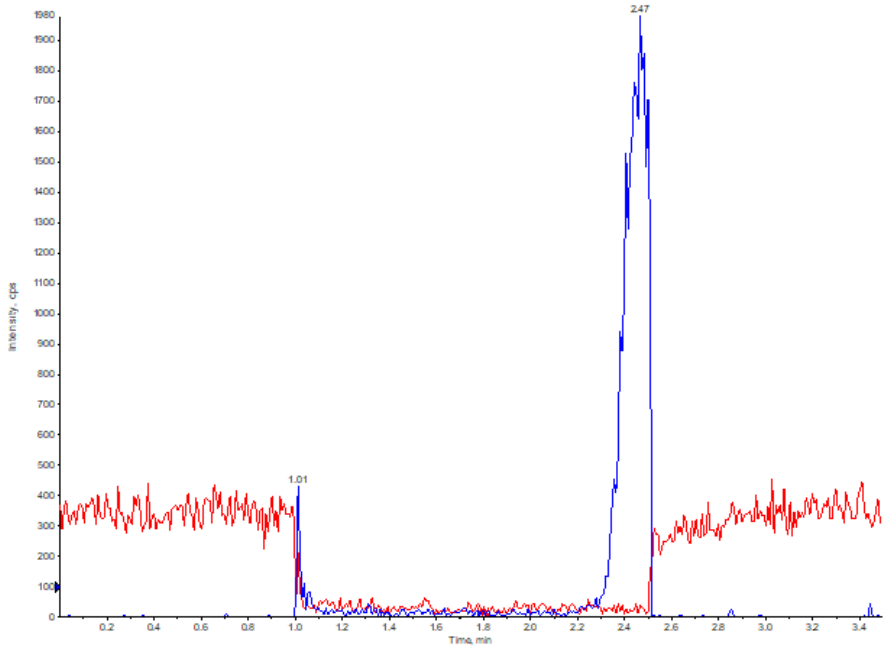
4.2.1. Selectivity and specificity

Figure 12 shows chromatograms of blank plasma, the IS (100 ng/ml), and metformin at LLOQ of 0.25 ng/ml. The potential interference from endogenous plasma components was inspected in plasma with low and high triglyceride levels. There was no interference from endogenous plasma components at the retention time for metformin and its labeled internal standard of 1.6 minutes in both low and high triglyceride plasma. The method showed an acceptable selectivity and specificity for the analyte and the internal standard.

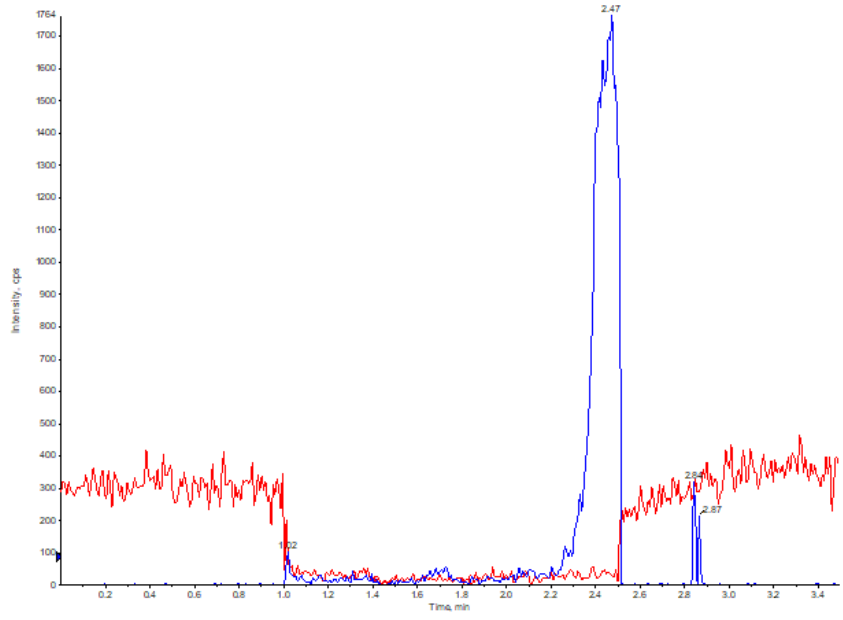
4.2.2. Sensitivity and carryover

The LLOQ was determined for metformin to be 0.25 ng/ml based on a response > 5 times of the baseline in blank plasma. The lower panel in **Figure 12** shows the chromatograms at LLOQ for metformin. Carryover was 6% of LLOQ signal for metformin in the 3 blank sample following 5 injections of samples at the highest standard concentration. Carryover acceptance criteria of < 20% of LLOQ signal was fulfilled for metformin.

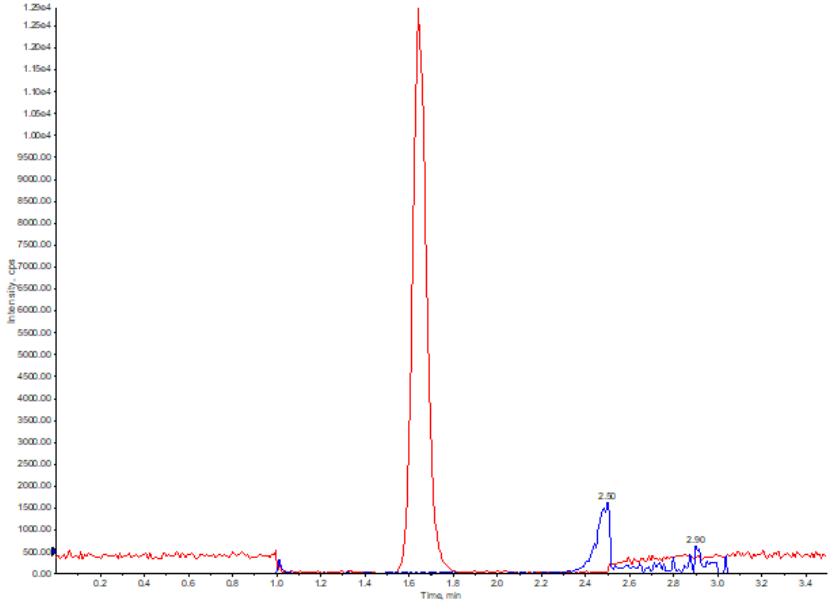
(a) Blank Plasma, Low TG Levels



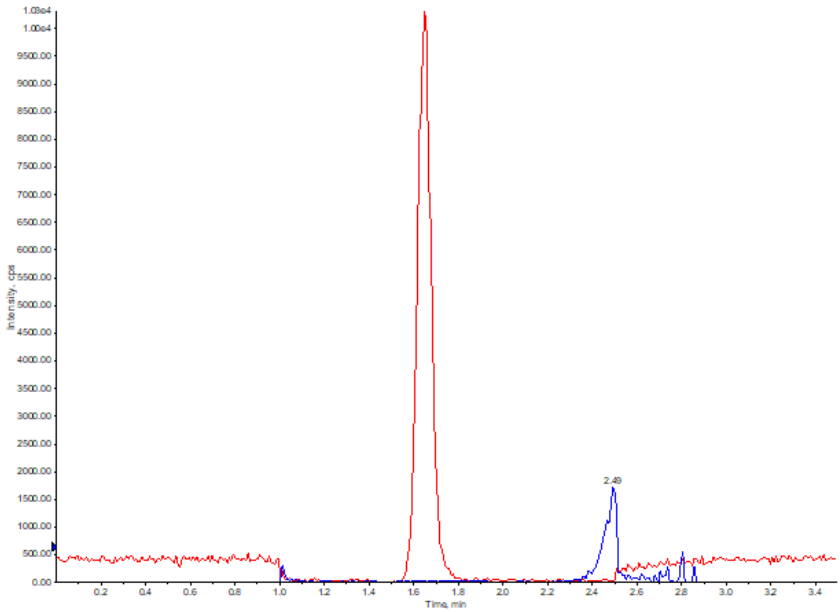
(b) Blank Plasma, High TG Levels



(c) IS, Low TG Levels



(d) IS, High TG Levels



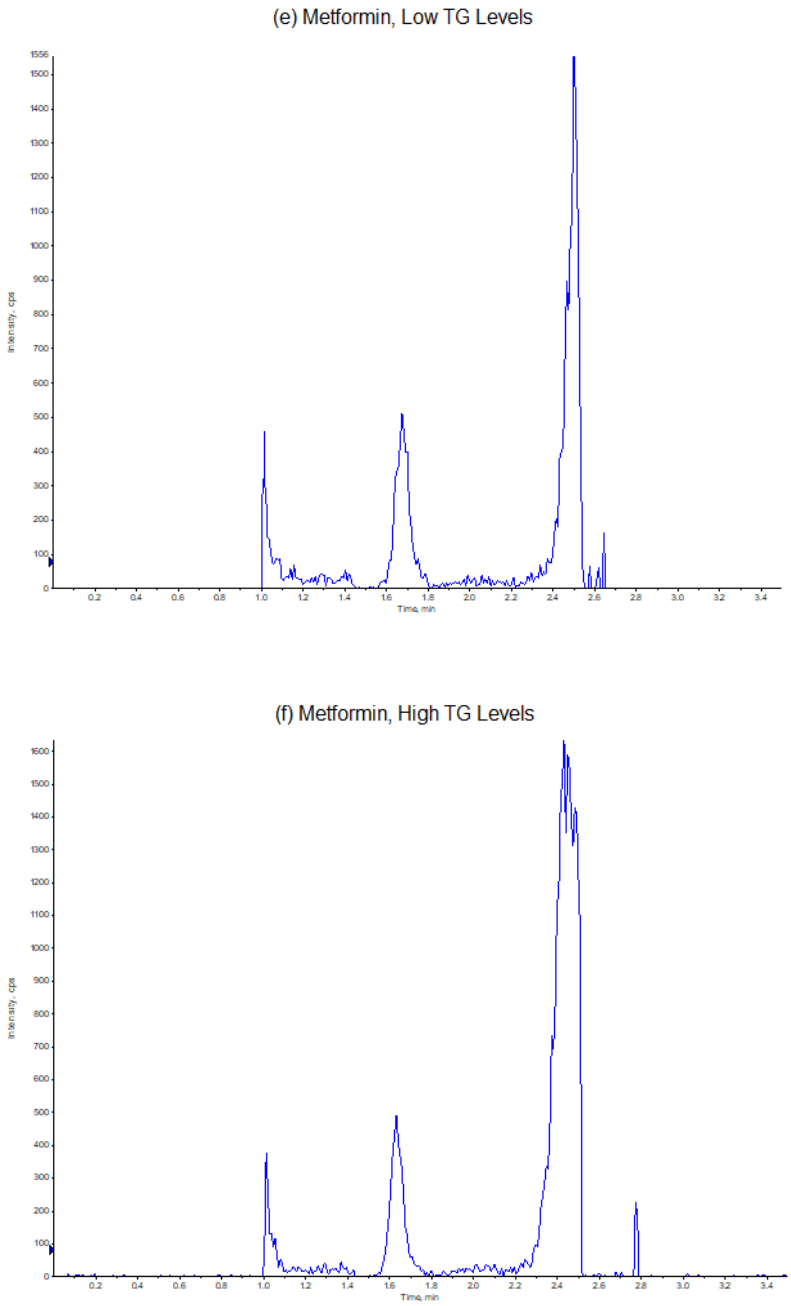


Figure 12. Representative chromatograms of (a, b) blank plasma, (c, d) IS at 100 ng/ml, and (e, f) metformin at LLOQ of 0.25 ng/ml in plasma with low (a, c, e) and high (b, d, f) triglyceride levels. The retention time for metformin and the labeled IS is 1.6 min.

4.2.3. Linearity

The linearity over the concentration range of 0.25-1,000 ng/ml for metformin was confirmed by constructing the calibration curves in plasma with both low and high triglyceride levels. Weighting factor of $1/x^2$ of metformin to the IS signals vs. metformin concentrations produced the best fit linear regression equations for the concentration-detector response relationship. **Table 10** present the mean ± standard deviation for the coefficients of determination (r^2) and slopes of the calibration curves from inter-day batches for metformin.

Table 10. Linearity of the calibration curves for metformin in plasma with low and high triglyceride levels

	Slope		r^2	
	Mean ± SD	%CV	Mean ± SD	%CV
Low TG (n=6)	0.025 ± 0.002	10.56	0.998 ± 0.002	0.162
High TG (n=4)	0.028 ± 0.003	12.14	0.992 ± 0.002	3.312

4.2.4. Accuracy and precision

The intra- and inter-day accuracy and precision values for plasma samples of metformin for plasma with low and high triglyceride levels are presented in **Table 11**. The intra-day accuracy and precision values for plasma with low triglycerides ranged 93-103% and < 13%, while those for plasma with high triglyceride were 95-112% and < 14%. The inter-day accuracy and precision values for plasma with low triglycerides were95-101% and < 11%, while those for plasma with high triglyceride

were 101-105% and < 11%. The intra- and inter-day accuracy and precision values of the method were not affected by high triglyceride levels in the plasma and were within the allowed deviation limit of 15% according to FDA guidelines (Food and Drug Adminisration, 2018).

Table 11. Intra-day (3 different days) and inter-day accuracy and precision of metformin at LLOQ and three QC concentration levels for plasma with low and high triglyceride levels

Concentration (ng/ml)	Day 1 (n=6)		Day 2 (n=6)		Day 3 (n=6)		Inter-day (n=18)	
	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)	Accuracy (%) Mean ± SD	Precision (%)
Low TG								
0.25	101.33 ± 5.47	5.39	102.67 ± 8.64	8.42	100.00 ± 13.39	13.39	101.33 ± 9.20	9.08
0.5	95.67 ± 11.76	12.29	97.33 ± 13.06	13.42	96.67 ± 9.35	9.67	96.56 ± 10.82	11.21
20	99.83 ± 10.08	10.09	98.67 ± 7.67	7.78	98.00 ± 8.25	8.41	98.83 ± 8.23	8.33
800	92.79 ± 3.61	3.89	96.90 ± 3.29	3.39	96.04 ± 4.22	4.39	95.24 ± 3.94	4.14
High TG								
0.25	112.33 ± 7.09	6.31	101.52 ± 7.90	7.78	100.48 ± 14.27	14.20	104.78 ± 11.11	10.60
0.5	103.97 ± 10.13	9.74	98.50 ± 10.46	10.61	105.32 ± 11.70	11.11	102.59 ± 10.57	10.31
20	99.53 ± 2.45	2.47	101.00 ± 7.82	7.75	103.12 ± 3.52	3.42	101.22 ± 5.07	5.01
800	95.13 ± 7.45	7.83	96.58 ± 9.99	10.34	110.83 ± 4.75	4.29	100.85 ± 10.27	10.18

4.2.5. Extraction recovery

The extraction recovery of metformin from plasma with low and high triglyceride levels was reproducible at the three QC concentration levels and above 89% and 90%, respectively **(Table 12).** The extraction recovery for the internal standard was tested only in plasma with high triglyceride levels and the recovery was 98.33 ± 4.27%. Since the extraction recovery of the IS from high TG plasma was above 85%, we did not test for the extraction recovery using low TG plasma.

Table 12. Extraction recovery (mean ± SD) of metformin at three levels of QC samples from human plasma with either low or high triglyceride levels using protein precipitation method

Concentration (ng/ml) n = 6	Low TG Levels	High TG Levels
0.5	88.89 ± 5.73	89.71 ± 4.16
20	92.74 ± 4.84	92.20 ± 10.09
800	94.84 ± 3.14	92.35 ± 9.74

4.2.6. Matrix effect

The matrix effect was not significant for metformin in plasma with either low or high triglyceride levels. The mean ± SD of the matrix effect for metformin in low and high triglyceride plasma are represented in **Table 13.** Matrix effect was in the range of 99-101 % in plasma with low triglyceride levels and in the range of 96-101% in plasma with high triglyceride levels. Matrix effect was tested for the internal standard in plasma with high triglyceride levels and was 88.35 ± 7.75 %.

Table 13. Matrix effect (mean ± SD) of metformin at three levels of QC samples from human plasma samples with low (n=6 at each QC level) and high (n=6 at each QC level) triglyceride levels

Concentration (ng/ml) n = 6	Low TG Levels	High TG Levels*
0.5		
20	101.46 ± 6.23	96.49 ± 8.89
800	100.64 ± 4.36	101.45 ± 6.78

* High TG levels in plasma samples were achieved by spiking with triglycerides to reach ≥ 300 mg/dl (352-403 mg/dl) triglyceride concentrations, t-test showed no statistically significant difference between the matrix effect at all concentration levels between plasma with low and high TG levels

4.2.7. Stability

The results from the stability experiments performed under different conditions for metformin are presented in **Table 14.** These conditions reflect the expected conditions during sample preparation, storage, and analysis. Results of freeze and thaw stability test showed that metformin was stable (96-100%) for three cycles when stored at -80ºC and thawed at room temperature. Bench-top stability test showed that metformin was stable (94-101%) for 4 hr at room temperature (the time required for sample preparation). Long-term stability test results showed that metformin was adequately stable (95-100%) for up to 1 month in plasma when stored at -80ºC. Processed samples can be analyzed overnight (24 hr) in the autosampler at 20ºC without any concern about the stability of metformin (92-100%). Lastly, the stock solution of metformin was stable (95-96%) up to one month when stored at -20ºC.

Table 14. Stability of metformin in human plasma under various conditions (n=6); RT, Room temperature; all data are presented as mean ± SD

Concentration (ng/ml)	Freeze and Thaw, three cycles, -80ºC to RT	Bench-top, RT for 4 hr	Long-term, -80ºC for 1 month	Processed sample, autosampler, 20ºC for 24 hr	Stock solution, -20°C for 1 month
0.5	100.21 ± 8.11	100.69 ± 8.58	99.88 ± 4.37	98.13 ± 6.12	94.96 ± 6.12
20	100.15 ± 2.95	94.13 ± 3.35	99.31 ± 4.86	93.40 ± 7.25	96.38 ± 5.94
800	95.79 ± 3.99	98.36 ± 6.29	95.15 ± 6.09	94.82 ± 9.36	95.57 ± 9.18

4.2.8. Conclusion

An LC-MS/MS method for the quantification of metformin was developed and validated for plasma with low (< 300 mg/dl) and high triglyceride (> 300 mg/dl) levels. Validation of the method according to the FDA guidelines proved it to be accurate, precise and reliable. Matrix effect was negligible even in plasma samples with high triglyceride levels. The method was used successfully for the analysis of clinical samples from Aim 3.

4.3. Statin study

4.3.1. Demographic data

Fifty (50) subjects, aged 25 to 68 years with severe obesity (BMI range of 32.7-62.0 kg/m^2) were recruited for the study. Thirty (30) of the patients were on one of the statins (statin group) as follows: nine (9) patients were on simvastatin (SMV group), five (5) on atorvastatin (ATV group), twelve (12) on rosuvastatin (RSV group), three (3) on pravastatin, and one (1) on lovastatin. Patients on pravastatin and lovastatin were not included in the statistical analysis among the statin group due to the limited sample size of these two groups. Twenty patients were not on any statin (non-statin group). **Table 15** shows the demographic data for the individual statin (SMV, ATV, and RSV) and the non-statin groups. Mean age was significantly older in RSV group (55.8 yr.) compared to the non-statin group (43.8 yr.). SMV and ATV groups showed older mean ages than that of the non-statin group (51.4 and 56.4 vs. 43.8 yr.), but it was not statistically significant. This can be attributed to insufficient power with the lower sample sizes (Nayak, 2010) of SMV (n=9) and ATV (n=5) groups.

Table 15. Demographic data in individual statin groups (SMV, ATV, and RSV) and non-statin group

		SMV (n=9)	ATV (n=5)	RSV (n=12)	Non-statin (n=20)
Mean age (yr.) ± SD		51.44 ± 8.44	56.40 ± 10.81	55.75 ± 9.13 *	43.80 ± 11.67
Mean BMI (kg/m^2) at baseline ± SD		42.86 ± 5.29	40.91 ± 4.28	41.90 ± 6.16	45.57 ± 6.97
Mean excess weight (lb.) at baseline ± SD		112.47 ± 38.15	102.13 ± 22.41	111.95 ± 42.73	125.19 ± 45.99
Racial/Ethnic Breakdown n (%)	Caucasian	5 (55.6)	2 (40.0)	10 (83.3)	9 (45.0)
	African American	2 (22.2)	2 (40.0)	1 (8.3)	5 (25.0)
	Hispanic	1 (11.1)	0 (0.0)	1 (8.3)	2 (10.0)
	Other	1 (11.1)	1 (20.0)	0 (0.0)	4 (20.0)
Sex n (%)	Male	3 (33.3)	2 (40.0)	6 (50.0)	2 (10.0)
	Female	6 (66.7)	3 (60.0)	6 (50.0)	18 (90.0)

***** Mean age was significantly older in RSV group compared to non-statin group (ANOVA followed by Tukey's test, p-value 0.015)

Mean BMI and excess weight at baseline were comparable among individual statin and non-statin groups. The racial/ethnic distribution was also comparable among the groups, with the highest number of subjects being Caucasian in all groups (more than 40%), and less than 5 subjects in all the other racial categories (African American, Hispanic and Other). Sex composition showed no statistically significant difference among the groups with more of female subjects (50-90%).

Since the three individual statin groups (SMV, ATV, and RSV) showed comparable mean age, mean BMI and excess weight at baseline, race distribution, and sex composition, the data from these three individual statins were also combined in one group (statin group). Demographic data of combined statin group in comparison to the non-statin group is presented in **Table 16**. The mean age in combined statin group was older than that of the non-statin group (54.4 vs. 43.8 yr.). This may be due to the fact that statins are prescribed to decrease the risk of atherosclerotic cardiovascular diseases, which is usually increased with age (Dhingra and Vasan, 2012). Mean BMI and excess weight at baseline, and race distribution were comparable between the two groups. Sex composition showed a statistically significant difference between the combined statin and the non-statin groups. The number of male subjects was significantly higher in statins group compared to the non-statin group (11 and 2, respectively), while females had comparable subject counts in the statin and non-statin groups (15 and 18, respectively). This difference could be explained by the higher incidence of

cardiovascular diseases in males compared to females, in which case statin treatment is recommended (Vitale et al., 2010).

Table 16. Demographic data in combined statins and non-statin groups

		Statins (n=26)	**Non-statin (n=20)**
Mean age (yr.) ± SD		54.38 ± 9.11 *	43.80 ± 11.67
BMI (kg/m^2) at baseline		42.04 ± 5.39	45.57 ± 6.97
Excess weight (lb.) at baseline		110.24 ± 36.95	125.19 ± 45.99
Racial/Ethnic Breakdown n (%)	Caucasian	17 (65.4)	9 (45.0)
	African American	5 (19.2)	5 (25.0)
	Hispanic	2 (7.7)	2 (10.0)
	Other	2 (7.7)	4 (20.0)
Sex n (%)	Male	11 (42.3) †	2 (10.0)
	Female	15 (57.7)	18 (90.0)

***** Mean age was significantly higher in statins group compared to non-statin group (Student's t-test, p-value 0.001)
† Statins group has a significantly different sex composition from non-statin group (Fisher's Exact test, p-value 0.022)

4.3.2. % Follow-up

The % follow-up at each time point of 1 week, 1, 3, 6, and 12 months in the statin study groups is represented in **Table 17**. The % follow-up was 84.8-93.5% for all the patients who were included in the study at 1-week, 1-, 3-, and 6-month follow-up visits. The % follow-up decreased drastically at 12-month follow-up visit (63.0%). Individual statin groups (SMV, ATV, and RSV groups) showed the same trend with % follow-up range 66.7-100.0% at 1-week, 1-, 3-, and 6-month, followed by a decrease at 12-month visit to 40.0-75.0%. The combined statin and the non-statin groups also showed the similar trend, with % follow-up ≥ 84.6% at all follow-up visits except at 12-month, when the % follow-up decreased to 61.5-65.0%. The drastic decrease in % follow-up at 12-month follow-up visit is common in bariatric surgery patients (Belo et al., 2018). Several predictors for loss of patients' follow-up have been reported such as treatment failure (Puzziferri et al., 2014), pre-surgery excess weight greater than 110 lb. (Belo et al., 2018), residing at a greater distance from the follow-up appointment location, and sex (males have more tendency to miss their follow-up appointments) (Sockalingam et al., 2013, Khorgami et al., 2015). Bariatric surgeries, in general, require life-long medical and psychological care post-surgery. Studies have shown that adherence to follow-up appointments after RYGB results in better weight loss outcomes. Strategies to improve patients adherence to follow-up appointments with their physicians are needed (Compher et al., 2012).

Table 17. % Follow-up (n) of patients at each time point of follow-up visits in the statin and non-statin groups

	1 Week	1 Month	3 Months	6 Months	12 Months
Total (n=46)	89.1 (41)	93.5 (43)	89.1 (41)	84.8 (39)	63.0 (29)
SMV (n=9)	88.9 (8)	100.0 (9)	77.8 (7)	66.7 (6)	55.6 (5)
ATV (n=5)	100.0 (5)	100.0 (5)	100.0 (5)	100.0 (5)	40.0 (2)
RSV (n=12)	75.0 (9)	75.0 (9)	100.0 (12)	91.7 (11)	75.0 (9)
Statins (n=26)	84.6 (22)	88.5 (23)	92.3 (24)	84.6 (22)	61.5 (16)
Non-statin (n=20)	95.0 (19)	100.0 (20)	85.0 (17)	85.0 (17)	65.0 (13)

4.3.3. Weight loss outcomes

4.3.3.1. BMI reduction from baseline

The reduction in BMI from baseline had similar patterns among the three individual statin and the non-statin groups. The reduction started at 1.6-2.3 unit of reduction in BMI at 1-week, with continuous reduction in BMI at 1-month (3.9-4.6 units), 3-month (6.1-7.6 units), and 6-month (8.6-10.3 units). At 12-month visit, the range was 12.3-12.7 unit reduction in BMI, except for ATV group in which a seemed to have less mean BMI reduction of 10.3 units with only 2 subjects at that time point. There was no significant difference in BMI reduction among the three individual statin groups at any time point post-surgery. However, statistical analysis among

the three groups at this time point post-surgery could not be performed in the original data set due to the low subject count (n=2) in ATV group at 12-month follow-up visit **(Figure 13, a)**. The similar pattern of BMI reduction post-surgery prompted the combination of these three groups into one group (statin group). The comparison of statin and non-statin groups indicated that the BMI reduction post-surgery was similar between combined statin and non-statin groups at all follow-up visits except at 3-month. Non-statin group had a significantly greater reduction in BMI at 3-month visit compared to that of the statin group (7.6 vs. 6.6 unit reduction in BMI, respectively). However, this difference was resolved at 6- and 12-month follow-up visits, with similar BMI reduction between the two groups at these follow-up visits. Statin group started showing a significant BMI reduction at 6-month follow-up visit, while non-statin group started showing reduction in BMI significantly at 3-month visit **(Figure 14, a)**. Multiple imputation (MI) statistical analysis results confirmed the outcomes from the original data set and added to the power of the analysis **(Figures 13, b and 14, b)**. Both combined statin and non-statin groups after MI started showing a significant reduction at-3 month visit and further significant reduction at 12-month follow-up visits **(Figure 14, b).** The similar pattern of BMI reduction post-surgery in statin and non-statin groups confirms that the weight loss was RYGB related and not affected by the statin treatment. These observations agree with the results from a published study (Perna et al., 2011), that concludes the weight loss between statin (atorvastatin,

simvastatin, rosuvastatin, pravastatin, lovastatin and fluvastatin) and non-statin groups is comparable in an average follow-up time of 5.6 months (range 1-36 months). On the other hand, our results disagree with that from another published study which reports patients on statins lose weight 12 months post-surgery more than patients who are not taking any statin (Taylor et al., 2017); however, the type of statin was not specified in this study. Both studies did not examine the weight loss pattern in individual statins.

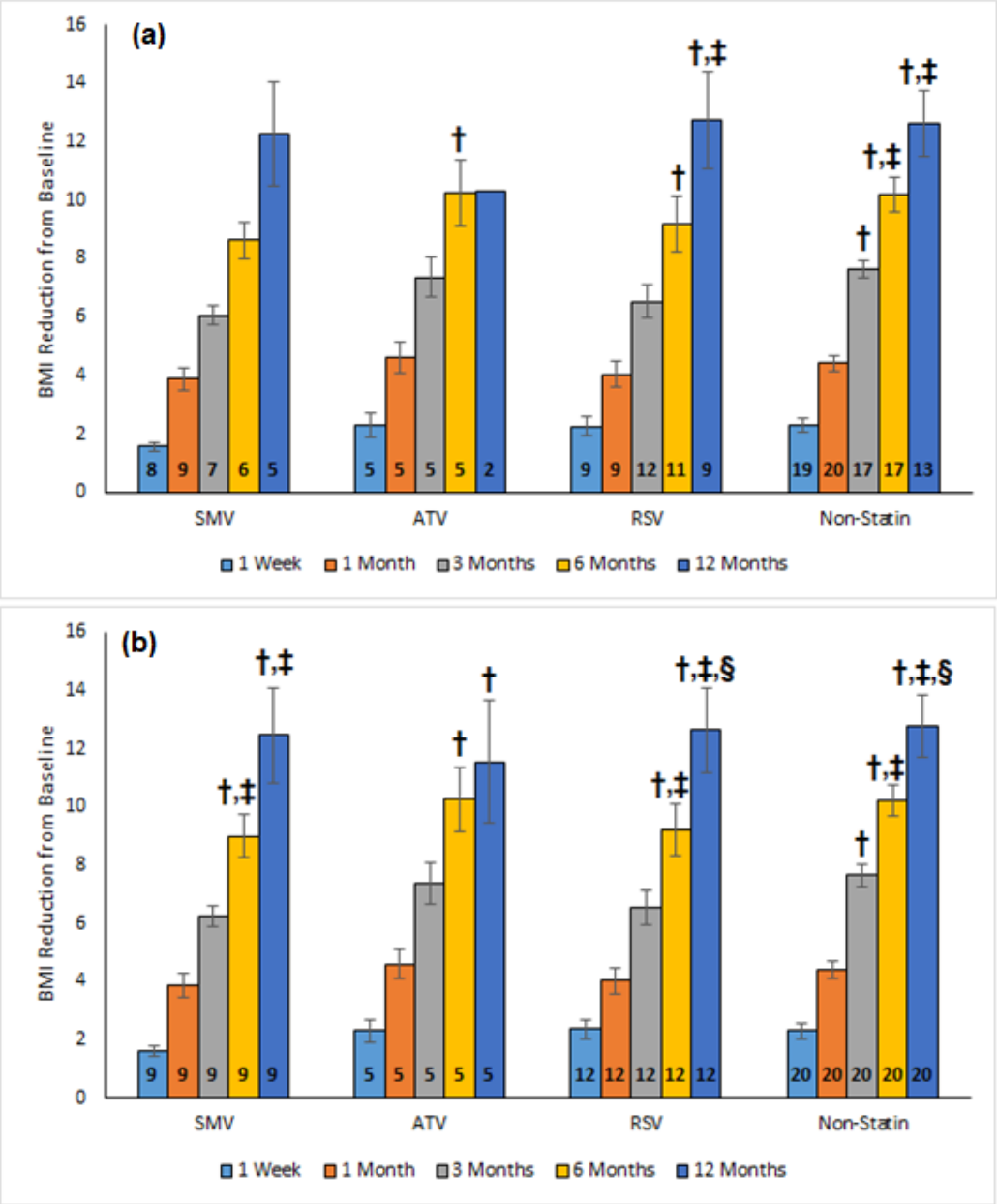


Figure 13. BMI reduction from baseline (mean ± SE) after RYGB at 1-W, 1-, 3-, 6-, and 12-M follow-up visits in individual statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
Statistically significant difference from (†) 1 W, (‡) 1 M, and (§) 3 M within each group.

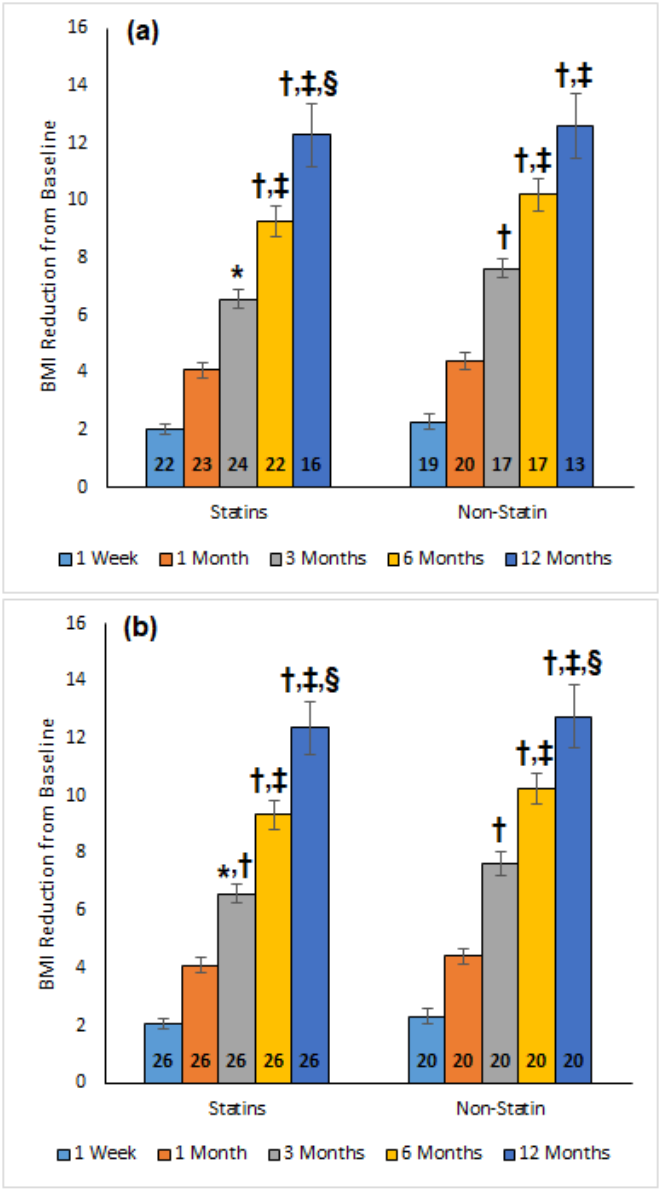


Figure 14. BMI reduction from baseline (mean ± SE) after RYGB at 1-W, 1-, 3-, 6-, and 12-M follow-up visits in combined statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
Statistically significant difference from (†) 1 W, (‡) 1 M, and (§) 3 M within each group.

(*) Statistically significant difference from non-statin group at 3 M.

4.3.3.2. Percentage excess weight loss (%EWL)

The pattern of weight loss after RYGB represented by %EWL was similar among the individual statin and non-statin groups **(Figure 15, a)**. %EWL started at 1-week with 10.6-15.3%, and patients lost excess weight continuously and significantly after surgery to reach 70.4-76.5 %EWL at 12-month follow-up visit. Individual statin groups did not show any statistically significant difference in excess weight loss among SMV, ATV and RSV groups at any time point post-surgery. When the three groups were combined in one group (statin group) and compared to the non-statin group **(Figure 16, a)**, the two groups showed similar trends in weight loss after surgery with %EWL reaching 12.2 and 12.5 by 1 week post-surgery in the statin and the non-statin groups, respectively. Patients in both groups continued to lose weight up to 12 months post-surgery to 75.4 %EWL in the statin group and 70.4 %EWL in the non-statin group. By using MI to increase the power of the statistical analysis, similar results were obtained to those from the original data set **(Figures 15, b and 16, b)**. Patients lost excess weight significantly starting at 3 months post-surgery in both statin and non-statin groups. Further significant excess weight loss by 12 months post-surgery was observed in both statin and non-statin groups. The results from %EWL post-surgery confirmed the results from BMI reduction after surgery. The similarity in weight loss pattern in statin and non-statin groups indicates the weight loss after RYGB is not affected by statin intake.

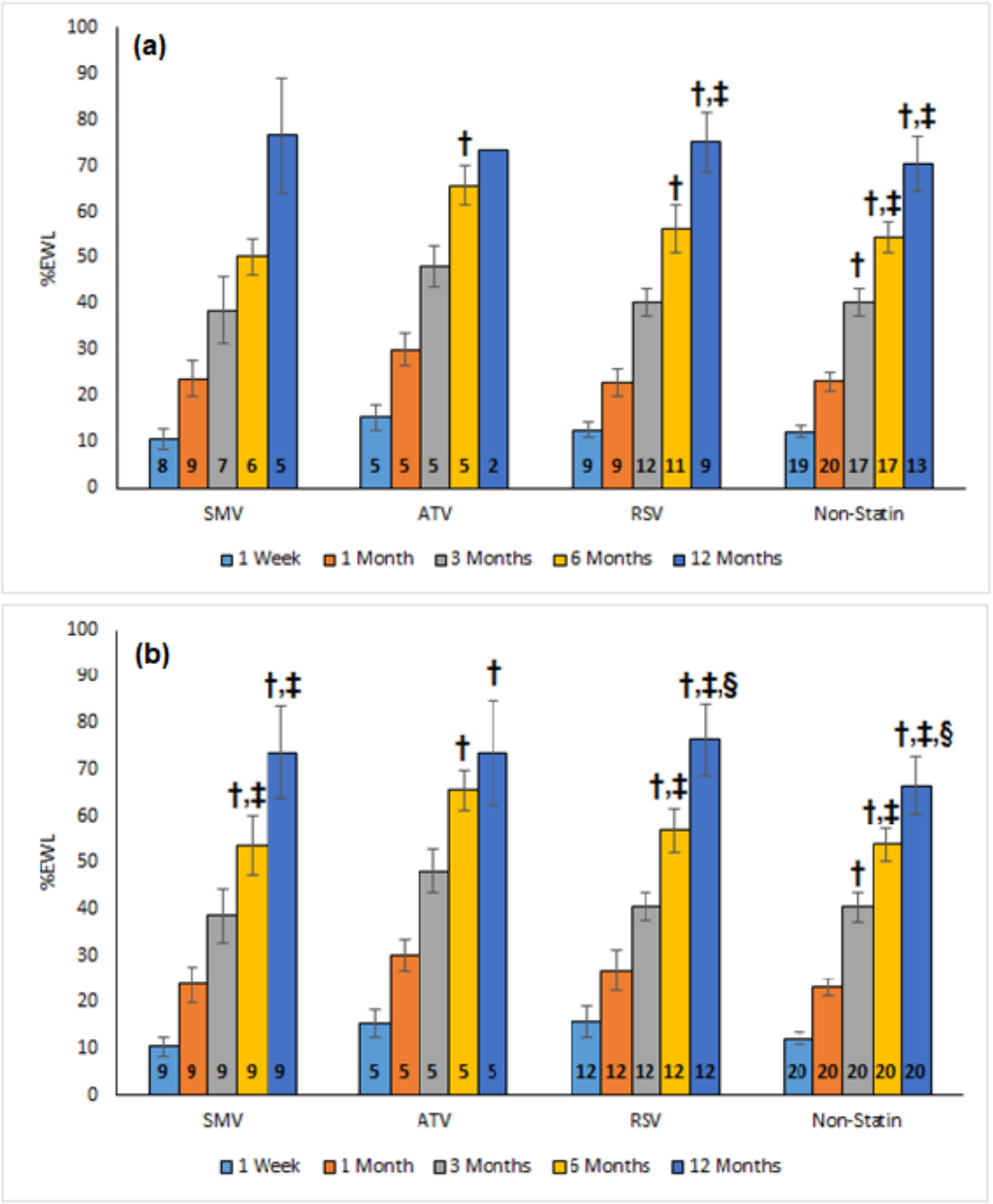


Figure 15. %EWL (mean ± SE) after RYGB at 1-W, 1-, 3-, 6-, and 12-M follow-up visits in individual statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
Statistically significant difference from (†) 1 W, (‡) 1 M, and (§) 3 M within each group.

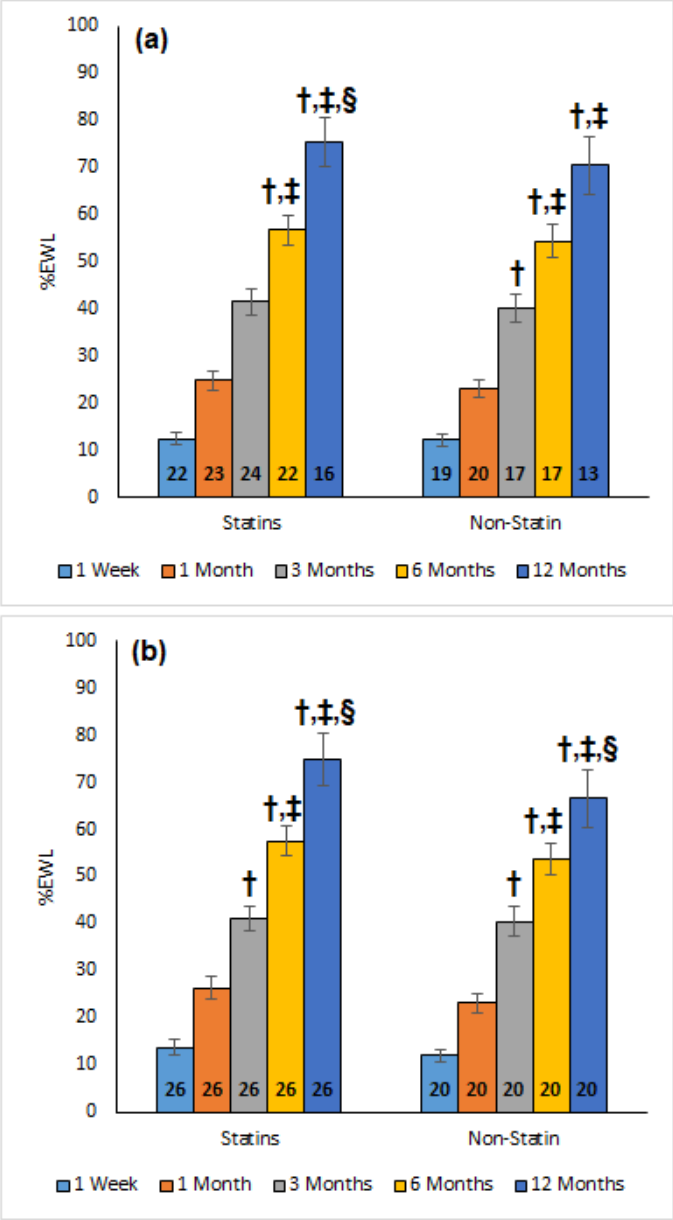


Figure 16. %EWL (mean ± SE) after RYGB at 1-W, 1-, 3-, 6-, and 12-M follow-up visits in combined statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
Statistically significant difference from (†) 1 W, (‡) 1 M, and (§) 3 M within each group.

4.3.4. Pharmacodynamic or response biomarkers

According to the FDA-NIH Biomarker Working Group, a pharmacodynamic or response biomarker is used to confirm a biological response happened in an individual after exposure to a medical or environmental agent (FDA-NIH Biomarker Working Group, 2016). To evaluate the biological response in patients to lipid lowering medications such as statins, serum LDL levels can be used as a pharmacodynamic/response biomarker (FDA-NIH Biomarker Working Group, 2016). However, other lipid parameters such as TC, TG and HDL are also considered useful in determining the overall biological effect of statin therapy in patients (Wiklund et al., 2013).

4.3.4.1. Low-density lipoprotein (LDL)

Mean LDL levels showed a decreasing trend at 3-month follow-up visit in all individual statin and non-statin groups. ATV and RSV groups had a trend of continuous increases in mean LDL levels at 6- and 12-month visits, reaching levels similar to or higher than baseline by 12 months post-surgery. SMV group also showed a trend of increase in LDL mean levels at 6-month follow-up visit, followed by a decrease at 12-months visit. However, it is worth mentioning that SMV and ATV groups had small number of subjects (3 and 2 subjects, respectively) at 12-month visit compared to baseline (n= 9 and 5, respectively). The later increase in LDL levels in ATV and RSV groups could be attributed to the discontinuation of

statins use post-surgery in some patients **(Table 18).** The trend in non-statin group was a decrease in mean LDL levels at 3-month visit, and it was stabilized afterwards **(Figure 17, a)**.

Table 18. LDL (mg/dl) in patients who were on statins and patients who discontinued statins after RYGB

	On statins				**Discontinued statins**		
	Baseline (n=26)	3M (n=13)	6M (n=13)	12M (n=7)	3M (n=4)	6M (n=4)	12M (n=3)
Mean	90.00	64.23	79.38	63.71	92.75	104.25	127.00
SD	24.98	21.37	29.61	29.40	26.11	24.88	42.14

Since individual statin groups showed a similar trend in LDL reduction up to 6 months post-surgery, they were combined into one group (statin group) and compared to the non-statin group. A significantly higher mean LDL level at baseline was observed in the non-statin group compared to the statin group (108.8 vs. 90.0 mg/dl). The levels of mean LDL became comparable in the non-statin (88.6 mg/dl) and statin (89.7 mg/dl) groups by 12 months post-surgery as shown in **Figure 18, a**. These results are in agreement with a published study that reports a significantly higher LDL levels in non-statin group (113.3 mg/dl) compared to statin group (98.9 mg/dl) pre-surgery (Taylor et al., 2017). By using MI to predict and impute the values for missing LDL data, the trend was similar to the original data in all groups except for SMV group. After MI, SMV group showed a higher mean LDL value at

12-month visit for 9 subjects (85.6 mg/dl) compared to the original data from 3 subjects only (60.7 mg/dl) **(Figure 17, b)**. Comparison of statin vs. non-statin groups after MI confirmed that the non-statin group has a significantly higher mean LDL level at baseline compared to the combined statin group. The non-statin group showed a significant decrease in LDL level post-surgery at 3- and 6-month follow-up visits compared to baseline **(Figure 18, b)**. This indicates that the high baseline LDL levels in the non-medicated group were corrected by RYGB.

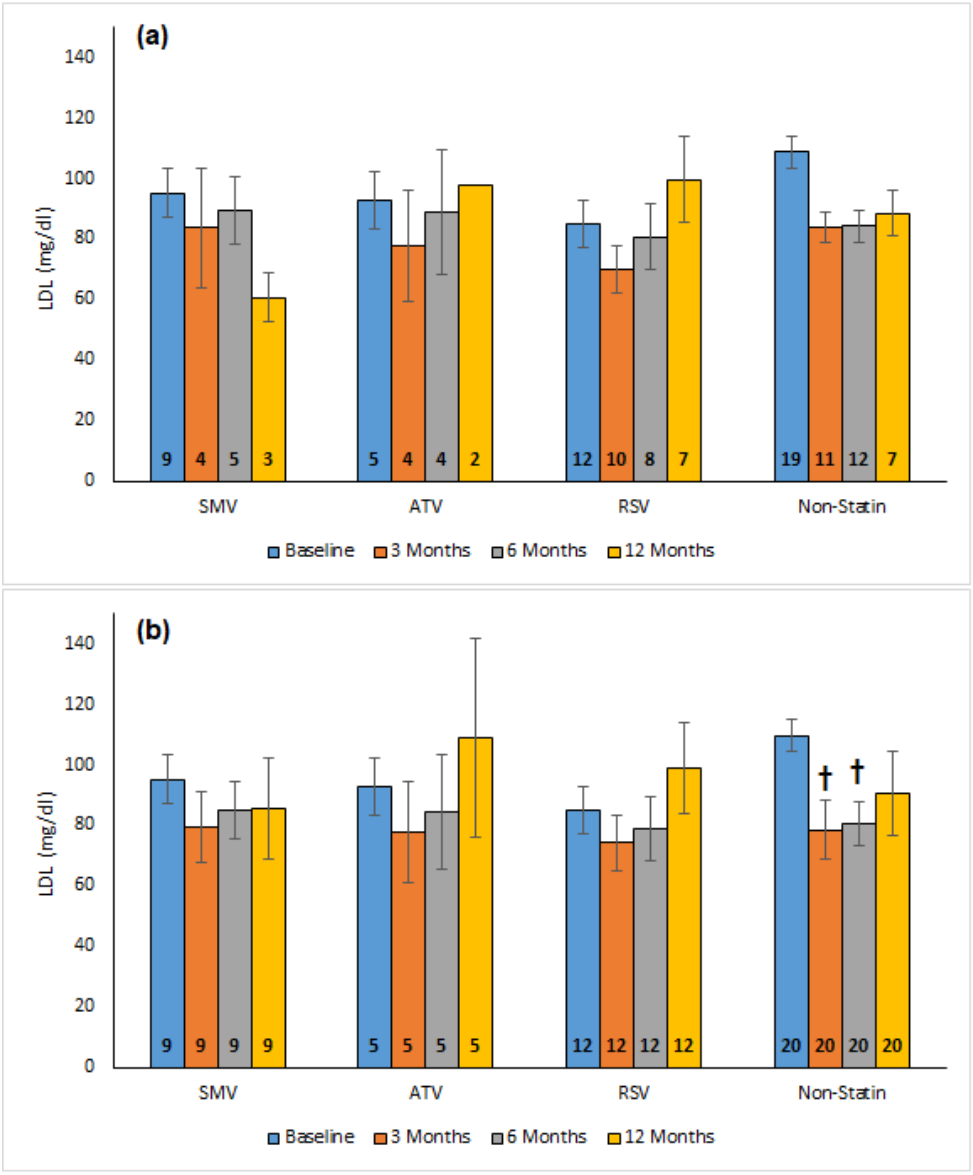
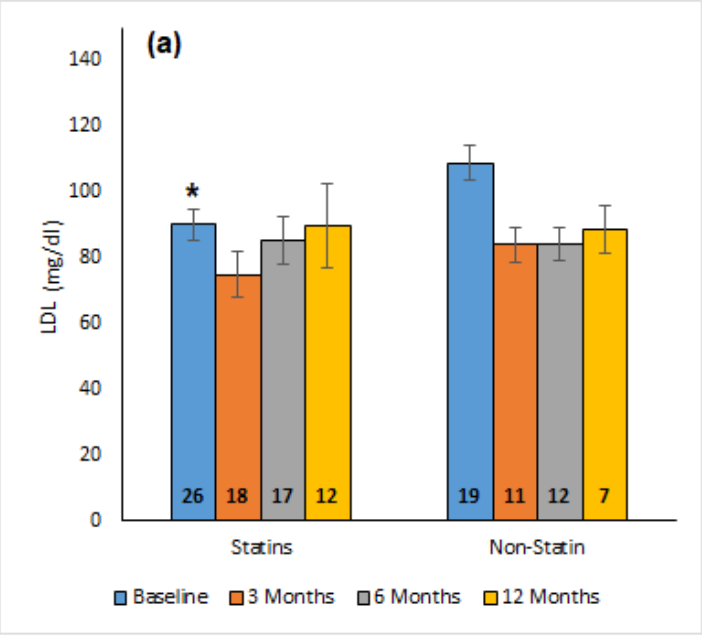


Figure 17. LDL in mg/dl (mean ± SE) at baseline, and 3-, 6-, and 12-M follow-up visits after RYGB in individual statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
(†) Statistically significant difference from baseline in the same group



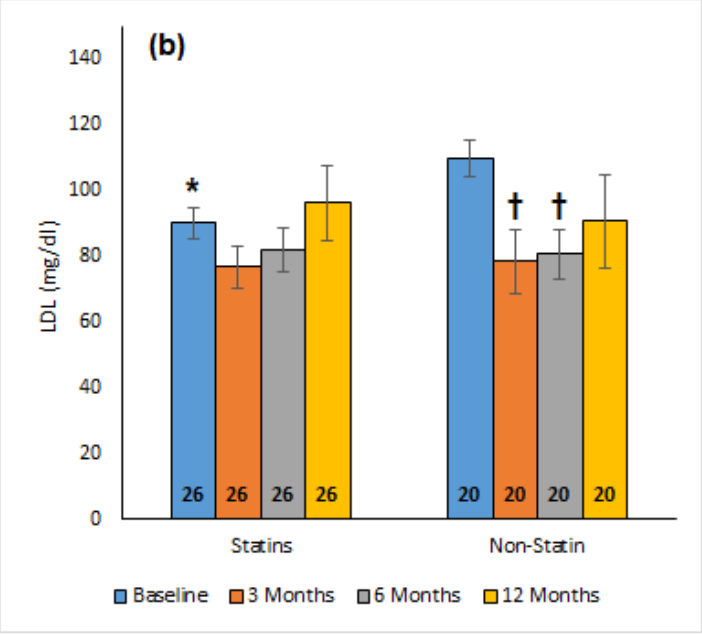


Figure 18. LDL in mg/dl (mean ± SE) at baseline, and 3-, 6-, and 12-M follow-up visits after RYGB in combined statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
(†) Statistically significant difference from baseline in the same group.
(*) Statistically significant difference from non-statin group at baseline.

4.3.4.2. High-density lipoprotein (HDL)

Mean HDL levels had a trend of slight decrease at 3-month follow-up visit, followed by a continuous increase at 6- and 12-month visits in ATV, RSV, and non-statin group. The increase at 12-month visit in mean HDL was significant in RSV and non-statin group (63 and 60 mg/dl, respectively) compared to 3-month levels in the same group (48 and 43 mg/dl, respectively). ATV group had only 2 subjects' data at 12-month visit, which hindered the inclusion of 12-month visit data in the statistical analysis of the different time points within this group. In SMV group, HDL levels at 12 months (49 mg/dl) were comparable to 6 months post-surgery (50 mg/dl) **(Figure 19, a)**. When individual statin groups were combined, a similar trend was observed of slight decrease in mean HDL levels at 3-month visit (45 from 47 mg/dl at baseline), followed by a continuous increase at 6- and 12-month visits (51 and 59 mg/dl, respectively). The increase becomes significant in statin group at 12-month compared to 3-month visit, similar to the non-statin group **(Figure 20, a)**. RSV and ATV groups had a similar pattern after-surgery in MI and original data, while SMV group had a higher mean HDL at 12-month visit in MI data (58 mg/dl, n=9) compared to the original available data (49 mg/dl, n=3). MI results for the individual statin groups are presented in **Figure 19, b**. MI confirmed the pattern of significant increase in mean HDL levels at 12-month visit in both statin and non-statin groups **(Figure 20, b)**. These results agree with a published study that reports a significant increase in HDL levels at one-year post-surgery in women

underwent RYGB (Asztalos et al., 2010). The study did not mention the medications these women might have been taking; however, the trend of change in HDL levels (-16, -2, +8, +15 unit change in HDL from baseline at 1, 3, 6, and 12 months post-surgery, respectively) was comparable to our results, regardless of what statin was used. Our results lead to the conclusion that RYGB causes a significant increase in HDL levels 6 months and one year after surgery regardless if subjects are under statin treatment.

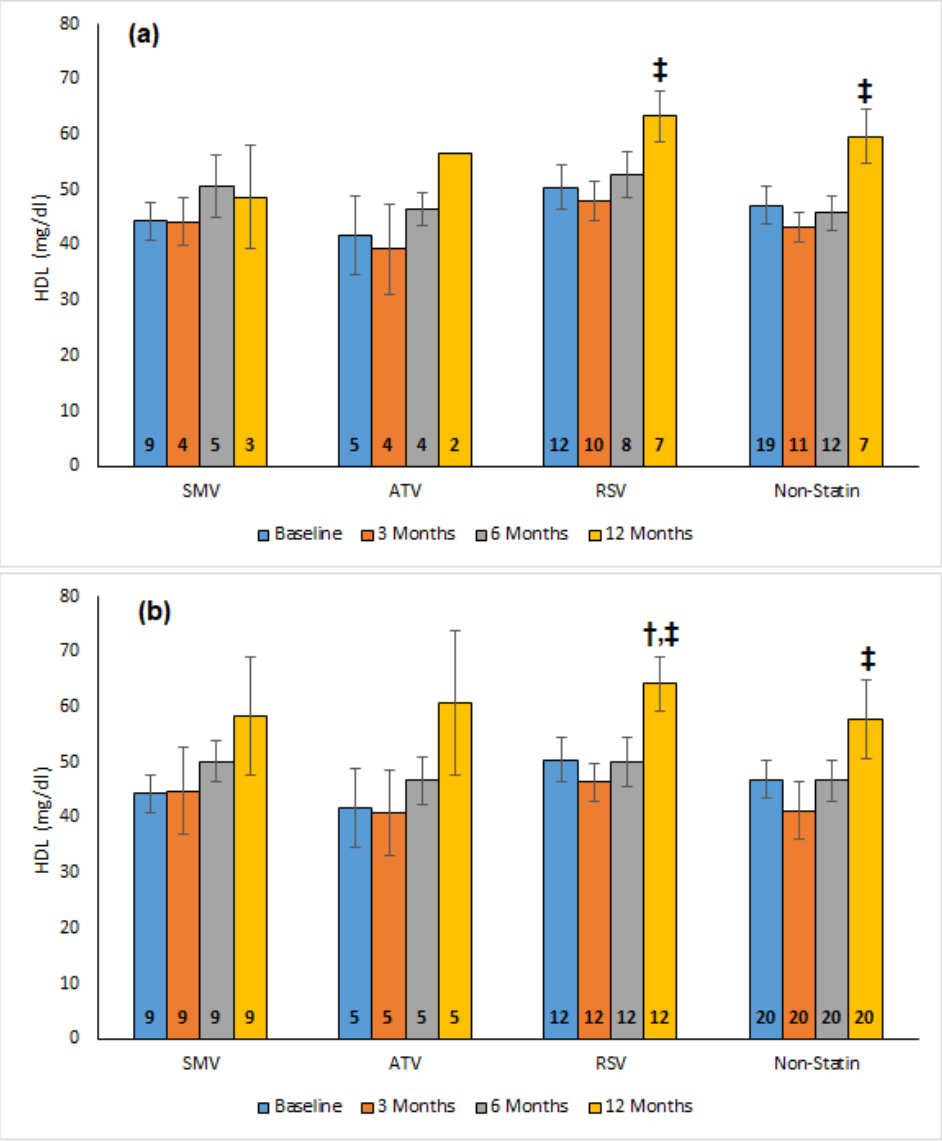


Figure 19. HDL in mg/dl (mean ± SE) at baseline, and 3-, 6-, and 12-M follow-up visits after RYGB in individual statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
Statistically significant difference from (†) baseline, and (‡) 3 M within each group.

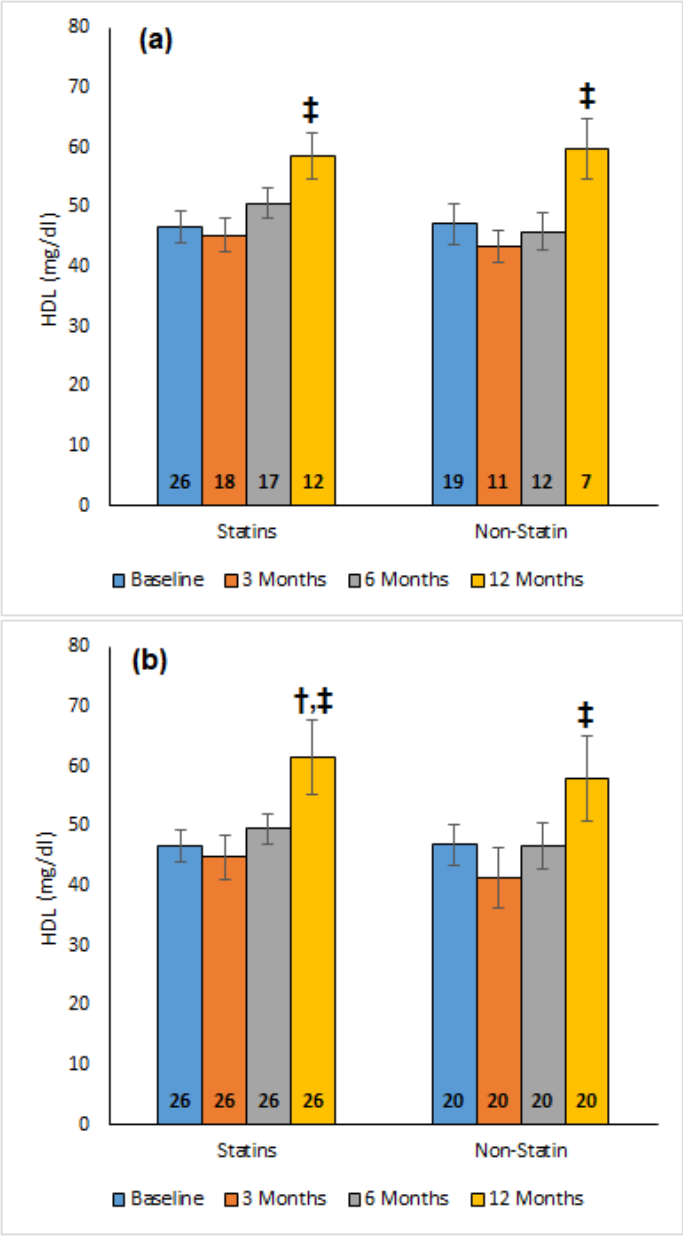


Figure 20. HDL in mg/dl (mean ± SE) at baseline, and 3-, 6-, and 12-M follow-up visits after RYGB in combined statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
Statistically significant difference from (†) baseline, and (‡) 3 M within each group.

4.3.4.3. Triglycerides (TG)

SMV and RSV groups had a higher mean TG values at baseline (182.9 and 164.8 mg/dl, respectively) compared to those post-surgery visits (≤ 124.6 and ≤ 116.6, respectively). On the other hand, ATV group had comparable levels of mean TG at baseline and post-surgery follow-up visits. ATV at 10 mg daily dose reduces TG levels by 14-19%, while SMV and RSV reduce TG by 14% with 40 mg daily dose and 17% with 20 mg daily dose, respectively (Heart Protection Study Collaborative, 2002, Sever et al., 2003, Colhoun et al., 2004, Ridker et al., 2008). ATV has a comparable percentage TG reduction to SMV and RSV with a lower daily dose. The mean ± SD of doses at baseline for patients on statins in our study were 36 ± 29, 32 ± 21, and 20 ± 15 mg for ATV, SMV and RSV, respectively. Since ATV had the higher mean daily dose, it could explain the lower TG levels at baseline in ATV group. Mean TG levels for the 5 patients on ATV appeared to be under control at baseline, resembling those of non-statin group. Non-statin group showed a trend of decrease in mean TG values, although not statistically significant **(Figure 21, a)**. Mean TG values in combined statin groups showed an immediate decrease from baseline values at 3-month visit, then stabilized at 6- and 12-month follow-up visits **(Figure 22, a)**. MI data confirmed the trends of decrease in TG levels in SMV and RSV groups post-surgery. Combined statin group showed a significantly higher mean TG level at baseline compared to that of the non-statin group. The levels of mean TG became comparable between the combined and non-statin

groups post-surgery. Mean TG value in combined statin group showed an immediate decrease from baseline value at 3-months visit and significant decreases from baseline value at 6- and 12-month follow-up visits **(Figures 21, b and 22, b)**. This might be explained by the effect of surgery itself on TG levels in combined statin group and individual SMV and RSV groups.

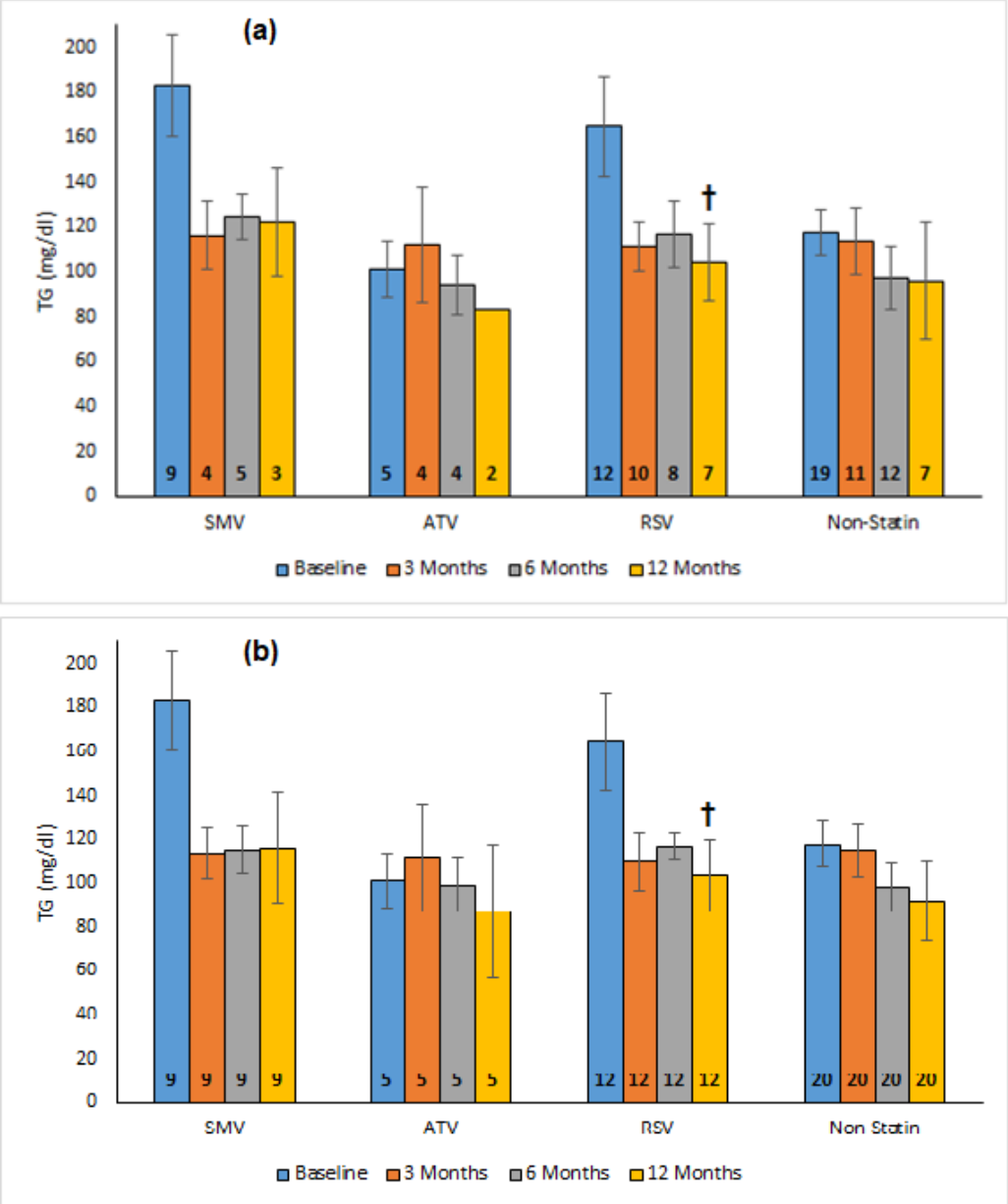
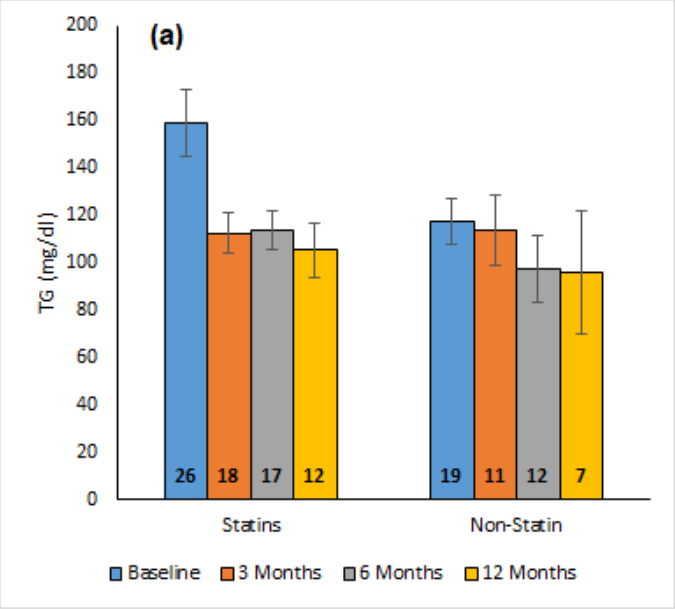


Figure 21. TG in mg/dl (mean ± SE) at baseline, and 3-, 6-, and 12-M follow-up visits after RYGB in individual statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
(†) Statistically significant difference from baseline in the same group.



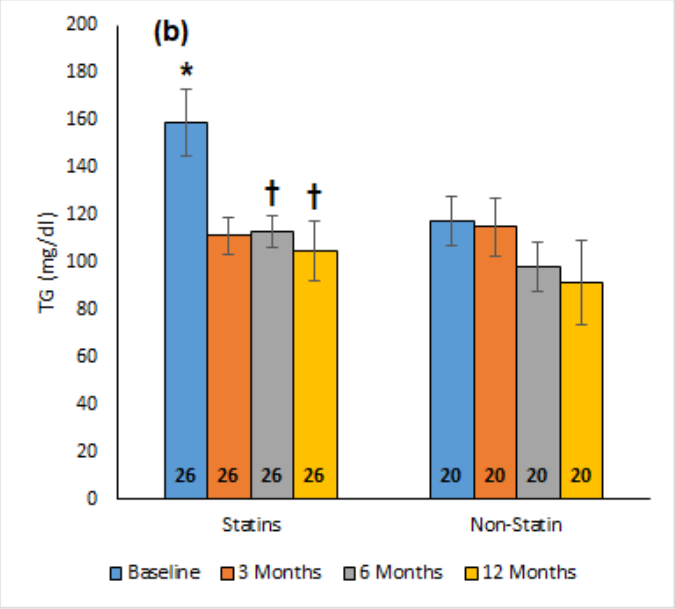


Figure 22. TG in mg/dl (mean ± SE) at baseline, and 3-, 6-, and 12-M follow-up visits after RYGB in combined statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
(†) Statistically significant difference from baseline in the same group.
(*) Statistically significant difference from non-statin group at baseline.

4.3.4.4. Total cholesterol (TC)

The trends in mean TC levels post-surgery in ATV and RSV groups were similar, with an initial decrease at 3-month follow-up visit, followed by a continuous increase at 6- and 12-month follow-up visits. SMV group showed a similar trend of decrease by 3 months, followed by an increase by 6 months post-surgery, but the levels at 12 months (n=3) were lower than those at 3 and 6 months, unlike ATV and RSV groups **(Figure 23, a)**. Comparison of individual groups of statins (SMV, ATV, and RSV groups) showed almost similar time profiles of TC; therefore, they were combined (statin group). Statin group showed a trend of decrease in TC mean value from 167 mg/dl at baseline to 142 mg/dl at 3-month visit. Mean TC values in statin group increased gradually at 6-month (159 mg/dl) and 12-month (164 mg/dl) follow-up visits. The increase in TC levels by 6 and 12 months post-RYGB could be attributed to the increase in HDL levels at 6- and 12- month follow-up visits, since TC = LDL + HDL + (TG/5). For non-statin group, the trend in TC levels was similar with a decrease in TC mean value from baseline (182 mg/dl) to 3-month follow-up (152 mg/dl). At 6-month follow-up visit, the level of mean TC in non-statin group stabilized at 152 mg/dl and showed a significant difference from baseline value, then increased to 167 mg/dl at 12-month visit **(Figure 24, a)**. Mean TC levels were not significantly different between statin and non-statin groups at baseline nor any time point post-surgery. This indicates that the observed trend of change in TC levels post-surgery is attributed to the surgery rather than statin

treatment. MI data presented in **Figures 23, b and 24, b** confirmed the trends of changes in TC levels post-surgery in statin and non-statin groups.

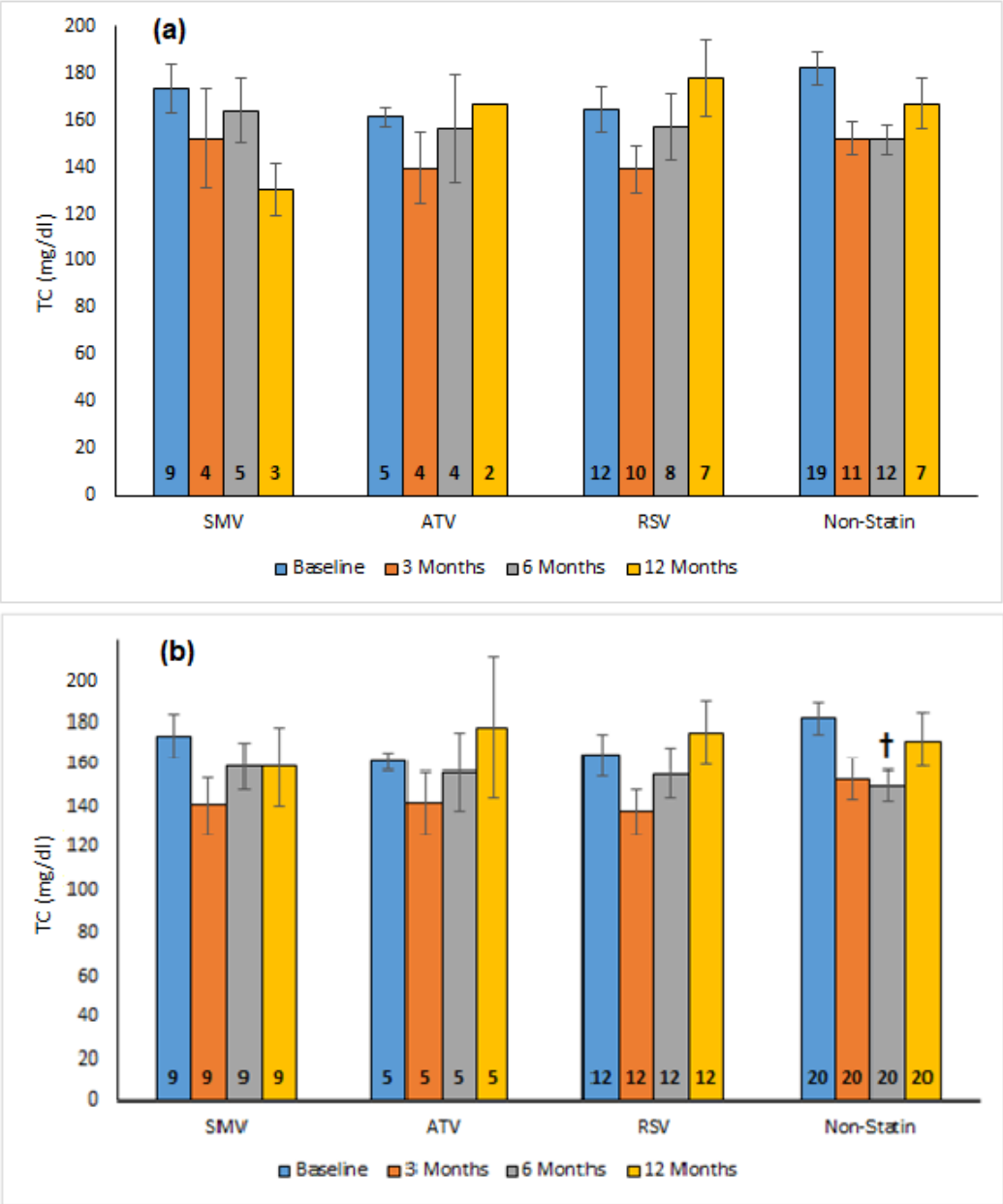


Figure 23. TC in mg/dl (mean ± SE) at baseline, and 3-, 6-, and 12-M follow-up visits after RYGB in individual statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
(†) Statistically significant difference from baseline in the same group

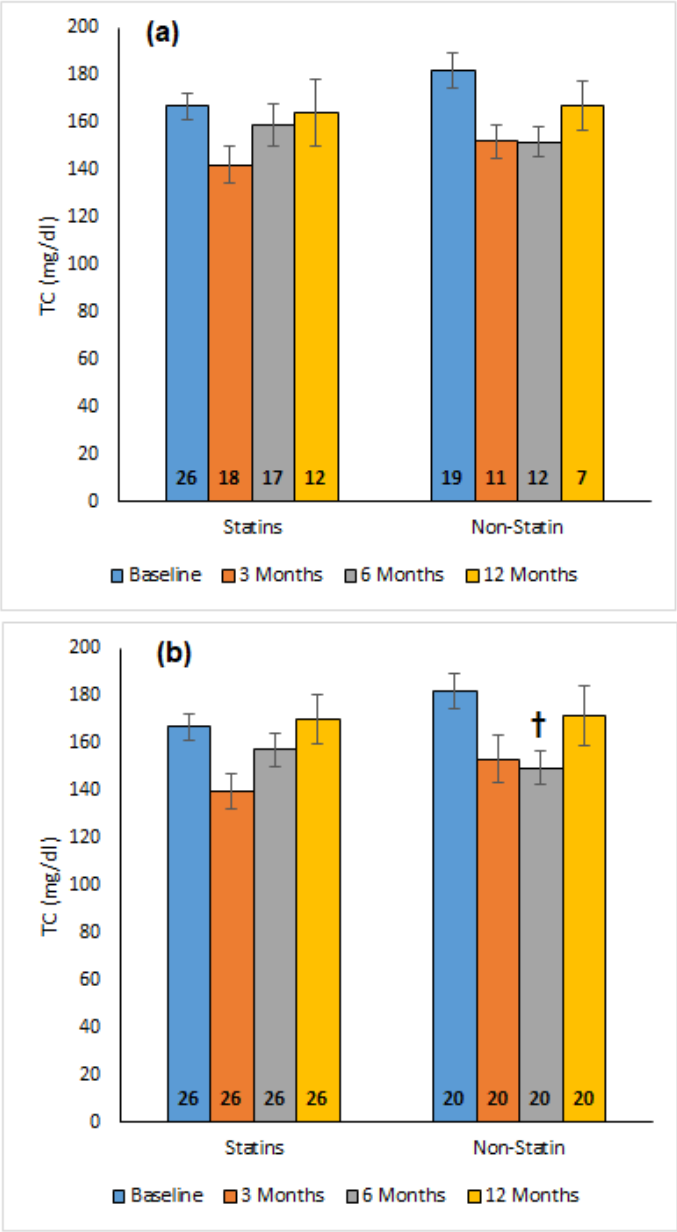


Figure 24. TC in mg/dl (mean ± SE) at baseline, and 3-, 6-, and 12-M follow-up visits after RYGB in combined statins and non-statin groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
(†) Statistically significant difference from baseline in the same group

4.3.5. Relating LDL Levels to Statin Discontinuation and Dose Reduction Post-Surgery

Figure 25 represents the individual LDL levels (mg/dl) in patients on ATV, RSV, or SMV pre- and post-RYGB. Individual LDL levels were analyzed in relation to statin discontinuation and dose reduction post-RYGB. Patients who discontinued using ATV (n=2) and RSV (n=5) had rebounds in their LDL levels higher than those in patients who continued using these two statins. Reducing the dose to half seems to have an effect of causing the increasing LDL levels post-RYGB in patients on ATV (n=2) and RSV (n=1). Patients who discontinued SMV (n=2) did not have available information about their LDL levels. One Patient on SMV with a reduced dose to half after surgery had the LDL levels remaining under the control post-RYGB. The discontinuation or reduction of the dose of ATV or RSV post-RYGB exhibited rebounds of LDL levels in some subjects, but the rebound was not apparent with patients on SMV pre-surgery. Discontinuation of statin treatment post-surgery led to increases in LDL values higher than that at baseline (2 patients on ATV and 2 patients on RSV). Reduction of dose to half seemed to have better results in controlling LDL than discontinuation of the therapy with ATV or RSV. The surgery itself seemed to have an immediate lowering effect on LDL, but the effect of surgery may be reversed at later time points (12M) post-RYGB in some patients even if the dose remained the same (3 patients on RSV and 2 patients on SMV).

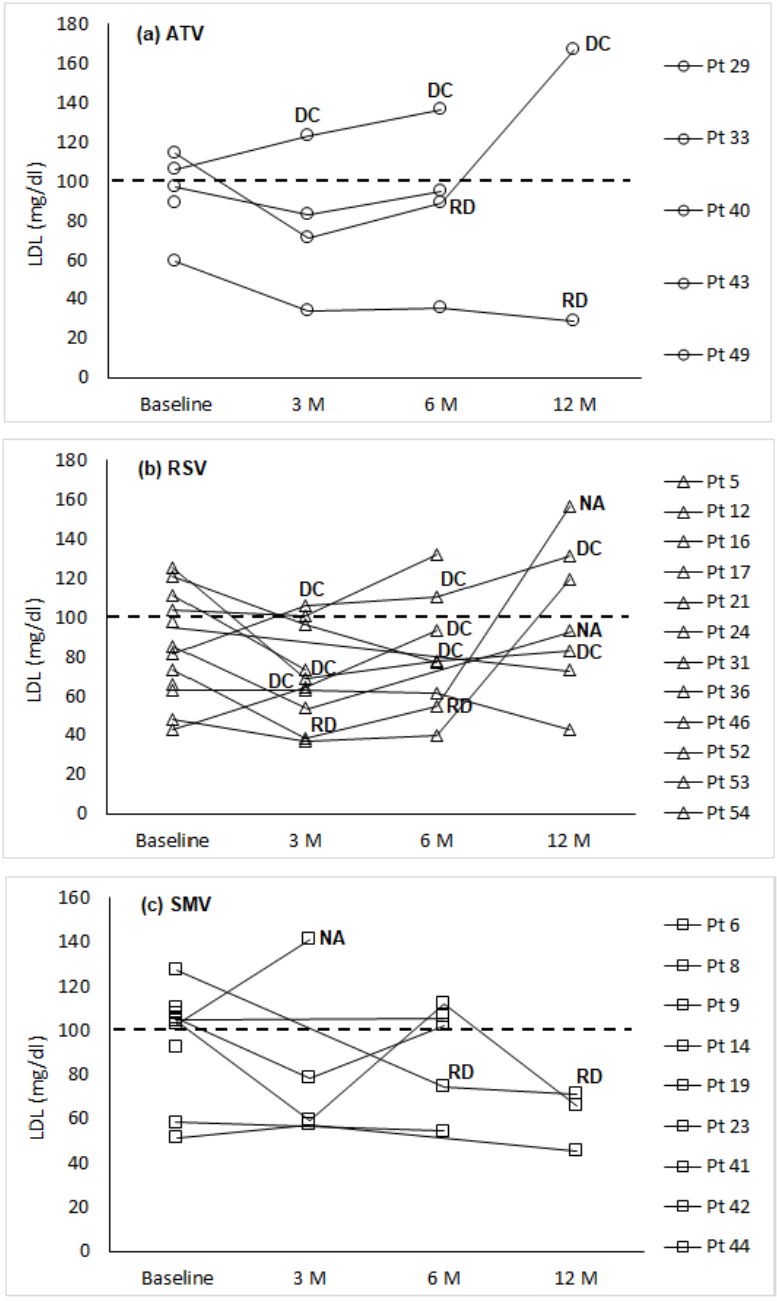


Figure 25. Individual LDL levels in patients on (a) ATV, (b) RSV, and (c) SMV pre- and post-surgery. DC = discontinuation of statin. RD = reduced dose of statin to half. NA = No information of the dose was available. Dotted line: represent 100 mg/dl (optimal level of LDL is < 100 mg/dl)

4.3.6. Pharmacokinetics

4.3.6.1. Atorvastatin (ATV)

The individual concentrations normalized by dose/bodyweight, [(nM)/(mg/kg)], of ATV and its two active metabolites showed similar trends of significant decreases by 3 months, then at a slower rate of decline up to 6 months post-surgery **(Figure 26)**. The mean concentrations of the ATV and the two metabolites at each time point were tabulated **(Table 19)** and showed a significant decrease by 6-month post-RYGB. This trend could be attributed to the pH-dependent equilibrium of ionized: unionized form of the ATV acid. It has been reported that gastrointestinal pH post-RYGB increases (Smith et al., 2011, Giuliano et al., 2012), which leads to a shift in the equilibrium towards the ionized form of the weak acid, ATV. The ionized form is more soluble, but less permeable. In addition, the surface area for passive absorption was reduced post-RYGB. These factors might lead to the decrease in the absorption of ATV, which is absorbed by passive diffusion. ATV is metabolized by intestinal CYP3A4 that is mainly expressed in the duodenum, which is bypassed in RYGB. This is expected to reduce the metabolism and increase its bioavailability. However, ATV is also a substrate of P-gp efflux transporter (Klotz, 2003). P-gp is mainly expressed in the distal segment of the small intestine, which is still available post-RYGB and expected to decrease the absorption of its substrates. The effects of gastrointestinal pH change and the

reduction in the surface area for passive absorption seem to dominate over the decreased metabolism for the effect of the RYGB on decrease of observed ATV concentrations. ATV is relatively lipophilic and expected to distribute to the fatty mass in the human body. However, studies have shown that statins have a selective distribution to the liver, their main site of action (Garcia et al., 2003). Loss of fatty mass after-RYGB could reduce the distribution of lipophilic drug, which might cause an increase in their plasma concentration. This was not observed in the case of ATV in this patient population of our study.

The sampling time were different between subjects; however, the ranges of sampling time for individual patients pre- and post-surgery were very similar as follows and made the description of the trends in profiles valid: patient 29 (13.5-19 hr post-dose), patient 33 (12-18 hr post-dose), and patient 40 (9.5 hr post-dose, only baseline sample is available). Patient 43 had a sampling time of 11-11.5 hr post-dose at baseline and 6 months follow-up, but a sampling time of 3 hr at 3 months visit. We anticipate that our conclusion from the RYGB impacts on PK data observation is valid, due to the long half-lives of ATV (14 hr) and its two active metabolites (20 hr).

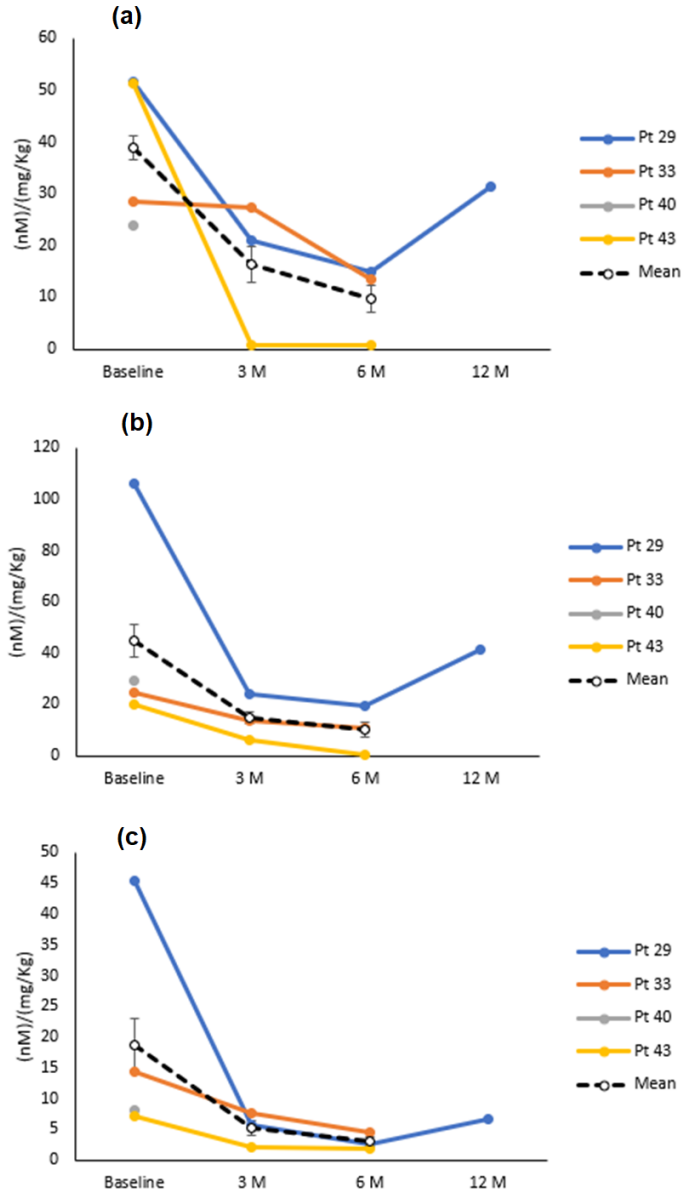


Figure 26. Concentrations [(nM)/ (mg/kg)] of (a) ATV, (b) 2-OH-ATV, and (c) 4-oh-ATV in individual patients pre- and post-RYGB

Table 19. Mean concentrations [(nM)/ (mg/kg)] of statins and their active metabolites pre- and at various time points post-RYGB (data are presented as mean ± SE)

	ATV	2-OH-ATV	4-OH-ATV	RSV	SMV	SMV-A
Baseline	38.81 ± 2.36 (n=4)	44.82 ± 6.11 (n=4)	18.75 ± 4.18 (n=4)	213.07 ± 22.87 (n=10)	8.52 ± 3.04 (n=6)	9.96 ± 3.99 (n=6)
3 Months	16.30 ± 3.41 (n=3)	14.62 ± 2.40 (n=3)	5.12 ± 1.20 (n=3)	122.56 ± 9.67 (n=4)	11.29 ± 1.96 (n=5)	24.89 ± 6.90 (n=5)
6 Months	9.75 ± 2.52 * (n=3)	10.26 ± 2.95 * (n=3)	2.94 ± 0.80 * (n=3)	83.28 ± 6.39 (n=3)	28.45 ± 3.54 (n=4)	39.32 ± 7.95 (n=4)
12 Months	31.31 (n=1)	41.09 (n=1)	6.55 (n=1)	195.94 (66.24, 325.65)	7.09 ± 0.42 (n=3)	10.45 ± 2.39 (n=3)

(*) Significantly lower than baseline concentration at $p < 0.05$ using Friedman test with Bonferroni correction.

ATV pre-surgery was metabolized to 2-OH-ATV and 4-OH-ATV, with 63-78% vs 22-37% of the metabolites, respectively **(Table 20).** The metabolite ratios of 2-OH-ATV/ATV and 4-OH-ATV/ATV at baseline among the 4 subjects varied significantly, with 0.39-2.06 and 0.14-0.88, respectively. The metabolism of ATV to 2-OH-ATV and 4-OH-ATV was decreased appreciably post-RYGB, as expressed by the metabolite ratios of [2-OH-ATV/ATV] and [4-OH-ATV/ATV]

(Table 20). Among the subjects who completed the follow-up visits, the ratios decreased by 3 months post-surgery, then remained relatively constant at the lower levels. The observed decrease in metabolism of ATV to the metabolites (ratios of metabolites to ATV) 3 months post-surgery and leveled off is consistent with the known reduction of available intestinal CYP 3A4 after RYGB, in which the proximal small intestine (duodenum and part of the jejunum) is bypassed, where most of the intestinal CYP3A4 enzyme is expressed (Thorn et al., 2005).

Table 20. Ratios of [2-OH-ATV/ATV] and [4-OH-ATV/ATV] pre- and post-surgery in individual patients

	Pt ID							
	29		33		40		43*	
	2-OH-ATV/ATV	4-OH-ATV/ ATV	2-OH-ATV/ ATV	4-OH-ATV/ ATV	2-OH-ATV/ ATV	4-OH-ATV/ ATV	2-OH-ATV/ ATV	4-OH-ATV/ ATV
Baseline	2.06 (70%)**	0.88 (30%)	0.86 (63%)	0.50 (37%)	1.22 (78%)	0.34 (22%)	0.39 (74%)	0.14 (26%)
3 M	1.16 (81%)	0.27 (19%)	0.50 (64%)	0.28 (36%)				
6 M	1.29 (88%)	0.17 (12%)	0.81 (71%)	0.33 (29%)				
12 M	1.31 (86%)	0.21 (14%)						

* Patient 43 had ATV levels below LLOQ of 0.25 ng/ml at 3- and 6-month follow-up visits, so the ratios could not be reliably derived.

** (%) of metabolites between 2-OH-ATV and 4-OH-ATV

A preliminary PK/PD correlation was attempted by plotting the LDL values with the summation of the molar concentrations of ATV, 2-OH-ATV and 4-OH-ATV at baseline, 3-month and 6-month follow-ups, respectively **(Figure 27).** It appeared that the threshold of effective ATV with active metabolites decreased from 40 nM pre-surgery to 20 nM at 3 and 6 months post-RYGB.

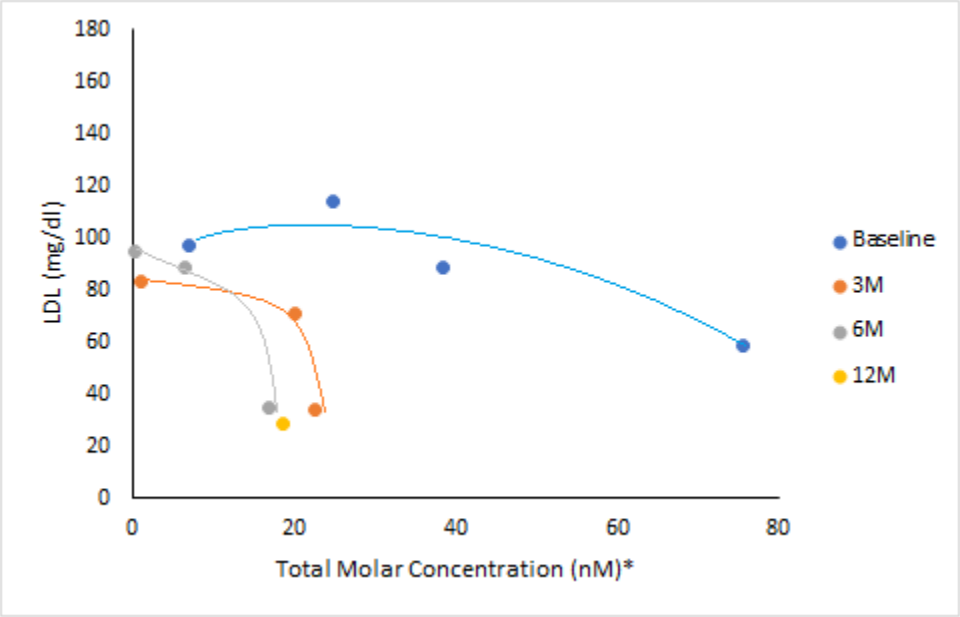


Figure 27. LDL (mg/dl) correlation with summation of molar concentrations of ATV and two active metabolites, 2-OH-ATV and 4-OH-ATV.

(*) Total molar concentration was calculated as [ATV + 2-OH-ATV + 4-OH-ATV] in nM

An attempt was made to develop a model correlating LDL with the total concentration of ATV and its two active metabolites, and patients' BMI, using DesignExpert 11. A linear model was found to be the best fit model with significant p-value of 0.0167 **(Table 21).**

Table 21. ANOVA for the linear model using total concentration of atorvastatin and its two active metabolites and BMI

Source	Sum of squares	df	Mean square	F-value	p-value
Model	6066.50	2	3033.25	10.19	0.0063
Total Concentration	931.90	1	931.90	3.13	0.1148
BMI	5669.46	1	5669.46	19.04	0.0024

The r^2 of the model was 0.7181. The predicted r^2 of the model was 0.5764 and was in reasonable agreement with the adjusted r^2 of 0.6476; i.e. the difference is less than 0.2. Adequate precision measures the signal to noise ratio, and it is desirable to be greater than 4. The model ratio was 7.27, which indicates an adequate signal. The predicted vs actual LDL values as well as a 3D plot of the total concentration, BMI and LDL were plotted **(Figures 28 and 29).** The diagnostic plot showed a

good predictability of LDL using the total concentration of ATV and its two active metabolites and BMI with a final equation of LDL = 46.16 – 0.48*Total Concentration + 3.69*BMI.

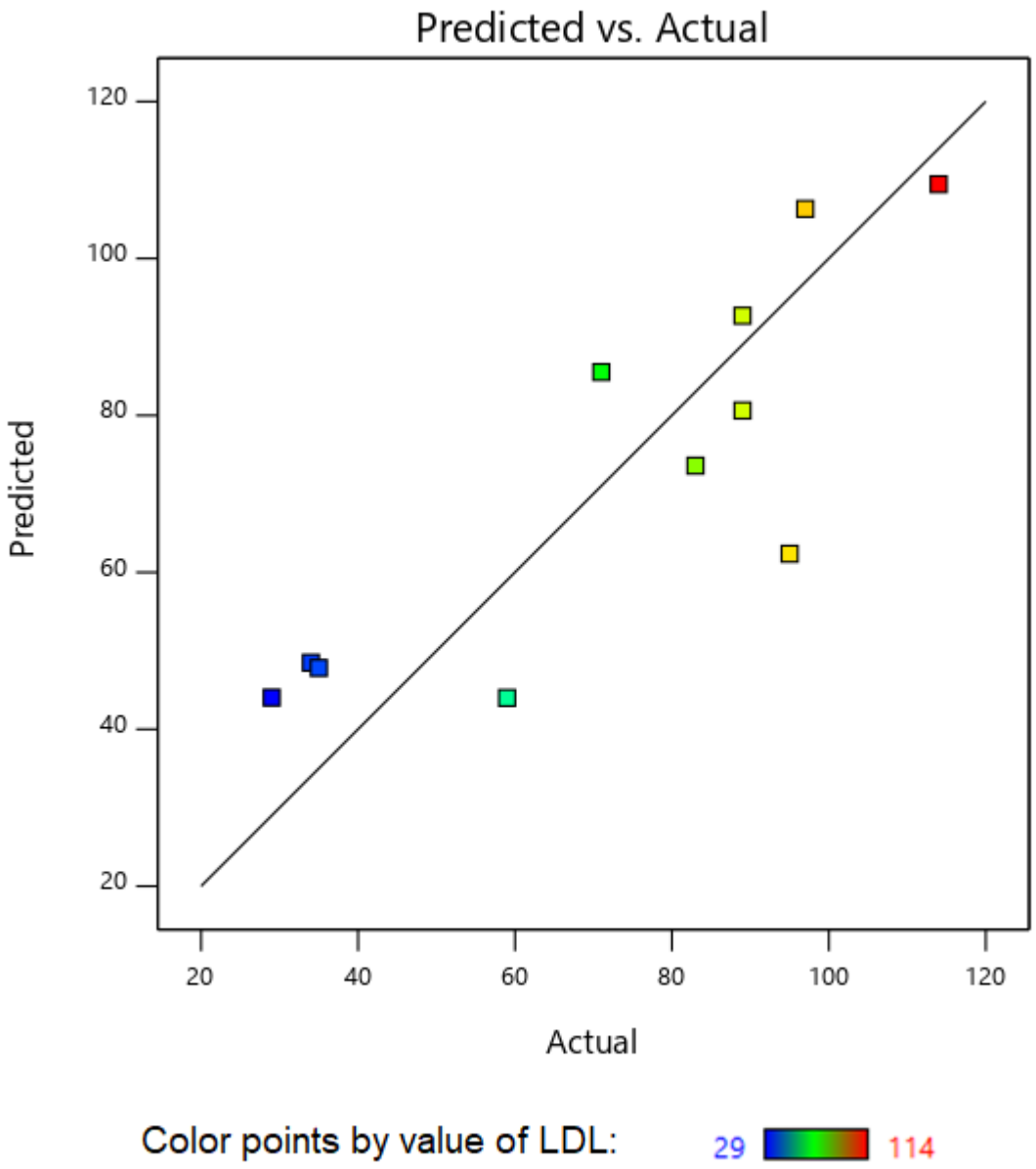


Figure 28. The predicted vs. actual LDL values from a linear model incorporating ATV and its two metabolites total concentration and BMI

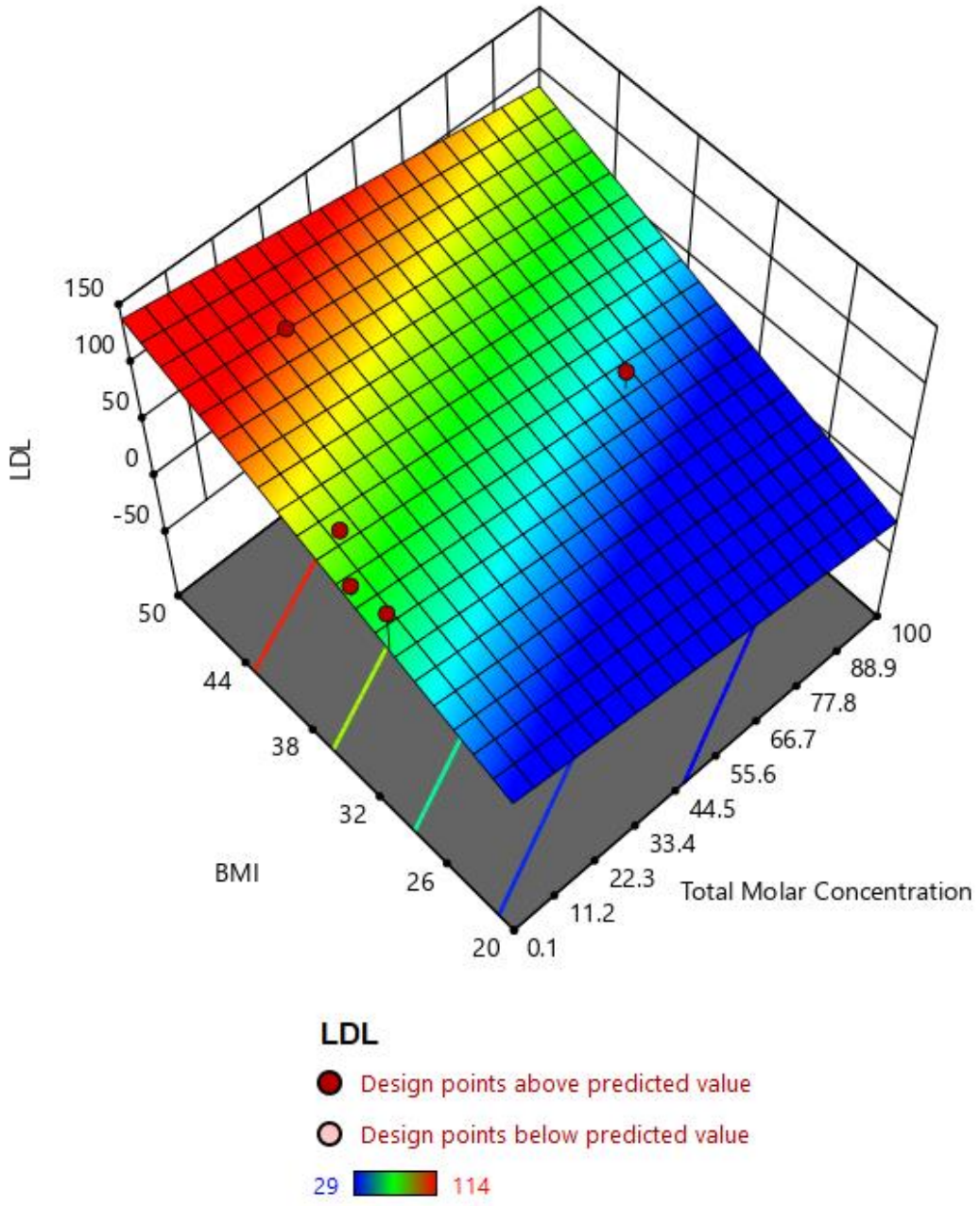


Figure 29. 3D plot of correlation of LDL with ATV and its two metabolites total concentration and BMI

4.3.6.2. Rosuvastatin (RSV)

RSV is considered a relatively hydrophilic statin compared to SMV and ATV. It is administered in the acid form, and its metabolism and renal excretion is minimal, with most of the dose eliminated in the bile (Schachter, 2005). The individual's concentrations of RSV [(nM)/(mg/kg)] showed a trend of decrease post-surgery at 3-month visit compared to baseline, then the levels stabilized by 6 months post-surgery **(Figure 30)**. This can be explained by a decrease in RSV absorption post-surgery. RSV absorption is facilitated by organic anion transporter (OATP2B1) (Johnson et al., 2017), which is expressed along the small intestine at similar levels in all segments (Meier et al., 2007). Since the proximal segment of the small intestine is bypassed after RYGB, the absorption of RSV is expected to decrease after the surgery. OATP2B1 is also a pH-dependent transporter with the optimal function at acidic pH ≤ 5.5, so the increase in gastrointestinal pH is expected to reduce the transporter capability. The distribution of RSV (a relatively hydrophilic drug) is not expected to be affected by weight loss after surgery. In addition, metabolism of RSV is minimal and any changes that might happened in its metabolism post-surgery will not have a major effect on its PK.

The inter- and intra-individual sampling time differences are not expected to affect our conclusion, because of the relatively long half-life of RSV (19 hr). Patient 53 had a decrease in the normalized plasma concentrations at 3- and 6-month follow-up visits compared to baseline with similar sampling time pre- and post-surgery in

the range of 4-4.5 hr post-dose. Patient 46 also showed a decrease in the normalized plasma concentrations at 3 and 6 months post-surgery compared to baseline with sampling times of 25, 12.5, and 9 hr post-dose at baseline, 3, and 6 months after-surgery, respectively. Patient 17 had a slightly lower concentrations at 3 and 12 months compared to baseline with sampling time range of 10-16 hr post-dose. Patient 54 is the only patient who showed a higher concentration at 3-month visit compared to baseline, but the sampling times were very different with 23 hr post-dose at baseline and 4 hours post-dose at 3 months visit. Patient 52 had a missing sample at 3-month visit. The values of normalized concentrations for patient 52 were comparable at baseline and 6 months post-surgery, then the concentration increased at 12-month visit. The sampling time range for this patient was 6-9.5 hr post-dose pre- and post-surgery. All these observations from individual patients' profiles confirm that there is a decrease in normalized plasma concentrations of RSV post-RYGB. This could be attributed to a decrease in RSV absorption by the decrease in the availability and capability of OATP2B1 intestinal transporter. The mean concentrations of RSV [(nM)/(mg/kg)] demonstrated the trend of decreased RSV concentration on the same dose basis post-RYGB as compared to that of the pre-operative therapy **(Table 19).**

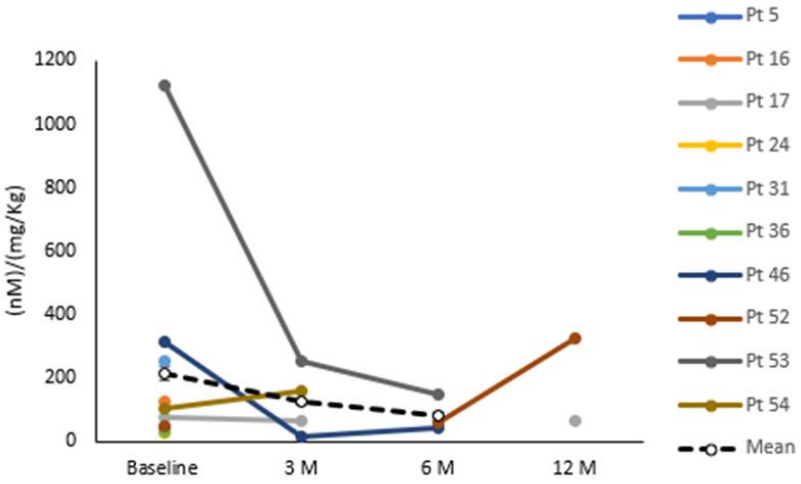


Figure 30. Concentrations [(nM)/ (mg/kg)] of RSV in individual patients pre- and post-RYGB

4.3.6.3. Simvastatin (SMV)

The individual normalized concentration profiles of SMV and its active metabolite, SMV acid (SMV-A), [(ng/ml)/(mg/kg)], are presented in **Figure 31**. Three out of the six subjects with follow-up visits showed a trend of increase in SMV and SMV-A concentrations post-RYGB with comparable sampling times (0.5-4 hr for patient 9 and 14-16 hr for patients 23 and 42). Two patients showed a trend of decrease in SMV and SMV-A concentrations at 6- and 12-month compared to 3-month visits (no baseline concentrations were available). The sampling time range for these two patients (14 and 19) was 11-15 hr. Patient 44 was the only patient with a complete profile at all time points pre- and post-RYGB. The trend of the

concentrations in this patient followed a zig-zag pattern, with a decrease at 3-month visit from baseline, followed by an increase at 6-month visit and a decrease at 12-month visit. The sampling times post-dose for this patient were comparable with a range of 11.5-13 hr post each dose. The mean profiles of SMV and SMV-A, showed a trend of increase after RYGB up to 6 months, and at 12 months post-surgery, the mean plasma concentration levels were similar to the baseline levels **(Table 19).** This may be partly explained by the increase in gastric pH after RYGB. SMV is a prodrug, administered in the inactive lactone form. It converts *in vivo* into the active acid form through chemical and enzymatic hydrolyses. The interconversion of lactone to acid is minimal at pH 4-5 (Nigovic et al., 2012), which might lead to an increase in absorption and bioavailability due to the SMV lactone form. This explanation agrees with studies that demonstrated an increase in the bioavailability of SMV after administration of multiple doses of antacid (AlAkhali and Alavudeen, 2014, Alakhali et al., 2018). Another potential explanation is the bypass of the intestinal CYP3A4 enzyme that leads to an increase in the bioavailability of SMV. The return of plasma levels 1-year post-RYGB to the baseline levels suggests an adaptation of the gastrointestinal tract. The adaptation mechanism could be due to the increase in the gastric acid secreting cells in the small stomach pouch that was created during the surgery, or by an increase in the expression of CYP3A4 enzyme at the more distal segments of the small intestine. SMV is considered a lipophilic statin; hence, its absorption is by passive diffusion.

The bypass of the proximal small intestine leads to a decrease in the intestinal surface area available for passive diffusion, which is expected to decrease the levels of lipophilic drugs post-surgery, but not apparent in our study observations. The interplay between all these factors leads to a large intra- and inter-individual variability in the PK results of SMV and SMV-A.

The hydrolysis and metabolism of SMV to SMV-A decreased appreciably post-surgery among 5 of 9 subjects, who had the follow-up visits. The metabolism decreased, as expressed by the ratios of [SMV-A/SMV], at 3 months or 6 months post-RYGB, then remained relatively constant at levels of lower ratios afterwards **(Table 22).** Only one subject (Patient ID 9) showed an increase in the metabolism post-RYGB compared to baseline, which could not be easily rationalized.

Table 22. The ratios of [SMV-A/SMV] pre- and post-RYGB in individual patients

	Pt ID								
	6	8	9	14	19	23	41	42	44
Baseline	0.10	0.43	2.13				1.12	7.85	1.34
3 M				7.67	1.92	17.14		0.28	0.42
6 M				0.72	1.02	2.15			0.55
12 m			3.09		1.09				0.51

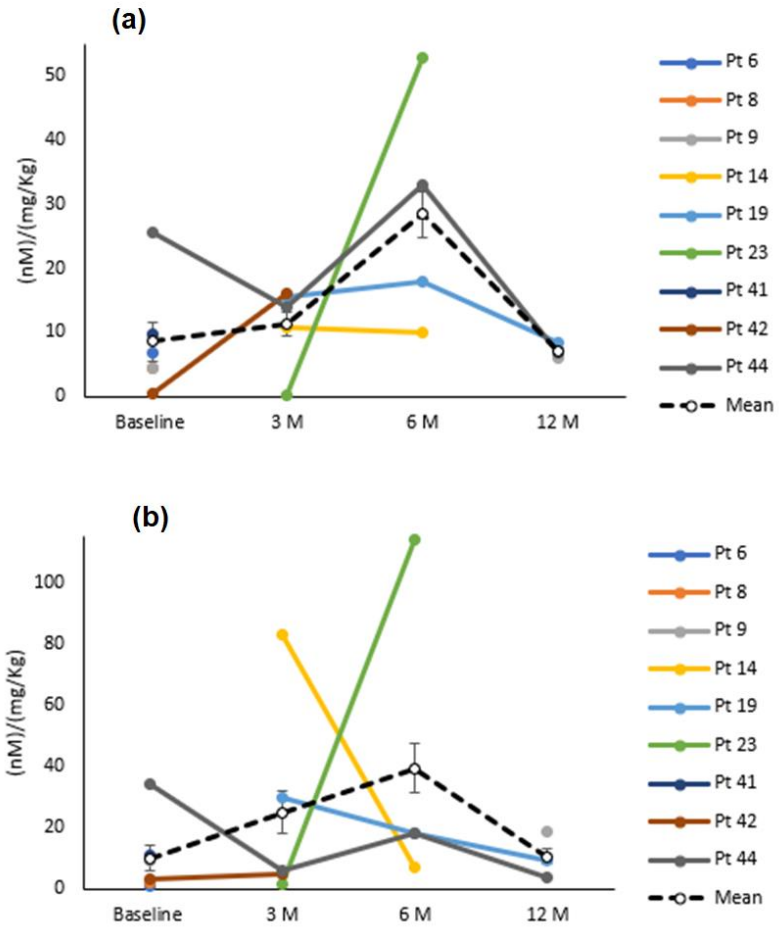


Figure 31. Concentrations [(nM)/ (mg/kg)] of (a) SMV and (b) SMV-A in individual patients pre- and post-RYGB

Since SMV in the lactone form is inactive, SMV-A (the active metabolite) concentrations were utilized for an attempt of preliminary PK/PD correlation. At baseline, there was not an observed trend of correlation between SMV-A concentrations (nM) and LDL levels (mg/dl). There was a trend of decrease in LDL levels with increase in SMV-A concentrations at 3 and 6 months post-surgery. However, LDL reduction seems to level off at SMV-A concentrations higher than 5-10 nM. This could be due to the saturation of HMG-CoA reductase enzyme (the target of SMV-A) **(Figure 32).** The same SMV-A concentrations appear to produce a better reduction in LDL levels at 3 months compared to 6 months post-surgery.

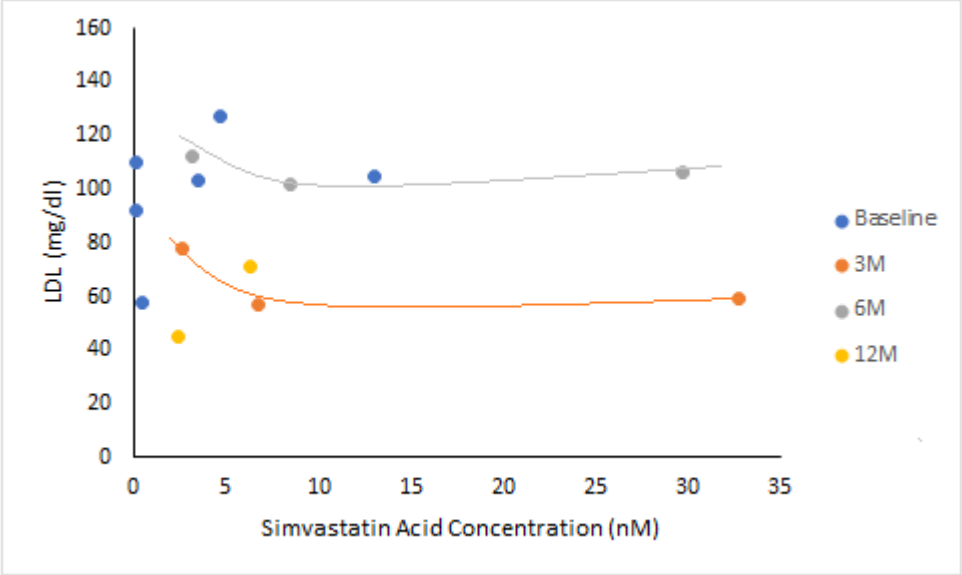


Figure 32. LDL (mg/dl) correlation with molar concentration of SMV-A

Another attempt was made to develop a model correlating LDL with the concentrations of SMV and SMV-A, and patients' BMI using DesignExpert 11. Multiple trials were made using SMV-A concentration only, the total concentration of SMV and SMV-A, and the ratio of SMV-A/SMV. A linear model was found to be the best fit model using the ratio of SMV-A/SMV with significant p-value of 0.0192 **(Table 23).**

Table 23. ANOVA for the linear model using ratios of the molar concentrations of SMV-A/SMV and BMI

Source	Sum of squares	df	Mean square	F-value	p-value
Model	4390.22	2	2195.11	5.78	0.0192
SMV-A/SMV	2335.98	1	2335.98	6.15	0.0306
BMI	2333.18	1	2333.18	6.14	0.0306

The r^2 of the model was 0.5124. The predicted r^2 of the model was 0.2956 and was in reasonable agreement with the adjusted r^2 of 0.4238; i.e. the difference is less than 0.2. Adequate precision measures the signal to noise ratio, and it is desirable to be greater than 4. The model ratio was 7.034, which indicates an adequate signal. The predicted vs actual LDL values and a 3D plot of the total

concentrations, BMI and LDL were plotted **(Figures 33 and 34).** The diagnostic plot showed a good predictability of LDL using the ratio of the molar concentrations of SMV-A to SMV and BMI with a final equation of LDL = 17.55 – 5.36*(SMV-A/SMV) + 2.15*BMI.

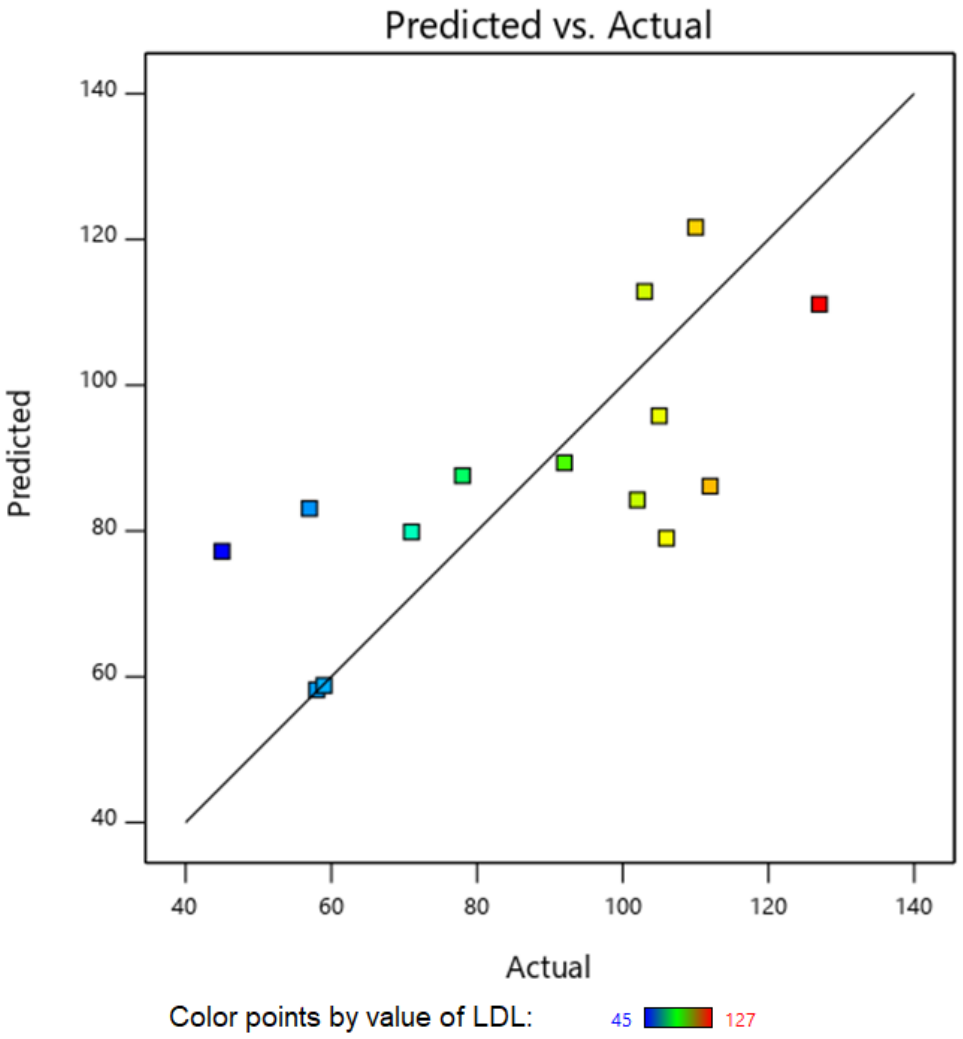


Figure 33. The predicted vs. actual LDL values from a linear model incorporating the ratio of SMV-A/SMV molar concentrations and BMI

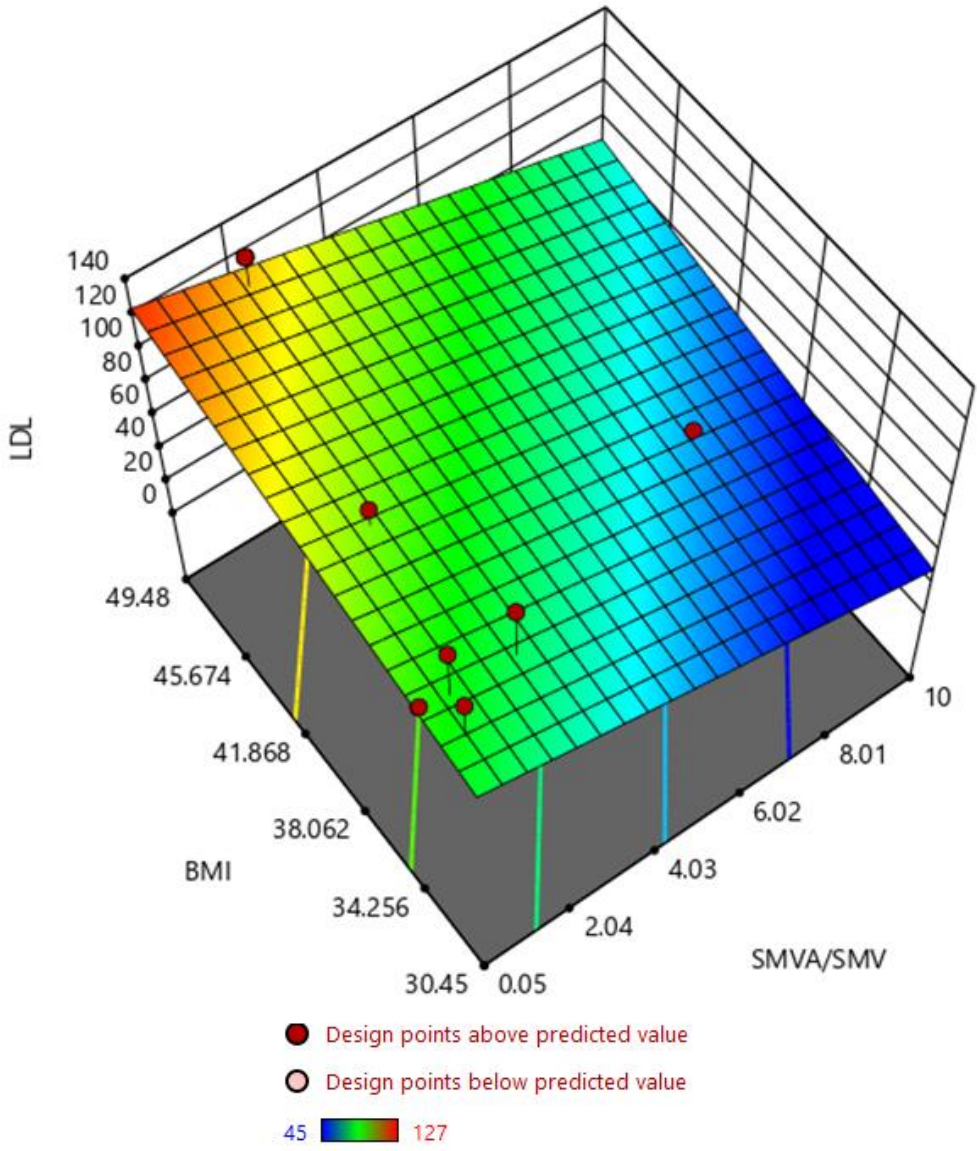


Figure 34. 3D plot of correlation of LDL with ratios of SMV-A/SMV molar concentrations and BMI

4.4. Metformin study

4.4.1. Demographic data

Fifty (50) subjects, aged 25 to 68 years with severe obesity (BMI range of 32.7-62.0 kg/m^2) were recruited for the study. Thirty-one (31) of the subjects had been taking metformin prior to the surgery (metformin group). Seven (7) of the 50 subjects were diabetics on other antidiabetic medications, such as insulin and sulfonylurea (other antidiabetics group). The other twelve (12) were non-diabetics and not on any antidiabetic medications (non-diabetics group). **Table 24** shows the demographic data for metformin, other antidiabetics, and non-diabetics groups. Mean age, BMI, and excess weight at baseline did not show statistically significant difference among the three groups. However, other-antidiabetics group showed a relatively higher mean excess weight at baseline than metformin and non-diabetics groups. The higher excess weight at baseline in the other antidiabetics group could be attributed to the well-known weight gain side effect of insulin or sulfonylurea (the antidiabetic medications) (Russell-Jones and Khan, 2007, Sola et al., 2015). Metformin and non-diabetics groups constituted mostly of Caucasian subjects (17 and 8 subjects, respectively, with all other races less than 6). The other antidiabetics group had 4 African Americans, 2 Caucasians, and 1 Hispanic. However, the statistical analysis on racial/ethnic distribution did not show any statistically significant difference among the three groups. Comparison of sex

showed no significant difference among the groups, with all groups having more females (57-75%) than males.

Table 24. Demographic data in metformin, other antidiabetics, and non-diabetics groups

		Metformin (n=31)	Other antidiabetics (n=7)	Non-diabetics (n=12)
Mean age (yr.) ± SD		49.8 ± 10.4	54.9 ± 16.5	47.4 ± 11.7
Mean BMI (kg/m^2) at baseline ± SD		43.3 ± 6.9	46.5 ± 5.6	41.7 ± 3.9
Mean excess weight (lb.) at baseline ± SD		113.0 ± 44.8	144.2 ± 34.4	104.8 ± 22.7
Racial/Ethnic Breakdown n (%)	Caucasian	17 (54.8)	2 (28.6)	8 (66.7)
	African American	6 (19.4)	4 (57.1)	1 (8.3)
	Hispanic	4 (12.9)	1 (14.3)	0 (0.0)
	Other	4 (12.9)	0 (0.0)	3 (25.0)
Sex n (%)	Male	9 (29.0)	3 (42.9)	3 (25.0)
	Female	22 (71.0)	4 (57.1)	9 (75.0)

4.4.2. % Follow-up

The % follow-ups at each time point of 1 week, 1, 3, 6, and 12 months in metformin, other antidiabetics, and non-diabetics groups are represented in **Table 25**. The % follow-ups for all the 50 patients who were included in the study at 1 week, 1, 3, and 6 months were in the range 82.0-94.0%. At 12 months visit, there was a decrease to 64.0%. Metformin, other antidiabetics, and non-diabetics groups had

a % follow-up of >80.0% up to 3 months post-surgery. At 6 months, % follow-up was 77.4 and 71.4 % for metformin and other antidiabetics group, while all patients in the non-diabetics group attended their 6 months follow-up appointment. Non-diabetics group also had the greatest % follow-up at 12 months visit (75.0%), compared to 61.3 and 57.1 % for metformin and other antidiabetics groups, respectively. These results agree with a published study that reports patients with diabetes mellitus have less adherence to follow-up after RYGB (Khorgami et al., 2015).

Table 25. % Follow-ups (n) of patients at each time point of follow-up visits in the metformin study groups

	1 Week	1 Month	3 Months	6 Months	12 Months
Total (n=50)	90.0 (45)	94.0 (47)	88.0 (44)	82.0 (41)	64.0 (32)
Metformin (n=31)	87.1 (27)	90.3 (28)	90.3 (28)	77.4 (24)	61.3 (19)
Other Antidiabetics (n=7)	85.7 (6)	100.0 (7)	85.7 (6)	71.4 (5)	57.1 (4)
Non-diabetics (n=12)	100.0 (12)	100.0 (12)	83.3 (10)	100.0 (12)	75.0 (9)

4.4.3. Weight loss outcomes

4.4.3.1. BMI reduction from baseline

In metformin group, there was a significant decrease in BMI at 3 M follow-up visit (7.0 units reduction) compared to 1-week follow-up (2.1 units reduction). The BMI stabilized at 6 M follow-up visit (9.7 units reduction), and reduced further significantly at 12 M visit (12.3 units reduction). The same trend of decrease was observed in mean BMI of the non-diabetic group, with significant reduction in BMI at 3 months visit compared to 1 week visit (6.7 and 2.2 unit reductions, respectively). BMI stabilized in the non-diabetics group at 6- and 12-month visits with 9.6 and 12.0 units reductions, respectively. The similar pattern of decrease in BMI post-RYGB in metformin and non-diabetics groups suggests that the effect on weight loss was RYGB induced. The other antidiabetics group showed a similar pattern up to 6 months post-surgery (10.5 units reduction), with significant decrease in BMI compared to 1-month visit (2.4 units reduction). At 12 months post-surgery, the reduction in BMI was 10.9 units, lower than those in the metformin and the non-diabetics groups **(Figure 35, a)**. This could be attributed to the weight-gaining side effects of the other anti-diabetic medications such as insulin and sulfonylurea (Russell-Jones and Khan, 2007, Sola et al., 2015).

Using MI, confirmed the results from the original data except for the other antidiabetics group at 12 months **(Figure 35, b)**. The other antidiabetics group

showed 12.1 units mean reduction in BMI using MI date, comparable to metformin and non-diabetics group. The reason for this difference in mean BMI reduction between the original data and MI data could be attributed to the small sample size of the other antidiabetics group (n=7), with poor follow-up at 12 months (n=4). At baseline, there was no significant difference among the three groups in mean BMI **(Table 24)**. BMI reduction did not show any significant difference among the three groups at any time point post-RYGB except at 1 month follow-up visit when the other antidiabetics group had a significantly higher reduction in mean BMI than the metformin group. This difference was resolved at later time points post-RYGB.

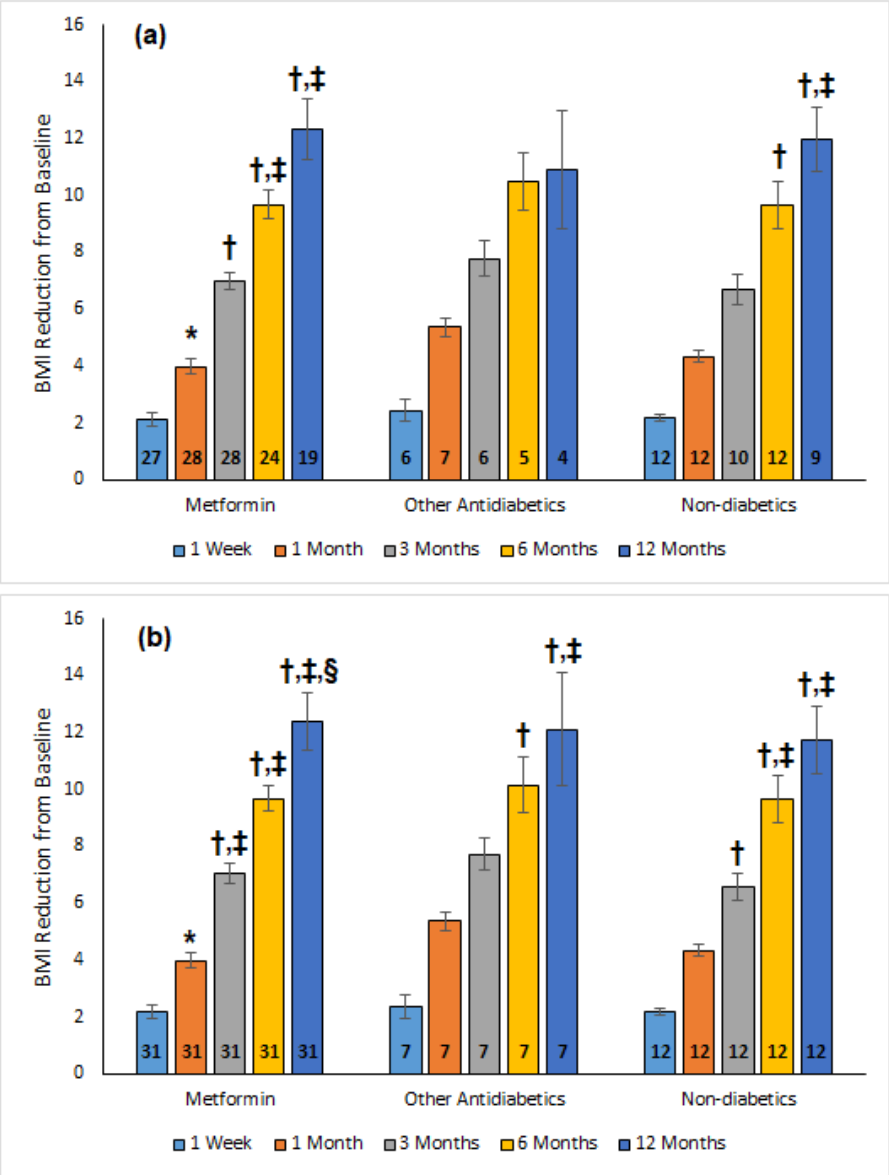


Figure 35. BMI reduction from baseline (mean ± SE) after RYGB at 1 W, 1, 3, 6, and 12 M follow-up visits in metformin, other antidiabetics, and non-diabetics groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits). Statistically significant difference within each group from (†) 1 W, (‡) 1 M, and (§) 3 M.

(*) Statistically significant difference from other antidiabetic group at 1 M.

4.4.3.2. Percentage excess weight loss (%EWL)

Metformin group had 12.2 % EWL at 1-week post-surgery, then a significant reduction in excess weight at 3 months visit (42.3 %). Patients on metformin continues to lose excess weight at 6- and 12-month visits in this group, with %EWL of 57.7 % and 75.9 %, respectively. Non-diabetics group had a similar trend, with 13.8 %EWL at 1-week, then a significant loss of weight with 59.4 %EWL at 6-month visit and 70.5 %EWL at 12-month visit. Other antidiabetics group showed a similar %EWL to the other two groups at 1-week visit (13.0 %). However, the % EWL at 12 months visit (48.2%) was less compared to the other two groups, and did not show any statistically significant loss in excess weight among the follow-up visits post-RYGB **(Figure 36, a)**. No significant difference in %EWL was observed among the three groups at any time point post-surgery. Using MI confirmed the results from the original data, with similar trends in loss of excess weight between metformin and non-diabetics group **(Figure 36, b)**. This observation confirms the conclusion that the weight loss was surgery induced and not correlated with the metformin treatment.

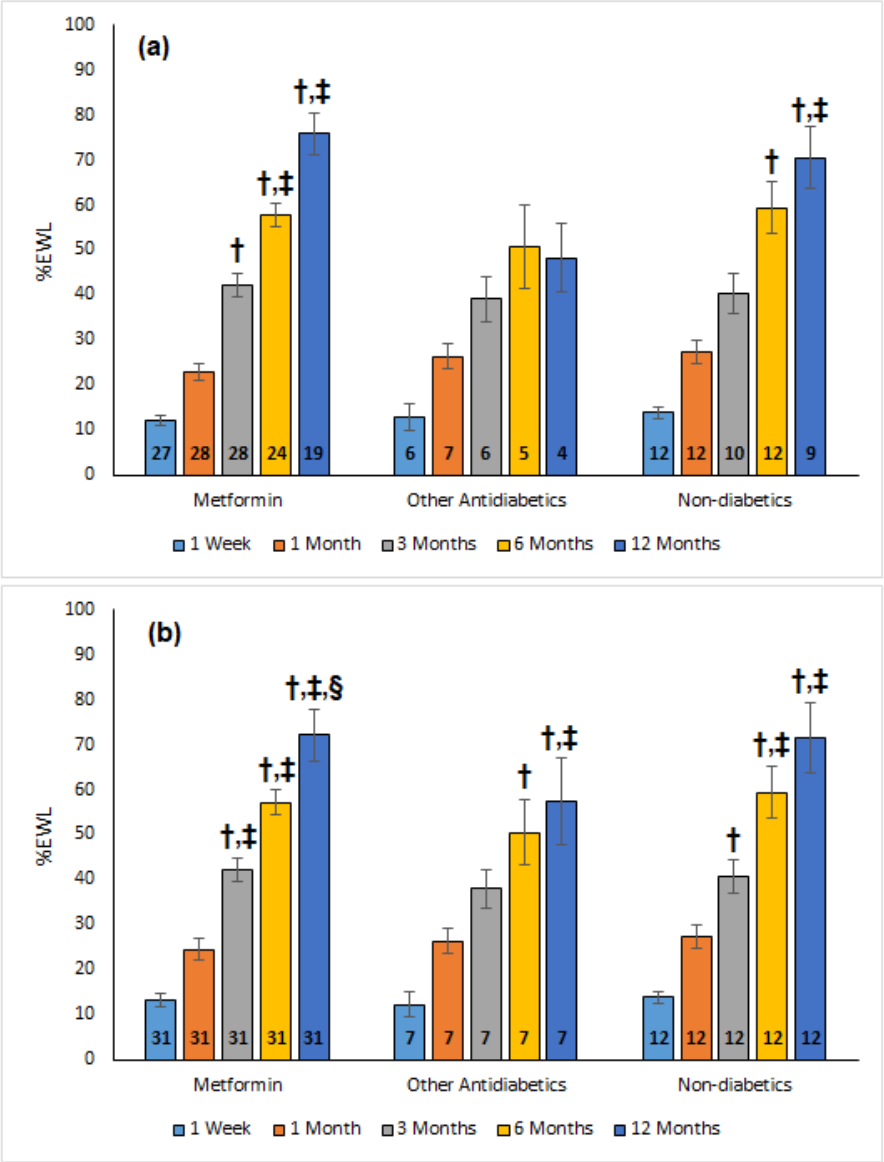


Figure 36. %EWL (mean ± SE) after RYGB at 1 W, 1, 3, 6, and 12 M follow-up visits in metformin, other antidiabetics, and non-diabetics groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits).
Statistically significant difference within each group from (†) 1 W, (‡) 1 M, and (§) 3 M.

4.4.4. Pharmacodynamics

4.4.4.1. HbA1c

At baseline, mean HbA1c levels in metformin and other antidiabetics groups (7.5 % and 8.1 %, respectively) were significantly higher than those in the non-diabetics group (5.5 %). The mean level of HbA1c continued to be significantly higher in metformin group at 3 months follow-up visit (6.7 %) compared to non-diabetics group (5.3 %). Mean HbA1c in the metformin group decreased significantly at 6 months follow-up visit compared to baseline, and then stabilized at a mean of 6.1 % at 12 months visit**.** In the non-diabetics group, mean HbA1c level remained in the normal range at the baseline and all follow-up visits. In other antidiabetics group, although the decrease in mean HbA1c was not statistically significant post-RYGB, but there was a trend of decrease from the baseline values **(Figure 37, a)**. Using MI confirmed the results from the original data, with diabetic patients in metformin and the other antidiabetics groups having significantly higher mean HbA1c levels at baseline even under medications, and the levels of HbA1c falling into similar range of the non-diabetic patients after RYGB **(Figure 37, b)**. This observation indicates that the surgery itself have a lowering effect on HbA1c levels in diabetic patients, and demonstrates the merit of surgery to offer a better control of diabetes with antidiabetic medications (metformin and other antidiabetics).

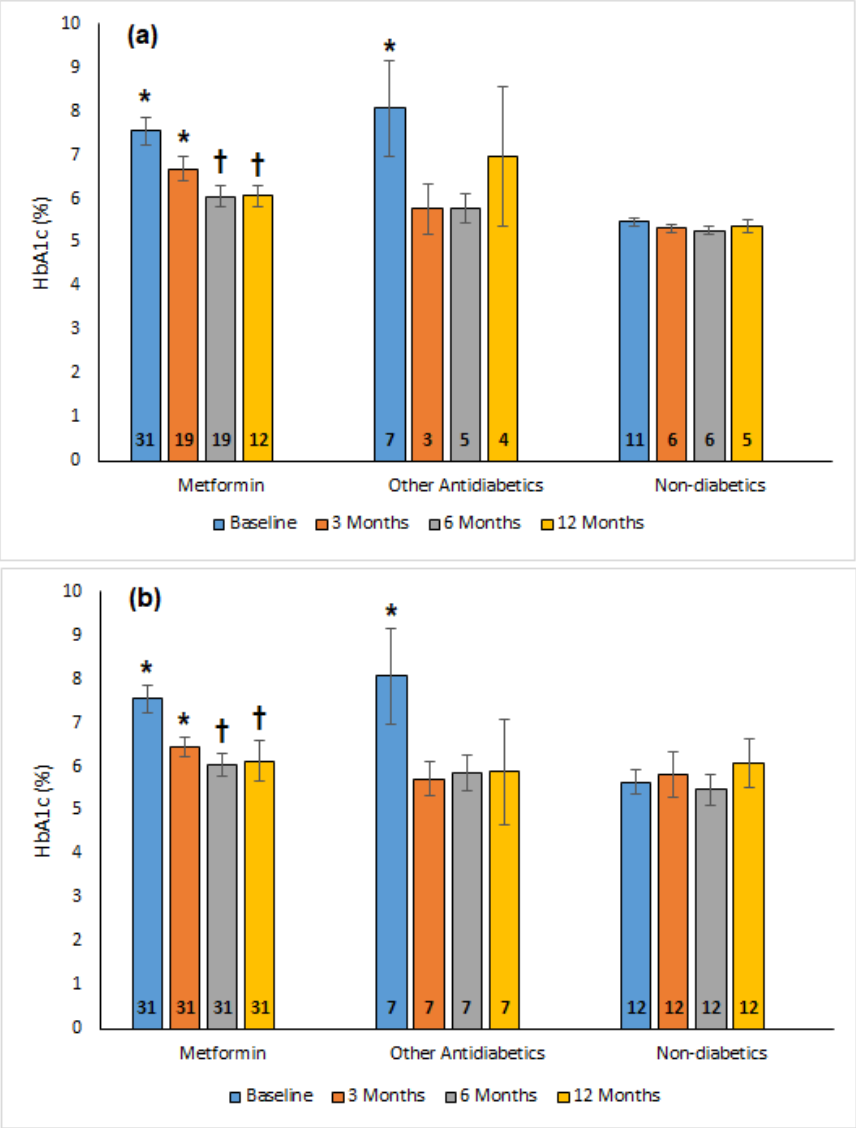


Figure 37. HbA1c in % (mean ± SE) at baseline and 3, 6, and 12 M follow-up visits after RYGB in metformin, other antidiabetics, and non-diabetics groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits). Statistically significant difference within each group from (†) baseline. (*) Statistically significant difference from the non-diabetics group at the same time point.

4.4.4.2. Glycemic Control

The percentage (%) of subjects with complete remission at 3, 6 or 12 months after surgery ranged from 26.3-47.3% **(Table 26)**. Glycemic control seemed to improve with time after surgery as the % of patients who had improvement in their glycemic outcomes increased from 3 to 12 months post-RYGB (5.3 to 25.0%). In addition, the recurrence percentage decreased with time post-RYGB from 52.6% at 3-month to 16.7% at 12-month follow-up visits. The results from many published studies record the complete resolution of type 2 diabetes in the range of 76.8-89 % of patients after surgery (Pories et al., 1995, Schauer et al., 2003, Buchwald et al., 2004, Alexandrides et al., 2007). However, only 26.3-47.3 of the patients in our study reached complete remission state at any time point post-RYGB. Our results disagreed with the general notion that RYGB causes remission or resolution of type 2 diabetes completely. In our tested patient population, the common claim that patients do not need metformin treatment after surgery was apparently inaccurate. Only 50 % of our patient population (either with complete or partial remission) discontinued metformin use at the 12-month follow-up visit after RYGB.

Table 26. Glycemic outcomes in metformin group represented as n (%)

Follow-Up Visit	Complete Remission	Partial Remission	Improvement	Unchanged	Recurrence
3 Months (n=19)	5 (26.3)	1 (5.3)	1 (5.3)	2 (10.5)	10 (52.6)
6 Months (n=19)	9 (47.3)	1 (5.3)	3 (15.8)	1 (5.3)	5 (26.3)
12 Months (n=12)	4 (33.3)	2 (16.7)	3 (25.0)	1 (8.3)	2 (16.7)

4.4.4.3. Relating HbA1c Levels to Metformin Treatment

The trend of change in HbA1c levels was linked to metformin treatment and discontinuation. From the 31 diabetic patients on metformin, 16 (52%) had HbA1c levels higher than 7% at baseline **(Figure 38)**. The target HbA1c for non-pregnant adult diabetic patients is <7% (American Diabetes Association, 2019). After the surgery, 10 of those 16 patients reached the target HbA1c at the latest time point of follow-up for each patient. However, four patients did not reach the target after RYGB and two patients have no data of HbA1c levels at any time point post-surgery. From the four patients who did not reach the target HbA1c, two were still on metformin and the other two discontinued metformin treatment. These observations mean that patients who discontinue metformin treatment post-RYGB might be at risk of hyperglycemia and should be monitored closely.

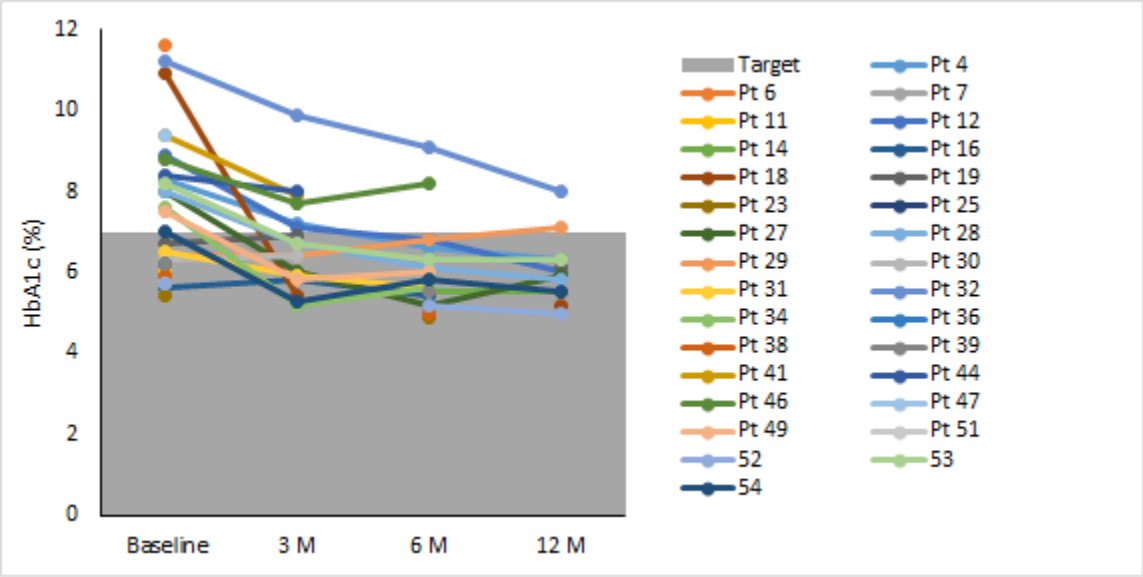


Figure 38. HbA1c levels (%) in individual patients in the metformin group pre- and post-RYGB

4.4.4.4. Glucose levels

Mean glucose concentrations at baseline were significantly higher in metformin and other antidiabetics groups (143.7 and 161.6 mg/dl, respectively) compared to the non-diabetics group (83.8 mg/dl). Mean glucose concentrations showed a trend of decrease after RYGB in metformin and other antidiabetics groups, to 108.1 and 108.8 mg/dl, respectively, at 12-month visit. Although the decrease in both groups was not statistically significant, the levels of glucose post-surgery are approaching levels similar to the non-diabetics group. In the non-diabetics group, mean glucose concentration did not significantly change from the baseline level at any follow-up visit **(Figures 39)**. The comparison of the glucose levels before and after RYGB was not attempted due to the unreliable information about the certainty of the patients' fasting state at each visit.

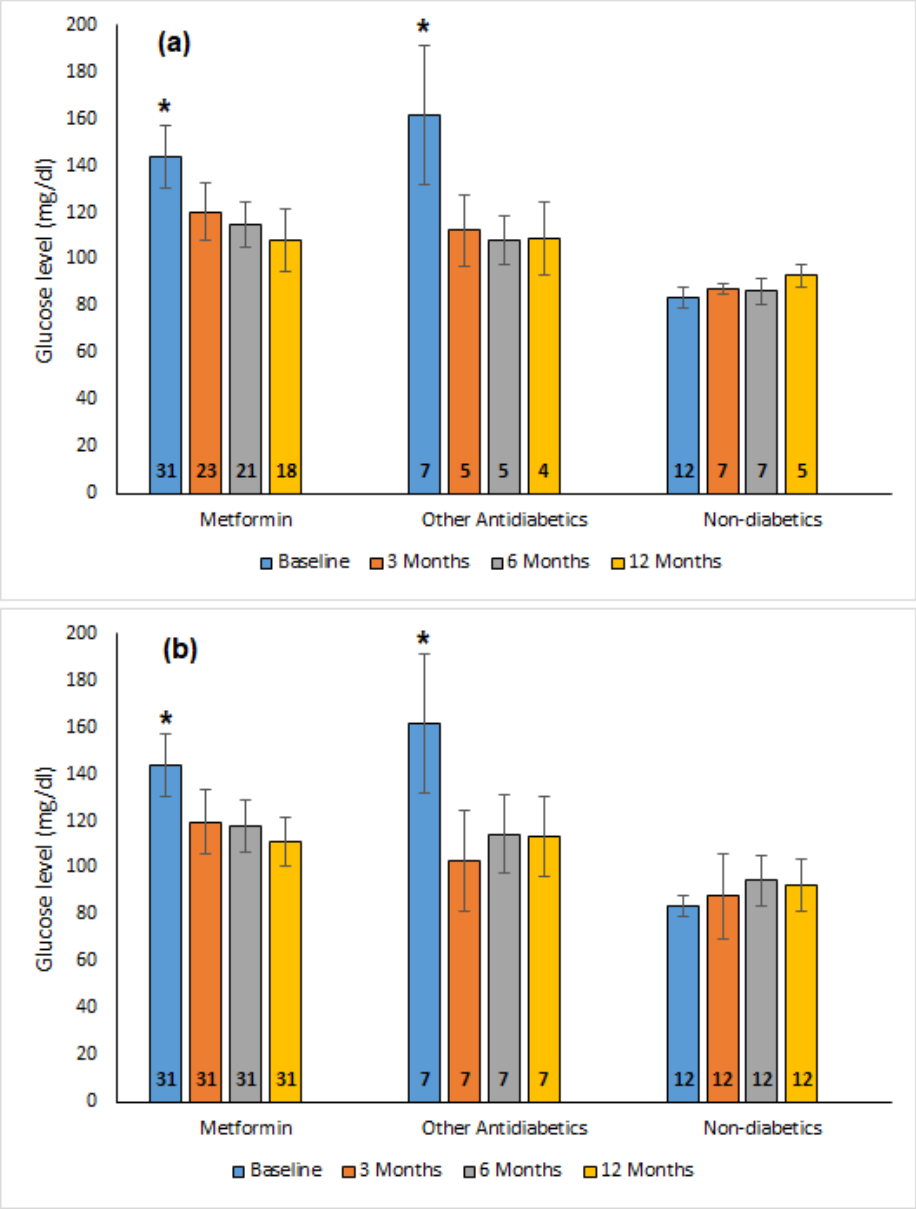


Figure 39. Glucose levels in mg/dl (mean ± SE) at baseline and 3, 6, and 12 M follow-up visits after RYGB in metformin, other antidiabetics, and non-diabetics groups from (a) original and (b) MI pooled data (Numbers inside the bars represent the datum points available at individual follow-up visits). (*) Statistically significant difference from the non-diabetics group at baseline.

4.4.5. Pharmacokinetics (PK)

The individual concentrations normalized by dose/bodyweight, [(ng)/(mg/kg)], of metformin are presented in **Figure 40**. Individuals who had similar sampling times before and after surgery (presented by a solid line) showed a trend of decrease in metformin normalized concentrations after RYGB. Patients 27, 46, and 53 showed a trend of decrease in metformin concentrations at 3 months compared to baseline with similar sampling times before and after RYGB (0.5-1.5 hours difference). Only one patient (Pt 28) showed a trend of increase in metformin concentration at 3 months visit compared to baseline. By comparing 3 months and 6 months visit, the trend was a decrease in concentrations in all patients who had similar sampling times (patients 28, 32, and 53 with 0.5-2 hours difference in sampling times). The trend from 6 to 12 months visit was also a decrease in concentrations from two patients (19 and 32), with sampling time range of ±2 hours.

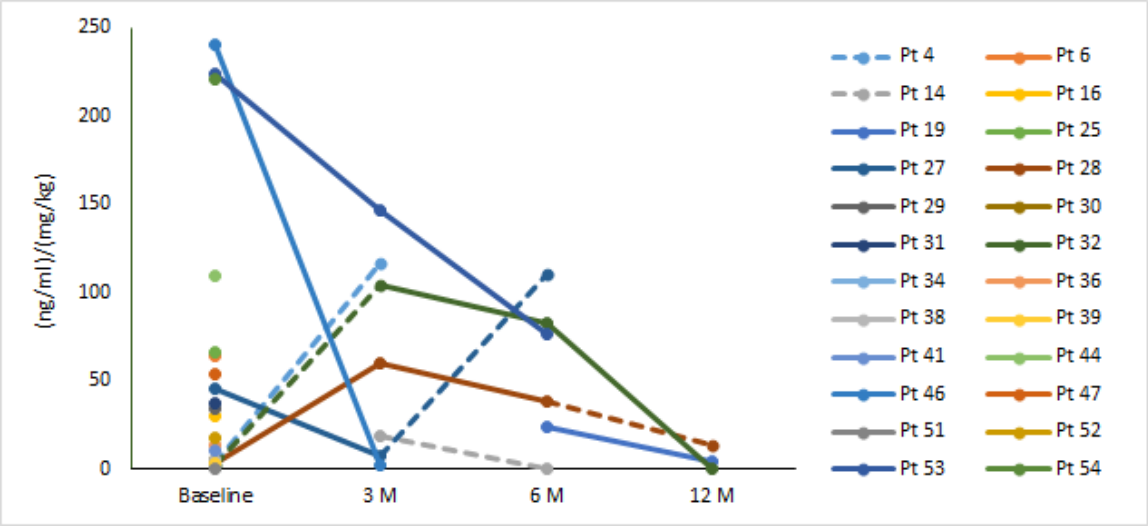


Figure 40. Concentrations [(ng/ml)/ (mg/kg)] of metformin in individual patients pre- and post-RYGB. Solid lines represent similar sampling time

These results are supported by the fact that metformin absorption occurs mainly in the proximal small intestine (Song, 2016), which is bypassed after RYGB. Our results are in contrary to the only published study about metformin absorption and bioavailability after RYGB, which reports that the $AUC_{0-\infty}$ increased by 21% in gastric bypass surgery patients compared to BMI matched individuals (Padwal et al., 2011). However, we believe our study have the merit of using the same individual as own control, and comparing results pre- and post-RYGB to monitor and characterize the impacts of RYGB longitudinally, and to avoid any concern of neglecting the inter-individual variability on Padwal's study.

4.5. Limitations and Merits

We recognized the limitations of the study design and approach. The small subject number in total (n=50) and especially in each group reduces the power of the statistical analysis and could increase the chance of type II error (false negative). In addition, the missing data due to patients missing their follow-up appointments or simply failure to record the data properly could also add to the loss in power of the statistical analysis. Multiple imputation is the recommended approach to handle data with high proportion of missing data (> 5%) and small samples (≤ 50). However, caution was taken by using multiple imputation, because the gain in power and accuracy of parameter estimates from any imputation method could be cancelled out by the additional level of error introduced by imputation. This is usually encountered in mean imputation method rather than multiple imputation, because the gain in power is most prominent in multiple imputation (Cheema, 2014).

Another limitation of the study is the availability of one blood sample only for PK data analysis at each time point pre- or post-RYGB. This limits the possibility of determining the individual PK parameters such as elimination rate constant (k_e). The sampling time was different between subjects and between different time points in the same subject pre- and post-RYGB. The comparison of normalized concentrations at different time points becomes difficult especially for drugs and metabolites with short half-lives (SMV, SMV-A and metformin). However, for drugs

and metabolites with relatively long half-lives (ATV, its two active metabolites, and RSV), the comparison becomes possible if the sampling times were comparable.

However, the merits of the study are significant. This is the first longitudinal study that monitor and characterize the impacts of RYGB on the PK and PD of three statins, ATV, RSV and SMV, and metformin, from the individual subjects pre-RYGB and from 1 week to 12 months post-RYGB, using their own pre-surgery controls. In addition, the PK of the active metabolites of ATV and SMV were also monitored longitudinally to characterize the impacts of RYGB on metabolisms of these statins. Moreover, this study is the first PK/PD correlation study that provides the correlation of LDL with BMI and total active ATV concentrations, as well as the correlation of LDL with BMI and SMV-A/SMV ratios post-RYGB. The correlation models offer the potential for future rational dose adjustment post-RYGB.

Chapter 5. Summary

5.1. Development and validation of simultaneous LC-MS/MS quantification method for simvastatin, atorvastatin, and rosuvastatin along with their active metabolites in plasma with low and high triglyceride levels

This is the first report for the development and validation of an accurate, sensitive, and reliable simultaneous quantification method for simvastatin, its active metabolite, simvastatin acid, atorvastatin, its two active metabolites, 2-hydroxy and 4-hydroxy atorvastatin, and rosuvastatin in human plasma samples. The method was validated in plasma with low (52-103 mg/dl, < 300 mg/dl) and high triglyceride (352-403 mg/dl, > 300 mg/dl) levels to ensure the reliability in plasma samples obtained from obese individuals pre- and post-RYGB. The method was validated according to the FDA Guidelines for Bioanalytical Method Validation, and has satisfactory (1) selectivity and specificity, (2) sensitivity and carryover, (3) linearity, (4) accuracy and precision, (5) extraction recovery (6) matrix effect, and (7) stability. The method was successfully utilized in quantifying simvastatin, atorvastatin, and their active metabolites, as well as rosuvastatin in plasma samples obtained from subjects pre- and post-RYGB.

5.2. Development and validation of LC-MS/MS quantification method for metformin in plasma with low and high triglyceride levels

Similarly, a method for the quantification of metformin in human plasma was developed and validated according to FDA Guidelines for plasma with low and high triglyceride levels. The method has satisfactory (1) selectivity and specificity, (2) sensitivity and carryover, (3) linearity, (4) accuracy and precision, (5) extraction recovery (6) (negligible) matrix effect, and (7) stability in plasma with low and high triglycerides. The assay was successfully used for the quantification of plasma samples obtained from subjects pre- and post-RYGB.

5.3. Characterization of longitudinal effect of RYGB on the PK and PD of simvastatin, atorvastatin, and their active metabolites, as well as rosuvastatin, and PK/PD correlation using the molar concentrations, normalized by the unit dose per body weight [(nM/ml)/(mg/kg)], of statins and their active metabolites with LDL

The impacts of RYGB on statins and their active metabolites were characterized. To our knowledge, this is the first report of the longitudinal effects of RYGB on the PK and PD of simvastatin (n=9), atorvastatin (n=5), and rosuvastatin (n=12), along with their active metabolites. An important merit of this study is the follow-up of the same subject pre- and post-RYGB, which accounts for the effects of inter-

individual variability. The plasma concentrations normalized by the dose per unit body weight [(nM)/(mg/kg)] showed a trend of decreasing up to 6 months after RYGB for atorvastatin (38.81 ± 2.36 to 9.75 ± 2.52 (nM)/(mg/kg)) and its two active metabolites, 2- hydroxy atorvastatin and 4-hydroxy atorvastatin, (44.82 ± 6.11 and 18.75 ± 4.18 to 10.26 ± 2.95 and 2.94 ± 0.80 (nM)/(mg/kg), respectively), and rosuvastatin (213.07 ± 22.87 to 83.28 ± 6.39 (nM)/(mg/kg)). The trend was opposite for simvastatin and its active metabolite, simvastatin acid, where the normalized plasma concentrations increased up to 6 months post-RYGB from 8.52 ± 3.04 and 9.96 ± 3.99 at baseline to 28.45 ± 3.54 and 39.32 ± 7.95 (nM)/(mg/kg) at 6-month post-RYGB, respectively, then normalized back to the baseline levels at one-year post-surgery with mean plasma concentrations of 7.09 ± 0.42 and 10.45 ± 2.39 (nM)/(mg/kg), respectively.

The decrease in atorvastatin plasma concentration post-RYGB could be attributed to a decrease in its absorption because of the effect of increased gastric pH to >4 on the ionization state of atorvastatin which makes it less permeable. In addition, the decrease in intestinal surface available for absorption post-surgery that could decrease atorvastatin absorption which is by passive diffusion. On the other hand, the decrease in rosuvastatin normalized concentrations post-surgery could be explained by the decrease in the availability/functionality of OATP2B1 transporter in the reduced intestinal tract, which will decrease the rosuvastatin absorption.

The increase in simvastatin concentrations post-RYGB may be due to the predomination of the lactone form of the medication at the higher pH levels in the gastrointestinal tract. The lactone form is more permeable and has a greater absorption than the hydroxy acid form. The plasma concentrations of simvastatin and its active metabolite returns to similar levels of the pre-surgery concentrations at 12 months post-RYGB, which suggests an adaptation mechanism of the gastrointestinal tract.

Despite different trends in changes of plasma concentrations, the metabolic rates for both atorvastatin and simvastatin showed a decrease in their metabolism post-surgery as expressed by the ratio of each metabolite/parent concentrations. The ratios of 2-hydroxy atorvastatin/atorvastatin and 4-hydroxy atorvastatin/atorvastatin decreased by approximately 60% at 3 months post-surgery. The ratio of simvastatin acid/simvastatin also decreased by 3-60% at 3 or 6 months post-RYGB. The ratios of each metabolite/parent remained relatively constant at the lower levels after the initial decrease.

A preliminary PK/PD correlation between the total molar concentrations of atorvastatin and its two active metabolites with LDL were performed. The threshold of the effective total molar concentrations of atorvastatin and its two active metabolites decreased from 40 nM pre-surgery to 20 nM at 3 and 6 months post-RYGB. In addition, simvastatin acid molar concentrations were correlated with LDL levels. At baseline, there was no obvious correlation observed. However, there

was a trend of decrease in LDL with increased simvastatin acid concentration at 3 and 6 months post-RYGB; the trend of decrease in LDL seems to level off at concentrations higher than 5-10 nM.

Individual LDL levels were analyzed in relation to statin discontinuation and dose reduction post-RYGB. Discontinuations or reduction of statin dosing with atorvastatin and rosuvastatin require the monitoring of LDL, as rebound LDL were often observed, which is consistent with the PK finding of reduced absorption of atorvastatin and rosuvastatin post-RYGB. The rebound in LDL levels was not apparent when patients on simvastatin with a reduction in simvastatin dose post-RYGB.

The effects of surgery on PD parameters was characterized by comparing statin to non-statin groups. The surgery had a significant effect on the lipid panel of unmedicated patients with high LDL to levels within the optimal level of <100 mg/dl by 3 months post-RYGB. Patients on simvastatin and rosuvastatin had higher levels of TG at baseline than atorvastatin and non-statin groups, but the high TG levels were corrected post-RYGB. There was an increase in HDL levels post-RYGB in all groups with similar time profiles. On the other hand, the effect on weight loss outcomes was merely surgery related with 10.6-15.3% reduction in excess weight loss by 1 week post-RYGB, and reaching 70.4-76.5% by 12 months post-surgery, and statin therapy had no added effect on weight loss outcomes post-surgery.

5.4. Characterization of RYGB effect on PK/PD of metformin

The surgery effects on metformin PK and PD were characterized. The trend in metformin concentration change post-RYGB showed a continuous decrease in concentrations from baseline to 3, 6, and 12 months post-surgery with ranges of 2.1-240.2, 1.8-146.7, 0.6-110.6, and 0.1-12.9 (nM)/(mg/kg), respectively. This initial decrease could be attributed to a decrease in metformin absorption after surgery. The later continuous decrease could be explained by the continuous enhancement in cardiac and renal functions, which led to a more efficient elimination of metformin, as metformin is excreted almost entirely by the renal route.

The effect of surgery on HbA1c was appreciated and showed a lowering effect on HbA1c levels to 6.1% post-surgery. This relates to the fact that there was no observed treatment failure in diabetic patients even with the decrease in metformin concentrations post-surgery. However, complete remission was reached in only 26.3-47.3% of the patients in our study at any time point post-surgery, which is in contrast to the general believe that RYGB causes complete remission or resolution of type 2 diabetes.

5.5. Conclusion

The objectives of the specific aims in this study were successfully achieved, although this study had some limitations and a relatively small sample size. The

drastic changes in the anatomy and physiology of human body post-RYGB makes the study of patients after RYGB challenging. The observed longitudinal changes in concentrations normalized by the unit dose per body weight post-RYGB were distinct among the statins, in which opposite effects were observed between atorvastatin/rosuvastatin and simvastatin. The changes in statins and metformin concentrations post-RYGB confirm that patients should be monitored closely after surgery. At the end, the most important message is that the follow-up of patients on statins and/or metformin following-RYGB is crucial and the dosing should be rationally adjusted, to ensure the therapeutic success with minimal adverse effects. In addition, patients who discontinue these medications post-surgery need supervision to ensure no recurrence of hyperlipidemia or diabetes.

Bibliography

AlAkhali, K. and S. Alavudeen. "Multiple dose of liquid antacid enhance simvastatin bioavailability in Malaysian male volunteers." Asian Journal of Pharmaceutics **8**(2): 90-94. 2014.

Alakhali, K., E. Vigneshwaran and M. Shaik. "Effect of food and antacid on simvastatin bioavailability on healthy adult volunteers." J Health Res Rev **5**(1): 26-32. 2018.

Alexandrides, T. K., G. Skroubis and F. Kalfarentzos. "Resolution of diabetes mellitus and metabolic syndrome following Roux-en-Y gastric bypass and a variant of biliopancreatic diversion in patients with morbid obesity." Obes Surg **17**(2): 176-184. 2007.

American Diabetes Association. "6. Glycemic Targets: Standards of Medical Care in Diabetes-2019." Diabetes Care **42**(Suppl 1): S61-S70. 2019.

American Society for Metabolic and Bariatric Surgery. "Bariatric Surgery Procedures." Retrieved October 23, 2018, from https://asmbs.org/patients/bariatric-surgery-procedures.

American Society for Metabolic and Bariatric Surgery. (2018, June 2018). "Estimate of Bariatric Surgery Numbers, 2011-2017." Retrieved October 23, 2018, from https://asmbs.org/resources/estimate-of-bariatric-surgery-numbers.

Asztalos, B. F., M. M. Swarbrick, E. J. Schaefer, G. E. Dallal, K. V. Horvath, M. Ai, K. L. Stanhope, I. Austrheim-Smith, B. M. Wolfe, M. Ali and P. J. Havel. "Effects of

weight loss, induced by gastric bypass surgery, on HDL remodeling in obese women." J Lipid Res **51**(8): 2405-2412. 2010.

Bailey, C. J. "Metformin: historical overview." Diabetologia **60**(9): 1566-1576. 2017.

Belo, G., L. T. Siqueira, D. A. A. Melo Filho, F. Kreimer, V. P. Ramos and A. A. B. Ferraz. "Predictors of poor follow-up after bariatric surgery." Rev Col Bras Cir **45**(2): e1779. 2018.

Bennett, D. A. "How can I deal with missing data in my study?" Aust N Z J Public Health **25**(5): 464-469. 2001.

Bhutta, H. Y., N. Rajpal, W. White, J. M. Freudenberg, Y. Liu, J. Way, D. Rajpal, D. C. Cooper, A. Young, A. Tavakkoli and L. Chen. "Effect of Roux-en-Y gastric bypass surgery on bile acid metabolism in normal and obese diabetic rats." PLoS One **10**(3): e0122273. 2015.

Biering, K., N. H. Hjollund and M. Frydenberg. "Using multiple imputation to deal with missing data and attrition in longitudinal studies with repeated measures of patient-reported outcomes." Clin Epidemiol **7**: 91-106. 2015.

Brethauer, S. A., J. Kim, M. El Chaar, P. Papasavas, D. Eisenberg, A. Rogers, N. Ballem, M. Kligman, S. Kothari and A. C. I. Committee. "Standardized outcomes reporting in metabolic and bariatric surgery." Obes Surg **25**(4): 587-606. 2015.

Buchwald, H., Y. Avidor, E. Braunwald, M. D. Jensen, W. Pories, K. Fahrbach and K. Schoelles. "Bariatric surgery: a systematic review and meta-analysis." JAMA **292**(14): 1724-1737. 2004.

Centers for Disease Control and Prevention. (2018, September 12, 2018). "Adult Obesity Prevalence Maps." Retrieved October 23, 2018, from https://www.cdc.gov/obesity/data/prevalence-maps.html.

Cheema, J. "Some general guidlines for choosing missing data handling methods in educational research." JMASM **13**(2): 53-75. 2014.

Colhoun, H. M., D. J. Betteridge, P. N. Durrington, G. A. Hitman, H. A. Neil, S. J. Livingstone, M. J. Thomason, M. I. Mackness, V. Charlton-Menys, J. H. Fuller and C. investigators. "Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial." Lancet **364**(9435): 685-696. 2004.

Compher, C. W., A. Hanlon, Y. Kang, L. Elkin and N. N. Williams. "Attendance at clinical visits predicts weight loss after gastric bypass surgery." Obes Surg **22**(6): 927-934. 2012.

Dhingra, R. and R. S. Vasan. "Age as a risk factor." Med Clin North Am **96**(1): 87-91. 2012.

Dong, Y. and C. Y. Peng. "Principled missing data methods for researchers." Springerplus **2**(1): 222. 2013.

El-Zailik, A., L. K. Cheung, Y. Wang, V. Sherman and D. S. Chow. "Longitudinal Impacts of Gastric Bypass Surgery on Pharmacodynamics and Pharmacokinetics of Statins." Obes Surg. 2019.

El-Zailik, A., L. K. Cheung, Y. Wang, V. Sherman and D. S. Chow. "Simultaneous LC-MS/MS analysis of simvastatin, atorvastatin, rosuvastatin and their active metabolites for plasma samples of obese patients underwent gastric bypass surgery." J Pharm Biomed Anal **164**: 258-267. 2019.

Endo, A. "A historical perspective on the discovery of statins." Proc Jpn Acad Ser B Phys Biol Sci **86**(5): 484-493. 2010.

FDA-NIH Biomarker Working Group. Pharmacodynamic/Response Biomarker. BEST (Biomarkers, EndpointS, and other Tools) Resource. Silver Spring (MD), Food and Drug Administration (US) and National Institute of Health (NIH). 2016.

Fong, C. "Statins in therapy: Understanding their hydrophilicity, lipophilicity, binding to 3-hydroxy-3-methylglutaryl-CoA reductase, ability to cross the blood brain barrier and metabolic stability based on electrostatic molecular orbital studies." European Journal of Medicinal Chemistry **85**: 661-674. 2014.

Food and Drug Adminisration. Bioanalytical Method Validation: Guidance for Industry., U.S. Department of Health and Human Services. 2018.

Fujino, H. and J. Kojima (2006). Drug metabolism and transporter properties of statins. Focus on Statin Research. B. Wong. New York, Nova Science Publisher, Inc.

Gadde, K. M., J. W. Apolzan and H. R. Berthoud. "Pharmacotherapy for Patients with Obesity." Clin Chem **64**(1): 118-129. 2018.

Garcia, M. J., R. F. Reinoso, A. Sanchez Navarro and J. R. Prous. "Clinical pharmacokinetics of statins." Methods Find Exp Clin Pharmacol **25**(6): 457-481. 2003.

Giuliano, C., M. Wilhelm and P. Kale-Pradhan. "Pharmacokinetic changes secondary to Roux en Y gastric bypass " Adv Pharmacoepidem Drug Safety **S1**(001). 2012.

Golzarand, M., K. Toolabi and R. Farid. "The bariatric surgery and weight losing: a meta-analysis in the long- and very long-term effects of laparoscopic adjustable gastric banding, laparoscopic Roux-en-Y gastric bypass and laparoscopic sleeve gastrectomy on weight loss in adults." Surg Endosc **31**(11): 4331-4345. 2017.

Gong, L., S. Goswami, K. Giacomini, R. Altman and T. Klein. "Metformin pathways: pharmacokinetics and pharmacodynamics." Pharmacogenet Genomics **22**(11): 820-827. 2012.

Graham, G. G., J. Punt, M. Arora, R. O. Day, M. P. Doogue, J. K. Duong, T. J. Furlong, J. R. Greenfield, L. C. Greenup, C. M. Kirkpatrick, J. E. Ray, P. Timmins and K. M. Williams. "Clinical pharmacokinetics of metformin." Clin Pharmacokinet **50**(2): 81-98. 2011.

Graham, J. W., A. E. Olchowski and T. D. Gilreath. "How many imputations are really needed? Some practical clarifications of multiple imputation theory." Prev Sci **8**(3): 206-213. 2007.

Hachon, L., X. Decleves, P. Faucher, C. Carette and C. Lloret-Linares. "RYGB and Drug Disposition: How to Do Better? Analysis of Pharmacokinetic Studies and Recommendations for Clinical Practice." Obes Surg **27**(4): 1076-1090. 2017.

Hales, C. M., M. D. Carroll, C. D. Fryar and C. L. Ogden. "Prevalence of Obesity Among Adults and Youth: United States, 2015-2016." NCHS Data Brief(288): 1-8. 2017.

Heart Protection Study Collaborative, G. "MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial." Lancet **360**(9326): 7-22. 2002.

Higa, K., J. Himpens, R. Welbourn, J. Dixon, R. Kinsman and P. Walton (2017). Third IFSO Global Registry Report 2017. Henley on Thames, United Kingdom, Dendrite Clinical Systems Limited.

Jakobsen, G. S., I. B. Skottheim, R. Sandbu, H. Christensen, J. Roislien, A. Asberg and J. Hjelmesaeth. "Long-term effects of gastric bypass and duodenal switch on systemic exposure of atorvastatin." Surg Endosc **27**(6): 2094-2101. 2013.

Jarolimova, J., J. Tagoni and T. A. Stern. "Obesity: its epidemiology, comorbidities, and management." Prim Care Companion CNS Disord **15**(5). 2013.

Jensen, M. D., D. H. Ryan, C. M. Apovian, J. D. Ard, A. G. Comuzzie, K. A. Donato, F. B. Hu, V. S. Hubbard, J. M. Jakicic, R. F. Kushner, C. M. Loria, B. E. Millen, C. A. Nonas, F. X. Pi-Sunyer, J. Stevens, V. J. Stevens, T. A. Wadden, B. M. Wolfe, S. Z. Yanovski, G. American College of Cardiology/American Heart Association

Task Force on Practice and S. Obesity. "2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society." J Am Coll Cardiol **63**(25 Pt B): 2985-3023. 2014.

Johnson, M., D. Patel, C. Matheny, M. Ho, L. Chen and H. Ellens. "Inhibition of Intestinal OATP2B1 by the Calcium Receptor Antagonist Ronacaleret Results in a Significant Drug-Drug Interaction by Causing a 2-Fold Decrease in Exposure of Rosuvastatin." Drug Metab Dispos **45**(1): 27-34. 2017.

Kang, H. "The prevention and handling of the missing data." Korean J Anesthesiol **64**(5): 402-406. 2013.

Keating, G. M. and D. M. Robinson. "Rosuvastatin: a review of its effect on atherosclerosis." Am J Cardiovasc Drugs **8**(2): 127-146. 2008.

Khorgami, Z., C. Zhang, S. E. Messiah and N. de la Cruz-Munoz. "Predictors of Postoperative Aftercare Attrition among Gastric Bypass Patients." Bariatr Surg Pract Patient Care **10**(2): 79-83. 2015.

Klotz, U. "Pharmacological comparison of the statins." Arzneimittelforschung **53**(9): 605-611. 2003.

Laerd Statistics. (2018). "ANOVA with Repeated Measures using SPSS Statistics." Retrieved March 19, 2019, from https://statistics.laerd.com/spss-tutorials/one-way-anova-repeated-measures-using-spss-statistics.php.

Lager, C. J., N. H. Esfandiari, A. R. Subauste, A. T. Kraftson, M. B. Brown, R. B. Cassidy, C. K. Nay, A. L. Lockwood, O. A. Varban and E. A. Oral. "Roux-En-Y Gastric Bypass Vs. Sleeve Gastrectomy: Balancing the Risks of Surgery with the Benefits of Weight Loss." Obes Surg **27**(1): 154-161. 2017.

Lennernas, H. "Clinical pharmacokinetics of atorvastatin." Clin Pharmacokinet **42**(13): 1141-1160. 2003.

Lennernas, H. and G. Fager. "Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences." Clin Pharmacokinet **32**(5): 403-425. 1997.

Li, Q., S. W. Blume, J. C. Huang, M. Hammer and M. L. Ganz. "Prevalence and healthcare costs of obesity-related comorbidities: evidence from an electronic medical records system in the United States." J Med Econ **18**(12): 1020-1028. 2015.

Maciejewski, M. L., D. E. Arterburn, L. Van Scoyoc, V. A. Smith, W. S. Yancy, Jr., H. J. Weidenbacher, E. H. Livingston and M. K. Olsen. "Bariatric Surgery and Long-term Durability of Weight Loss." JAMA Surg **151**(11): 1046-1055. 2016.

Marterre, W. F., S. Hariharan, M. R. First and J. W. Alexander. "Gastric bypass in morbidly obese kidney transplant recipients." Clin Transplant **10**(5): 414-419. 1996.

Mauro, V. F. "Clinical pharmacokinetics and practical applications of simvastatin." Clin Pharmacokinet **24**(3): 195-202. 1993.

Meier, Y., J. J. Eloranta, J. Darimont, M. G. Ismair, C. Hiller, M. Fried, G. A. Kullak-Ublick and S. R. Vavricka. "Regional distribution of solute carrier mRNA expression along the human intestinal tract." Drug Metab Dispos **35**(4): 590-594. 2007.

Melissas, J., K. Stavroulakis, V. Tzikoulis, A. Peristeri, J. A. Papadakis, A. Pazouki, A. Khalaj and A. Kabir. "Sleeve Gastrectomy vs Roux-en-Y Gastric Bypass. Data from IFSO-European Chapter Center of Excellence Program." Obes Surg **27**(4): 847-855. 2017.

Milne, M. D. "Influence of acid-base balance on efficacy and toxicity of drugs." Proc R Soc Med **58**(11 Part 2): 961-963. 1965.

Mitra, A. and F. Kesisoglou. "Impaired drug absorption due to high stomach pH: a review of strategies for mitigation of such effect to enable pharmaceutical product development." Mol Pharm **10**(11): 3970-3979. 2013.

Motycka, C. A., E. St Onge and S. A. Miller. "Treatment options for obesity and potential therapies on the horizon." P T **36**(5): 282-301. 2011.

Must, A. and N. M. McKeown (2000). The Disease Burden Associated with Overweight and Obesity. Endotext. L. J. De Groot, G. Chrousos, K. Dungan et al. South Dartmouth (MA).

National Institutes of Health. "Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults--The Evidence Report. ." Obes Res **6 Suppl 2**: 51S-209S. 1998.

Nayak, B. K. "Understanding the relevance of sample size calculation." Indian J Ophthalmol **58**(6): 469-470. 2010.

Nayak, B. K. and A. Hazra. "How to choose the right statistical test?" Indian J Ophthalmol **59**(2): 85-86. 2011.

Nguyen, N. T., H. S. Ho, K. L. Mayer, L. Palmer and B. M. Wolfe. "Laparoscopic Roux-en-Y gastric bypass for morbid obesity." JSLS **3**(3): 193-196. 1999.

Nguyen, N. T. and J. E. Varela. "Bariatric surgery for obesity and metabolic disorders: state of the art." Nat Rev Gastroenterol Hepatol **14**(3): 160-169. 2017.

Nigovic, B., A. Mornar and M. Sertic (2012). A Review of Current Trends and Advances in Analytical Methods for Determination of Statins: Chromatography and Capillary Electrophoresis. Chromatography- The Most Versatile Method of Chemical Analysis**:** 385-428.

Padwal, R., D. Brocks and A. M. Sharma. "A systematic review of drug absorption following bariatric surgery and its theoretical implications." Obes Rev **11**(1): 41-50. 2010.

Padwal, R. S., R. Q. Gabr, A. M. Sharma, L. A. Langkaas, D. W. Birch, S. Karmali and D. R. Brocks. "Effect of gastric bypass surgery on the absorption and bioavailability of metformin." Diabetes Care **34**(6): 1295-1300. 2011.

Pang, K. S. "Modeling of intestinal drug absorption: roles of transporters and metabolic enzymes (for the Gillette Review Series)." Drug Metab Dispos **31**(12): 1507-1519. 2003.

Perna, M., M. Baker, T. K. Byrne and K. Morgan. "Statins and the bariatric patient: characterization and perioperative effects of statin therapy in the gastric bypass patient." Am Surg **77**(1): 44-47. 2011.

Pfizer Ireland Pharmaceuticals. (2009). "Lipitor (atorvastatin calcium)." Dublin, Ireland. Retrieved Jan 19, 2019, from https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/020702s057lbl.pdf.

Pories, W. J., M. S. Swanson, K. G. MacDonald, S. B. Long, P. G. Morris, B. M. Brown, H. A. Barakat, R. A. deRamon, G. Israel, J. M. Dolezal and et al. "Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus." Ann Surg **222**(3): 339-350; discussion 350-332. 1995.

Prueksaritanont, T., R. Subramanian, X. Fang, B. Ma, Y. Qiu, J. H. Lin, P. G. Pearson and T. A. Baillie. "Glucuronidation of statins in animals and humans: a novel mechanism of statin lactonization." Drug Metab Dispos **30**(5): 505-512. 2002.

Puzziferri, N., T. B. Roshek, 3rd, H. G. Mayo, R. Gallagher, S. H. Belle and E. H. Livingston. "Long-term follow-up after bariatric surgery: a systematic review." JAMA **312**(9): 934-942. 2014.

Quan, V., F. Cooper and M. Bekheit. "The influence of total bowel length on gastric bypass outcomes." Mini-invasive Surgery **1**: 95-98. 2017.

Rena, G., D. G. Hardie and E. R. Pearson. "The mechanisms of action of metformin." Diabetologia **60**(9): 1577-1585. 2017.

Ridker, P. M., E. Danielson, F. A. Fonseca, J. Genest, A. M. Gotto, Jr., J. J. Kastelein, W. Koenig, P. Libby, A. J. Lorenzatti, J. G. MacFadyen, B. G. Nordestgaard, J. Shepherd, J. T. Willerson, R. J. Glynn and J. S. Group. "Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein." N Engl J Med **359**(21): 2195-2207. 2008.

Roberts, M. S., B. M. Magnusson, F. J. Burczynski and M. Weiss. "Enterohepatic circulation: physiological, pharmacokinetic and clinical implications." Clin Pharmacokinet **41**(10): 751-790. 2002.

Rogers, C. C., R. R. Alloway, J. W. Alexander, M. Cardi, J. Trofe and A. A. Vinks. "Pharmacokinetics of mycophenolic acid, tacrolimus and sirolimus after gastric bypass surgery in end-stage renal disease and transplant patients: a pilot study." Clin Transplant **22**(3): 281-291. 2008.

Russell-Jones, D. and R. Khan. "Insulin-associated weight gain in diabetes--causes, effects and coping strategies." Diabetes Obes Metab **9**(6): 799-812. 2007.

Sampsel, S. and J. May. "Assessment and management of obesity and comorbid conditions." Dis Manag **10**(5): 252-265. 2007.

Sasaki, J. "Pitavastatin approved for treatment of primary hypercholesterolemia and combined dyslipidemia." Vasc Health Risk Manag **6**: 997-1005. 2010.

Schachter, M. "Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update." Fundam Clin Pharmacol **19**(1): 117-125. 2005.

Schauer, P. R., B. Burguera, S. Ikramuddin, D. Cottam, W. Gourash, G. Hamad, G. M. Eid, S. Mattar, R. Ramanathan, E. Barinas-Mitchel, R. H. Rao, L. Kuller and D. Kelley. "Effect of laparoscopic Roux-en Y gastric bypass on type 2 diabetes mellitus." Ann Surg **238**(4): 467-484; discussion 484-465. 2003.

Sever, P. S., B. Dahlof, N. R. Poulter, H. Wedel, G. Beevers, M. Caulfield, R. Collins, S. E. Kjeldsen, A. Kristinsson, G. T. McInnes, J. Mehlsen, M. Nieminen, E. O'Brien, J. Ostergren and A. investigators. "Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial." Lancet **361**(9364): 1149-1158. 2003.

Skottheim, I. B., K. Stormark, H. Christensen, G. S. Jakobsen, J. Hjelmesaeth, T. Jenssen, J. L. Reubsaet, R. Sandbu and A. Asberg. "Significantly altered systemic exposure to atorvastatin acid following gastric bypass surgery in morbidly obese patients." Clin Pharmacol Ther **86**(3): 311-318. 2009.

Smith, A., B. Henriksen and A. Cohen. "Pharmacokinetic considerations in Roux-en-Y gastric bypass patients." Am J Health Syst Pharm **68**(23): 2241-2247. 2011.

Sockalingam, S., S. Cassin, R. Hawa, A. Khan, S. Wnuk, T. Jackson and A. Okrainec. "Predictors of post-bariatric surgery appointment attendance: the role of relationship style." Obes Surg **23**(12): 2026-2032. 2013.

Sola, D., L. Rossi, G. P. Schianca, P. Maffioli, M. Bigliocca, R. Mella, F. Corliano, G. P. Fra, E. Bartoli and G. Derosa. "Sulfonylureas and their use in clinical practice." Arch Med Sci **11**(4): 840-848. 2015.

Song, R. "Mechanism of Metformin: A Tale of Two Sites." Diabetes Care **39**(2): 187-189. 2016.

Srinivas, N. R. "Impact of Roux-en-Y Gastric Bypass Surgery on Pharmacokinetics of Administered Drugs: Implications and Perspectives." Am J Ther **23**(6): e1826-e1838. 2016.

Stancu, C. and A. Sima. "Statins: mechanism of action and effects." J Cell Mol Med **5**(4): 378-387. 2001.

Stephen, S., A. Baranova and Z. M. Younossi. "Nonalcoholic fatty liver disease and bariatric surgery." Expert Rev Gastroenterol Hepatol **6**(2): 163-171. 2012.

Stumvoll, M., H. U. Haring and S. Matthaei. "Metformin." Endocr Res **32**(1-2): 39-57. 2007.

Taylor, B. A., J. Ng, A. Stone, P. D. Thompson, P. K. Papasavas and D. S. Tishler. "Effects of statin therapy on weight loss and diabetes in bariatric patients." Surg Obes Relat Dis **13**(4): 674-680. 2017.

Thorn, M., N. Finnstrom, S. Lundgren, A. Rane and L. Loof. "Cytochromes P450 and MDR1 mRNA expression along the human gastrointestinal tract." Br J Clin Pharmacol **60**(1): 54-60. 2005.

Tobert, J. A. "Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors." Nat Rev Drug Discov **2**(7): 517-526. 2003.

Varma, M. V., C. J. Rotter, J. Chupka, K. M. Whalen, D. B. Duignan, B. Feng, J. Litchfield, T. C. Goosen and A. F. El-Kattan. "pH-sensitive interaction of HMG-CoA reductase inhibitors (statins) with organic anion transporting polypeptide 2B1." Mol Pharm **8**(4): 1303-1313. 2011.

Viollet, B., B. Guigas, N. Sanz Garcia, J. Leclerc, M. Foretz and F. Andreelli. "Cellular and molecular mechanisms of metformin: an overview." Clin Sci (Lond) **122**(6): 253-270. 2012.

Vitale, C., M. Fini, G. Speziale and S. Chierchia. "Gender differences in the cardiovascular effects of sex hormones." Fundam Clin Pharmacol **24**(6): 675-685. 2010.

Vree, T. B., E. Dammers, I. Ulc, S. Horkovics-Kovats, M. Ryska and I. Merkx. "Differences between lovastatin and simvastatin hydrolysis in healthy male and female volunteers:gut hydrolysis of lovastatin is twice that of simvastatin." ScientificWorldJournal **3**: 1332-1343. 2003.

White, C. M. "A review of the pharmacologic and pharmacokinetic aspects of rosuvastatin." J Clin Pharmacol **42**(9): 963-970. 2002.

Wiklund, O., C. Pirazzi and S. Romeo. "Monitoring of lipids, enzymes, and creatine kinase in patients on lipid-lowering drug therapy." Curr Cardiol Rep **15**(9): 397. 2013.

Yska, J. P., S. van der Linde, V. V. Tapper, J. A. Apers, M. Emous, E. R. Totte, B. Wilffert and E. N. van Roon. "Influence of bariatric surgery on the use and pharmacokinetics of some major drug classes." Obes Surg **23**(6): 819-825. 2013.