Determining the Longitudinal Compositional and Functional Changes to the Microbiota After RYGB Surgery

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Determining the Longitudinal Compositional and Functional Changes to the Microbiota after RYGB Surgery

Paul S. Vonck

Thesis in the Field of Biology
for the degree of Masters of Liberal Arts in Extension Studies

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Abstract

Obesity has become an epidemic with more deaths attributed to being overweight and obese than underweight. Bariatric surgery is an effective means to achieve sustained weight loss and metabolic improvements when performed on morbidly obese individuals. Other studies have suggested that metabolic improvements are associated with gut microbial changes therefore we investigated the longer duration (9 year) effects of bariatric surgery on the microbiome of Roux-en-Y gastric bypass (RYGB) patients or vertical banded gastroplasty patients compared to weight matched subjects. Additionally, we investigated the effects of RYGB surgery on the microbiome of individual morbidly obese patients within a one year timeframe. RYGB and VBG surgery led to altered relative phylogenic abundances and differential abundancies in functional potential found at the gene and metabolic pathway level. These functional potential differences included the ability to transfer and use multiple carbohydrate based energy sources, an additional ability to metabolize protein and fatty acids and an increased ability to perform aerobic respiration.
Acknowledgments

I would not have been able to complete this project without the help of many individuals and organizations. First, I would like to thank Dr. Ali Tavakkoli for agreeing to be my thesis director and whose work serves as an inspiration.

Also, thank you to my thesis advisor Dr. James Morris who has provided kind assistance in all aspects of the project and insured completion. I would also like to recognize the open source software community for making available all the wonderful software that makes us more productive.

The completion of my thesis was done in memory of my father.
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Chapter I

Introduction

Worldwide obesity has more than doubled since 1980 and has become an epidemic with more deaths attributed to being overweight and obese than underweight. More than 1.9 billion people were overweight and of these 600 million were obese in the year 2014 (WHO). The obesity epidemic started in the United States and Western Europe and is now widespread across all continents with the exception of sub-Saharan Africa and Asia. Obesity is a major risk factor for metabolic diseases such as cardiovascular disease and diabetes. Cardiovascular diseases, primarily heart disease and stroke, are the leading cause of death worldwide with an estimated 17 million deaths or 30% of all deaths in 2012 (WHO). Half of individuals with diabetes die of cardiovascular disease and overall the diabetic individual mortality rate is double when compared to normal individuals (Karlsson, et al., 2013).

At a fundamental level, obesity can be avoided by decreasing energy intake and increasing energy expenditure by exercise. However, obesity is a much more complicated issue with weight reduction attempts being countered by biologic responses. Morbid obesity is most often not a personal choice but a complex disease. Genetic and environmental factors, diet, energy extraction, and active and basal energy expenditure play a role in determining body weight and levels of biomarkers in the blood.

The mechanisms responsible for becoming overweight and the development of obesity are not fully understood although several genetic and environmental factors have been identified (Tremaroli et al., 2015). Although several pharmacological agents are available for the treatment of obesity related metabolic disorders, only a fraction of patients respond to treatment (Erejuwa
et al., 2014). The increase in the prevalence of obesity and its metabolic derangements combined with the lack of efficacy in available treatments has led to an increase in demand for surgical treatment options.

Bariatric surgery, including vertical banded gastroplasty (VBG) and Roux en-Y gastric bypass (RYGB) are weight loss surgical interventions usually utilized in morbid obesity (Figure 1). Bariatric surgery promotes and sustains weight loss, improves insulin sensitivity, improves pancreatic β-cell function, reduces adiposity, improves diabetes mellitus and other metabolic parameters, as well as improves cardiovascular risk factors and reduces mortality rate (Erejuwa, 2014). The mechanisms by which bariatric surgeries reduce body weight and fat as well as improve metabolic parameters are not known. Major changes of gut microbial communities have been hypothesized to mediate part of the beneficial metabolic effects observed after RYGB. RYGB-induced metabolic and microbial modifications can contribute to weight loss and metabolic improvements. Performing a descriptive metagenomics analysis of the difference between RYGB, VBG and OBS patients (study 1) or from the differences at subsequent timepoints after RYGB (study 2) may shed light on the contribution of the microbiota to this improvement and also draw attention to any potential negative effects.

Several recent metagenomic studies have demonstrated that the gut microbiota is altered in obesity (Le Chatelier et al., 2013). Furthermore, the gut microbiota is not only associated with obesity but can also transfer the obese phenotype by transplantation in mice (Tremaroli et al., 2015) and increase insulin sensitivity in humans. However, little is known whether the microbiome composition is longitudinally altered after bariatric surgery.

RYGB leads to metabolic improvements and metabolic changes are associated with gut microbial changes. An important question is whether functional effects following RYGB may be
due to the alterations of the microbiome after RYGB surgery. Several mechanisms for the
influence of the gut microbiota on obesity have been proposed. Increased energy harvest by the
breakdown of otherwise indigestible carbohydrates to short chain fatty acids has been proposed
(Palleja et al., 2016). The gut microbiota interacts with the signaling and regulatory network of
the host and thereby regulates energy balance. The characterization of the function and
metabolism of the microbiota is a problem that has begun to be solved by the bioinformatics
analysis of metagenomics sequences. This study will primarily use the open source Metaphlan2
and HUMAnN2 bioinformatics pipelines along with other statistical methods using the open
source programming language R and available R packages to analyze differences in the
metagenomes from two studies. Data from Trermorali et. al will be used to explore the long-term
effects of bariatric surgery from patients 9 years after being randomized to RYGB or VBG
surgery compared to patients matched for weight and fat mass loss. A second study from Palleja
et. al will be used to investigate the metagenomic and phenotypic changes of 13 morbidly obese
patients examined before, three months and 1 year after RYGB.

Figure 1. Anatomical changes in gastrointestinal tract
Source: Kaska 2017.
(A) VBG sleeve gastrectomy
(B) Roux-en-Y gastric bypass - distal (C) Roux en Y gastric bypass - long limb
Chapter II

Methods

Shotgun metagenomic facilitates the sampling of genes from all organisms present in a complex sample. Additionally, bacterial diversity can be evaluated and the relative abundance of microbes maybe determined. Shotgun metagenomics also provides a means to study unculturable microorganisms that are otherwise difficult or impossible to analyze. Next generation metagenomic sequencing can detect microbial community members present in very low abundance where other methods are unable to detect low abundance organisms and their genetic content. For this analytic study, shotgun metagenomics data has been downloaded from two similar studies: (Tremaroli, 2015) which was deposited with the European Nucleotide Archive under accession number ENA:ERP009310 and (Palleja, 2016) which was deposited with the European Nucleotide Archive under accession number ENA:ERP014480.

DNA extraction, and metagenomic sequencing

The data set from ENA:ERP009310 (Tremaroli) consists of Genomic DNA extracted from fecal samples using a repeated bead beating procedure (Salonen et al., 2010) and sequenced on Illumina HiSeq 2000. In total, the data consists of 63 Gbp of paired-end reads, with an average of 3.0 ± 3.0 (SD) Gbp for each sample. As an additional quality control step prior to running analysis, the KneadData tool was run to ensure that the data consisted of high quality bacterial reads free from contaminants. The data was used to analyze the gut microbiota of weight-stable women 9 years after randomization to either RYGB or vertical banded gastroplasty (VBG) and matched for weight and fat mass loss (Tremaroli, 2015). The dataset also consists of
two groups of non-operated women with body mass index (BMI) matched to the patients’ pre-surgical BMI (OBS).

The data set from ENA:ERP014480 (Palleja) consists of Genomic DNA extracted as described above. Whole genome shotgun sequencing was performed on the 33 fecal samples using the Illumina HiSeq 2000 platform and paired-end sequencing method (2 × 100 bp). Reads were quality controlled, accepting only reads with a quality trimming cutoff of 20 and a minimum length of 30 bp (Palleja). Before being deposited with the ENA, DNA sequences were screened against the human genome (hg19) to remove contaminants. Once again, the KneadData tool was run on the deposited data to ensure high quality data.

Taxonomic profiling of fecal metagenomes

To perform phyla, genera and species profiling of the data, the MetaPhlAn2 bioinformatics pipeline was downloaded and used on a standard PC hosting a virtual machine running Linux. The PC used was a multicore processor with 16GB available memory which is considerably less than many other analysis platforms require. Furthermore, the virtual machine with the installed tools could be run on a high performance multicore cloud computing environment if necessary. MetaPhlAn2 relies on ~1M unique clade-specific marker genes identified from ~17,000 reference genomes. (Truong, 2015). By identifying clade specific marker genes and including only those in a reference database, Metaphlan2 provides a significant speedup compared to alignment to a full database of microbial genomes (Segata et al., 2012).

As an additional step, composition data was verified using MOCAT2 which is a different open source bioinformatics pipeline available from the European Molecular Biology Lab (EMBL). The phyla, genera and species of the composition of the data were calculated using
both MetaPhlAn and MOCAT. The output was then postprocessed or used as an initial screen in functional profiling as described below.

This study relies on aligning sequence data to reference databases; however, an overwhelming majority of microbes that compose these microbial communities are not yet characterized in detail to any great level. Reads aligned by Metaphlan2 belonging to clades with no available sequenced genomes are reported as an ‘unclassified’ subclade of the closest ancestor for which there is available sequence data. Results indicate that unclassified reads were uniform across subjects and should not skew interpretation of relative abundancies between subjects.

Functional profiling

The functional content of the data was explored using HUMAnN2 which was run on the standard PC hosting the virtual machine described above. Functional profiling answers the question what the microbes are doing or rather what they are capable of doing. HUMAnN2 uses the UniRef database to provide gene family definitions and MetaCyc to provide pathway definitions by gene family. HUMAnN2 relies on several other open source programs such as BowTie, Diamond and MinPath to compute the abundance of gene families and metabolic pathways present.

HUMAnN2 performs a series of alignment steps. The first aligns reads to a custom nucleotide database created from the species computed by Metaphlan2. Reads remaining unaligned after the first step undergo a translated search against a UniRef database. HUMAnN2 reports an abundance of unmapped reads which represents reads remaining unmapped after both steps. The level of unmapped reads could affect the significance of relative abundancies between subjects. However, results indicate a uniform level of unmapped reads across samples. When
attempting to reconstruct pathways, HUMAnN2 reports unintegrated for the reads which were unable to be assigned a pathway. Once again, this value was not significantly different between subjects.

Statistical analysis

The output from MetaPhlAn2 and HUMAnN2 was modified using utility scripts and then further analyzed by LEFSe to determine significant differences between RYGB, VBG, and OBS data for the data from the first data set while the differences between Baseline, 3Mo and 1Year were explored in the second study. LEfSe determines the features (in this case taxonomy, genes, or pathways) most likely to explain differences between groups by coupling standard tests for statistical significance with additional tests for effect relevance.

We performed further analysis of the output from MetaPhlAn2 and HUMAnN2 using the vegan and edgeR packages in R. To have a better overview of how taxonomic profiles changed between the we projected their values onto the principal component analysis (PCA) space. Groups RYGB, VBG and OBS were used in the first study while groups Baseline, 3Mo and 1Yr were used in the second study. Paired Wilcoxon rank sum tests were performed to analyze changes in phenotypic parameters between time points after surgery. Additionally, permutational multivariate analysis of variance (PERMANOVA) was used to assess the effects of the surgery on fasting insulin, fasting glucose, BMI, postprandial GLP-1, postprandial glucose and postprandial insulin. The R package edgeR was used to assess the differential abundance of gene ontologies (GOs) and computed pathways between groups in both studies. The read counts for each GO or pathway were loaded into R and the dispersion was calculated with the function estimateDisp(). For further analysis, the tagwise dispersion was used in the glmFit() function and
the resulting tags were used by plotSmear() to plot the fold change against abundance. The
topTags() function was used to select entries which were written to an output spreadsheet using
the R package xlsx. The R package mgcv was used to fit a generalized additive model (GAM)
between phenotypic parameters and the phyla principal component axis. The R package akima was
used to produce the contour lines overlaid on the plot as described in the vegan package
documentation.
Chapter III

Results

Phylogeny composition changes

From Study1, we observed significant differences in microbiota composition for RYGB versus OBS samples, but not for VBG versus OBS or RYGB versus VBG (Figure 2A). From study 2, we observed significant differences in microbiota composition for samples 3 months after RYGB versus Baseline samples, for samples 1 year after RYGB versus Baseline but not for 1 year post surgery versus 3 months (Figure 3A). Previous short-term studies have demonstrated that women who have undergone RYGB had increased abundance of Proteobacteria in comparison to non-operated severely obese women. Proteobacteria are generally not considered to be beneficial due to their pro-inflammatory properties (Palleja et al., 2016). For study 1, the results of Metaphlan2 analysis at the phyla level and post processed confirm that the level of Proteobacteria is increased in RYGB and VBG subjects compared to OBS subjects (Figure 2A). For study 2, the results confirm that the level of Proteobacteria increased in RYGB patients 3 months and 1 year after surgery compared to baseline (Figure 3A).
Figure 2. Long-term Effects of Bariatric Surgery on Phyla Composition
(A) Histogram of phyla relative abundance for RYGB, VBG, and OBS
(B) PCA grouping on phyla
(C) PCA labeled grouping on phyla

We visualized the changes in overall gut microbial phyla composition induced by RYGB using a principal component analysis of the relative phyla abundances (Fig. 2B), which showed separation between OBS patients relative to RYGB and OBS patients in study 1. For study 2, there was a clearer separation between baseline relative to 3 months and 1 year post RYGB
surgery. The clearer separation in study 2 could be the result of the shorter duration after surgery or could be the result of following the same patient between groups.

Figure 3. 1 Year Effects of RYGB Surgery on Phyla Composition
(A) Histogram of phyla relative abundance for Baseline, 3Mo and 1Yr
(B) PCA grouping on phyla
(C) PCA labeled grouping on phyla

We labeled the PCA plots with patient IDs (Figures 2C, 3C) to help determine the patients which failed to separate from others. From study 2 (Figure 3C) several patients failed to show differentiation between baseline and 3 months.
At the genus level compared to the phyla level, the separation between RYGB or VBS vs OBS (Figure S1B) or for 3M vs Baseline (Figure S3B) was less clear. In particular, several patients failed to show separation after RYGB vs Baseline (Figure S3C). Several facultative anaerobes in the Proteobacteria (e.g., Escherichia, Klebsiella, and Actinomyces) were present at increased relative abundance in RYBG vs OBS patients (Table S1). The same facultative anaerobes from Proteobacteria had an increased relative abundance in 3 Months vs Baseline (Table S2). Additionally, other facultative anaerobes had an increased relative abundance the most noteworthy from the Clostridium genus.

We observed significant differences in microbiota species composition for RYGB versus OBS samples, and for VBG versus OBS (Figure S2A). The abundance of 10 species differed significantly between RYGB and OBS samples. In particular, the levels of several species in the Gammaproteobacteria class (*E. Coli, K. pneumoniae*) were higher while the levels of two species in the Firmicutes phylum (Eubacterium rectale, Clostridium bartletti) were lower in RYGB versus OBS samples (Figure S5A). Both RYGB and VBG patients showed significant increases in *E. coli* compared to OBS patients which could be the result of the surgical bypass of a large section of acid producing stomach by both procedures. VBG versus OBS, showed a decreased level of another species in the Firmicutes phylum (Roseburia intestinalis) as well as a similar shift to Gammaproteobacteria (Figure S5B). Additionally, the increase in Proteobacteria, including *E. coli, K. pneumoniae*, and *E. faecalis*, might result from a higher presence of oxygen in distal parts of the gut due to the surgical rearrangements as reported previously (Graessler, 2013). Intriguingly, other studies have found that a shift to Proteobacteria as well as the decline of butyrate producing Firmicutes, like *E. rectale* could have long-term effects on host health with the potential risk of bowel inflammation and colorectal carcinomas.
We also observed significant differences in microbiota species composition after RYGB surgery versus baseline (Figure S4A). We detected over 30 species that changed their relative abundance within the first 3 months (SFigure 6) and nearly 30 species that changed their relative abundance between baseline and 1 year (SFigure 7). Once again, we detected several species in the Gammaproteobacteria class (*E. Coli, K. pneumoniae* and others) with higher relative abundance at both time points post-surgery. We also observed the decline of the butyrate producing Firmicute, Faecalibacterium prausnitzii, three months (SFigure 6) but not 1 year post surgery while the level of the butyrate producing species Clostridium bartletti increased at 1 year but not 3 months post-surgery (SFigure 7). Interestingly, both studies showed a relative decrease in the *C. bartletti* species after surgical procedures. Recent studies have linked *C. bartletti* with inflammation in addition to being associated with autism in children (Autism speaks grant, 2016). In addition, we saw an increase in several oral microbiota, such as Streptococcus species., *F. nucleatum* and *B. dentium,* post-surgery. Other studies have postulated that a decrease in acid secretions could make the gastric barrier less stringent for oral microbiota species. The number of oral microbiota species present declined between 3 months and 1 year and showed no difference in study 1 which was 9 years after surgery between groups. One possibility is that the initial conditions allowing early colonization of oral bacteria are offset by other time dependent factors.

**Phenotypic changes after RYGB**

Gut microbial compositional changes occurred within short-term (within 3 months) or long-term following RYGB using samples collected 3 months and 1 year after RYGB. We sought to analyze the per patient phenotypic data available for this study and additionally divided
the analysis based on whether the patient had a pre-surgery diagnose as Type 2 Diabetes (T2D, 7 of 13 patients), Impaired Glucose Tolerance (IGT, 1 of 13) or Normal Glucose Tolerance (NGT, 5 of 13). To facilitate statistical analysis, we combined T2D and IGT into the group IT2D (8 of 13 patients) and plotted the changes in phenotypic outcomes between baseline, 3 months and 1 year after RYGB surgery for both IT2D and NGT groups (Figure 5).

To test significant difference, we performed the Wilcoxon paired-signed test between baseline metabolic measurements and 3 months post-surgery. Fasting glucose (p=0.016), fasting insulin (p=0.008), BMI (p=0.008), postprandial AUC glucose (p=0.016), and postprandial AUC GLP.1 (p=0.016) showed significant improvement between baseline and 3 months while only postprandial AUC insulin (p=0.937) did not show significant improvement. There was not enough statistical power to continue the analysis to 1 year post-surgery, however when combining all patients and normalizing by the number of months post-surgery the metabolic improvements were shown to have occurred predominantly within the first 3 months (data not shown).

We next sought to analyze the distribution of several phenotypic outcomes along the PCA ordination axes previously discussed. A Generalized Additive Model (GAM) was used to fit fasting glucose to the first two PCA coordinates (SFigure 5) and a GAM was used to fit AUC GLP.1 to the first two PCA coordinates (SFigure 6). Contour lines were drawn using the R package vegan for both analysis. SFigure6 shows separation between PCA variables between timepoints generally leads to an improved GLP.1 outcome. Previous studies have shown that GLP-1 increase may help improve and resolve diabetes based on the mechanism in which this hormone conveys improvements on glucose metabolism (Rhee 2012).
Figure 4. Metabolic improvements after RYGB surgery
Type 2 Diabetes (n=7) and Impaired Glucose Tolerant (n=1) patients grouped into IT2D (n=8) and compared with Normal Glucose Tolerant (NGT, n=5) patients. P Values for paired Wilcoxon rank sum tests.

Microbial gene content changes

To explore the genetic content of the gut microbiome after bariatric surgery, we used the HUMAnN2 pipeline to align reads in 2 phases to determine the relative abundance of genes aligning to the UniRef database. The UniRef aligned reads were remapped to produce the relative abundance of Gene Ontology (GOs) present. We observed differences in the genetic content of the microbiome of RYGB (Figure 5A) and VBG patients (Figure 5B) compared to OBS patients but relatively few differences when comparing RYGB and VBG patients (Figure 5C). 1218 GOs were enriched while 19 were depleted in RYGB versus OBS samples (Figure 5A, Table 3-Sheet1). 954 GOs were enriched while 23 were depleted in VBG versus OBS samples (Figure 5B, Table 3-Sheet2). Remarkably, only 44 GOs were enriched and 46 found depleted in RYGB versus VBG samples (Figure 5C, Table 3-Sheet3).

We also observed differences in the genetic content of the microbiome of RYGB patients 3 months (Figure 6A) and 1 year post-surgery (Figure 6B) compared to same patients before surgery but relatively few differences when comparing RYGB patients 3 months versus 1 year post-surgery (Figure 6C). 1661 GOs were enriched while 82 were depleted in RYGB 3 months versus baseline samples (Figure 6A, Table 4-Sheet1). 1583 GOs were enriched while 49 were depleted in RYGB 3 months versus baseline samples (Figure 6B, Table 4-Sheet2). As expected, only 25 GOs were enriched and 36 found depleted in 1 month versus 3 month samples (Figure 6C, Table 4-Sheet3).
To explore the changes in functional potential of the microbiome after bariatric surgery we determined the relative abundance of MetaCyc pathways in each sample by using the HUMAnN2 pipeline. We observed differences in the genetic content of the microbiome of RYGB (Figure SA) and VBG patients (Figure SB) compared to OBS patients but relatively few differences in the genetic content of the microbiome of OBS patients compared to VBG patients.

Figure 5. Gut Microbiome Gene Ontology (GO) changes of RYGB and VBG Women in Compared to OBS

Gene level differences in the microbiomes measured by the abundance of GOs. Each spot represents a GO, and red spots represent GOs whose abundance is significantly different. The number of GOs significantly increased (up) and depleted (down) is shown under each plot. The direction of change is defined as up being the first class in the title above and down being the second title. FC, fold change; CPM, counts per million. See Table 1 for complete list of differential GO changes from this study.

(A) RYGB vs OBS (B) VBG vs OBS (C) RYGB vs VBG
differences when comparing RYGB and VBG patients (Figure S10C). 114 Pathways were
enriched while 18 were depleted in RYGB versus OBS samples (Figure S10A, Table 5-Sheet1).
93 pathways were enriched while 3 were depleted in VBG versus OBS samples (Figure S10B, Table 5-Sheet2).

Figure 6. Gut Microbiome Gene Ontology (GO) changes of RYGB subjects
at two Timepoints in Comparison to Baseline
Gene level differences in the microbiomes measured by the abundance of GOs. Each spot
represents a GO, and red spots represent GOs whose abundance is significantly different. The
number of GOs significantly increased (up) and depleted (down) is shown under each plot. The
direction of change is defined as up being the first class in the title above and down being the second title. FC, fold change; CPM, counts per million. See Table 2 for complete list of differential GO changes from this study.

(A) 3Mo vs Baseline (B) 1Yr vs Baseline (C) 1Yr vs 3Mo

Only 2 pathways were enriched and 0 found depleted in RYGB versus VBG samples (Figure S10C, Table 5-Sheet3).

We also observed differences in the microbiome MetaCyc pathways of RYGB patients 3 months (Figure S11A) and 1 year post-surgery (Figure S11B) compared to same patients before surgery but relatively few differences when comparing RYGB patients 3 months versus 1 year post-surgery (Figure S11C). 179 pathways were enriched while 90 were depleted in RYGB 3 months versus baseline samples (Figure S11A, Table 6-Sheet1). 161 pathways were enriched while 77 were depleted in RYGB 3 months versus baseline samples (Figure S11B, Table 6-Sheet2). As expected, no pathways were enriched (FDR<0.05) and only 7 pathways were enriched and 9 found depleted (less stringent PValue<0.05) in 1 month versus 3 month samples (Figure S11C, Table 6-Sheet3).

Microbial functional potential changes

Carbohydrate phosphotransferase systems (PTS) catalyze the transport and phosphorylation of numerous monosaccharides, disaccharides, amino sugars, polyols, and other sugar derivatives (Deutschcer, 2006). We observed numerous PTS systems which were enriched in RYGB versus OBG (Table 3-Sheet1, Figure Figure8A) as well as VBG compared to OBS (Table 3-Sheet 2) indicating an increase potential for carbohydrate energy metabolism. We also observed numerous ATP-binding Cassette (ABC) transporters used to transport many amino acids and their derivatives, particularly lysine/arginine/ornithine, histidine, and putrescine in
RYGB compared to OBS patients (Table 3-Sheet1, Figure 8B). Additionally, we also observed an increased potential for ABC transport of iron, vitamin B12, thiamine, manganese, iron, and zinc (Figure 8C). We also observed increases in ABC transporters in VBG compared to OBS patients but the increase in putrescine transporters did not reach significance (Table3-Sheet2). We also observed an increase in putrescine metabolism pathways (Table5-Sheet1, Figure 7D) present in RYGB vs OBS and an increase in a subset of the putrescine pathways when comparing VBG vs OBS. The increase in PTS systems and ABC transporters was also seen when comparing patients 3 months and 1 year post RYGB compared to baseline although the increase tended to be less significant in patients after 1 year (Table4-Sheet1, Table4-Sheet2, Table6-Sheet1, Table6-Sheet2).

Bacteria use two-component sensory systems to sense and respond to changes in the surrounding environment (Palleja et al., 2016). Other studies have found that the two component systems responding to nitrogen availability, phosphoglycerate transport, and short-chain fatty acid (SCFA) metabolism were specifically enriched in RYGB compared to OBS patients (Palleja et al., 2016; Tremaroli et al., 2015). We observed numerous examples of increased two-component systems both in RYGB versus OBS and VBG versus OBS microbiomes as well as patients both 3 months and 1 year post surgery. (Table3, Table5, and Figure 8E – Study 2 shown)

We also observed an increase in fatty acid transport systems and fatty acid metabolism pathways present (Table 3, Table 5, and Figure 8F,8H) when comparing RYGB versus OBS patients and VBG versus OBS patients. We also saw significant increases in fatty acid transport and metabolism when comparing RYGB patients post-surgery versus baseline. Short chain fatty acids (SCFA) are produced when fiber is fermented in the colon and we detected an increase in
Short chain fatty acid (SCFA) transport genes and pathways. We also observed an increase in
Figure 7. Gut Microbiome Pathway changes after RYGB
Functional differences in the microbiomes measured by the abundance of MetaCyc pathways. Histogram of the LDA scores computed for pathway differentially abundant between RYGB and OBS patients.

Trimethylamine-N-oxide reductase Trimethylamine-N-oxide reductase (TMAO) in RYGB patients in both studies but not in VBG patients. We also detected an increase in bile acid catabolism in RYGB patients in both studies but not a significant increase in VBG patients after adjusting for FDR. We also observed a significant increase in a number of aerobic respiration pathways (Table 3, Table 5, and Figure 8G) in RYGB patients in both studies and also for VBG patients.
Chapter IV
Discussion

Differing effects of compositional changes

Several previous studies have shown that the regulation of energy and fat storage is effected by intestinal microbes may contribute to obesity and its complications (Karlsson et al., 2013; Qin et al., 2012). Obesity has been associated with reduced microbial diversity and reduced gene richness (Le Chatelier et al., 2013) while dietary weight loss interventions have been shown to shift genetic content and microbiota composition toward that of lean individuals (Cotillard et al., 2013). At the phyla level, others studies have reported a change in the Bacteroidetes/Firmicutes ratio and we observed no significant change in the ratio in study 1 but a decreased ratio in study 2. One possible explanation for this difference is that patients from study 2 started with a higher
Figure 8. Examples of functional potential changes between patients
Taken from both studies, RYGB vs OBS and RYGB vs baseline
(A) Carbohydrate PTS (RYGB vs OBS)
(B) Putrescine ABC transporter (RYGB vs OBS)
(C) Zinc ABC transporter (RYGB 3MO vs Baseline)
(D) Putrescine/SCFA metabolism (RYGB 3MO vs Baseline)
(E) Two Component System (RYGB 3MO vs Baseline)
(F,H) Fatty acid metabolism (RYGB vs OBS) and (RYGB 3MO vs Baseline)
(G) Aerobic respiration (RYGB 3MO vs Baseline)
ratio at baseline possibly because some patients (n=7 of 13) were taking Metformin at baseline which has been shown to alter the microbiota composition (Forslund, 2015). Specifically, a large proportion of Prevotella was observed from baseline patients in study 2 while study 1 had an overall lower proportion. Interestingly, VBG patients were observed with a low level of Prevotella compared to both groups. Prevotella has been observed to have a bimodal distribution and an open question is what factors caused the difference in VBG patients. From study 2, we observed a significant decrease in F. prausnitzii which is known for beneficial effects on host metabolism and the production of butyrate. The relative amount of F. prausnitzii was seen to increase by 1 year and was no longer significantly different than baseline levels. The F. prausnitzii levels in RYGB and VBG patients were not significantly different than OBS patients and RYGB patients actually showed an higher level 9 years after surgery. One possibility is that RYGB patients initially show a decline but that level recovers over time. Alternatively, the number of patients taking Metformin in study 2 may have confounded the result. Other studies have suggested a beneficial effect of Bifidobacterium species in the improvement of obesity-related metabolic parameters. However, The Bifidobacterium genus, however, is complicated with other studies showing differential species response. We also saw a lower proportion of species from the Bacteroides genus, which has been associated with a proinflammatory status (Boulangé, 2016), when comparing RYGB to OBS or baseline. However, VBG patients had a higher proportion of Bacteroides spp. compared to OBS.

The most significant phylogenetic alteration, observed in both studies, took place in the increase of Proteobacteria after bariatric surgery. This increase agrees with most studies and is interesting because Proteobacteria species such as E. Coli are not usually detected in healthy humans or rodents. Furthermore, E. Coli has been associated with inflammatory diseases of the
intestinal tract such as Colitis and negative metabolic outcomes. However, the *E. coli* species itself is complex with many differences between strains and a modification to *E. coli* Nissle 1917 protected mice against high-fat diet induced obesity and inflammation (Chen 2014). In addition, a recent study found gut *E. coli* proteins activated host satiety pathways after growth (Breton 2016). Additional studies have also shown that different strains of *E. Coli* maybe beneficial in fighting inflammation and positively affect metabolic outcomes.

**Metabolic potential differences are widespread**

We observed an increase in a number of facultative anaerobes, such as *E. coli*, and microbial pathways involving aerobic respiration in agreement with increased oxygen levels consistent with the shortening of the small intestine after surgery. We also saw an increase in glutathione transfer and metabolism (Table 3, Table 4). Glutathione is known for its antioxidant properties and its ability to counter the potential negative effects of reactive oxygen species and has been studied in the use of probiotics. Increased glutathione metabolism is consistent with the possible benefit of higher levels of Proteobacteria after RYGB surgery.

The increase in putrescine metabolism may represent the result of incomplete protein digestion in the stomach and small intestine after bariatric surgery although other studies have not detected problems with protein digestion. Putrescine may also be synthesized by bacteria and we found evidence for the transport, synthesize and metabolism of putrescine. We observed an increase in pathways which degrade putrescine to succinate and produce gamma-aminobutyric acid (GABA) as a byproduct. GABA is known to act on receptors in the hypothalamus to promote satiety and additionally promote an increase in GLP-1 which forms a closed loop by promoting the formation of GABA by pancreatic beta-cells (Palleja, 2016).
Future direction

The modelling done on same patient data over multiple time points was limited because of the statistical problems in working with a small number of samples. Larger studies with more participants and at different time increments after surgery would aid in the development of predictive models. Additional metabolic data, such as GABA levels, at these additional time points would also help characterize the adaptation of the gut microbiota to RYGB and VBG and help elucidate their role in metabolic improvements and raise concerns about and possible negative effects. Additional strain level characterization of species, especially *E. coli*, may reveal the contribution of *E. coli* to positive metabolic outcomes after bariatric surgery.
Terms and Abbreviations

“BLAST”: Basic Local Alignment Search Tool. Bioinformatics alignment tool.
“BMI”: Body mass index
“COG”: Cluster of orthologous groups
“CVD”: Cardiovascular disease
“Descriptive metagenomics”: the study of microbial relative abundance to determine structure and variation of the microbiome.
“DIAMOND”: Bioinformatics software for aligning translated DNA query sequences against a protein reference database. Functionally similar to BLAST but roughly an order of magnitude faster.
“Functional metagenomics”: the study of host–microbe and microbe–microbe pathway interactions to predict correlations between the microbiome and host phenotype.
“HbA1c”: Glycated hemoglobin
"HMP": Human microbiome project
“HUMAnN”: HMP Unified Metabolic Analysis Network. Bioinformatics pipeline used to analyze metagenomics sequence data.
“KEGG”: Kyoto Encyclopedia of Genes and Genomes
“KO”: KEGG orthology
“Metagenome”: all genetic material from the genomes of many individual organisms within a sample.
“Metagenomic sequencing”: the high-throughput sequencing of metagenome using next-generation sequencing technology.
“Metagenomics”: the study of genetic material sampled directly from environmental samples.
“Microbiome”: community of microorganisms living on or in a living organism.
“MOCAT”: Metagenomic gene assembly and prediction pipeline which can also be used to perform descriptive and functional metagenomics.
“OBS”: Obese (refers to a group of individuals with a high Body Mass Index)
“PCA”: Principal component analysis
"RYGB": Roux en-Y gastric bypass surgery
“T2D”: Type 2 diabetes
“TMA”: Trimethylamine
“TMAO”: Trimethylamine N-oxide
“VBG”: Vertical banded gastroplasty
SFigure 1. Long-term Effects of Bariatric Surgery on Genus Composition
(A) Histogram of genus relative abundance for RYGB, VBG, and OBS
(B) PCA grouping on genus
(C) PCA labeled grouping on genus
Figure 2. Long-term Effects of Bariatric Surgery on Species Composition
(A) Histogram of species relative abundance for RYGB, VBG, and OBS
(B) PCA grouping on species
(C) PCA labeled grouping on species
Figure 3. 1 Year Effects of RYGB Surgery on Genus Composition
(A) Histogram of genus relative abundance for Baseline, 3Mo and 1Yr
(B) PCA grouping on genus
(C) PCA labeled grouping on genus
SFigure 4. 1 Year Effects of RYGB Surgery on Species Composition
(A) Histogram of species relative abundance for Baseline, 3Mo and 1Yr
(B) PCA grouping on species
(C) PCA labeled grouping on species
SFigure 5. Differential species changes after Bariatric Surgery
(A) Histogram of RYGB vs OBS
(B) Histogram of VBG vs OBS
(C) Histogram of RYGB vs VBG
Figure 6. Differential species changes 3 Months after RYGB Surgery
LDA significant differences in species composition between baseline and 3 Months after RYGB
SFigure 7. Differential species changes 1 Year after RYGB Surgery
Significant differences in species composition between baseline and 3 Months after RYGB
SFigure 8. Fasting Glucose modeled on Phyla PCA
Patients labelled on Phyla PCA and fasting glucose model fit to PCA parameters and contour lines drawn to model.
Figure 9. Postprandial AUC GLP-1 modeled on Phyla PCA
Patients labelled on Phyla PCA and postprandial GLP-1 model fit to PCA parameters and contour lines drawn to model.
SFigure 10. Gut Microbiome MetaCyc pathway changes of RYGB and VBG Women in Compared to OBS

Functional differences in the microbiomes measured by the abundance of MetaCyc pathway. Each spot represents a pathway, and red spots represent pathways whose abundance is significantly different. The direction of change is defined as up being the first class in the title above and down being the second title. FC, fold change; CPM, counts per million. See table 3 for list of pathways differentially changed.

(A) RYGB vs OBS (B) VBG vs OBS (C) RYGB vs VBG
SFigure 11. Gut Microbiome MetaCyc pathway changes of RYGB subjects at two Timepoints in Comparison to Baseline

Functional differences in the microbiomes measured by the abundance MetaCyc pathways. Each spot represents a pathway, and red spots represent pathways whose abundance is significantly different. The number of pathways significantly increased (up) and depleted (down) is shown under each plot. The direction of change is defined as up being the first class in the title above and down being the second title. See table 4 for list of pathways differentially changed.

(A) 3Mo vs Baseline  (B) 1Yr vs Baseline  (C) 1Yr vs 3Mo
Figure 12. Gut Microbiome Pathway changes after VBG
Functional differences in the microbiomes measured by the abundance of MetaCyc pathways. Histogram of the LDA scores computed for pathway differentially abundant between VBG and OBS patients.
Figure 13. Gut Microbiome Pathway Differences RYGB vs VBG
Functional differences in the microbiomes measured by the abundance of MetaCyc pathways. Histogram of the LDA scores computed for pathway differentially abundant between RYGB and VBG patients.
SFigure 14. Gut Microbiome Pathway Differences RYGB 1Year vs Baseline

Functional differences in the microbiomes measured by the abundance of MetaCyc pathways.

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Histogram of the LDA scores computed for pathway differentially abundant between baseline and 1 year post surgery RYGB patients.

SFigure 15. Gut Microbiome Pathway Differences RYGB 1Year vs 3Mo
Functional differences in the microbiomes measured by the abundance of MetaCyc pathways. Histogram of the LDA scores computed for pathway differentially abundant between 3 month and 1 year post surgery RYGB patients.
Table 1. Genus Differences between Baseline and 3 Months

<table>
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<tr>
<th>Genus</th>
<th>LDA</th>
<th>MeanAbund</th>
<th>KW_Wilcox</th>
<th>Class</th>
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Table 2. Genus Differences between OBS and RYGB

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<th>KW_Wilcox</th>
<th>Class</th>
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Table 3. Gene Ontology Differences RYGB, VBG, OBS

attached as Study1.GO.xlsx

Table 4. Gene Ontology Differences Baseline, 3Mo, 1Year post RYGB

attached as Study2.GO.xlsx

Table 5. Pathway Differences RYGB, VBG, OBS

attached as Study1.Pathway.xlsx

Table 6. Pathway Differences Baseline, 3Mo, 1Year post RYGB.

attached as Study2.Pathway.xlsx
References


