DARLENE ANA SOUZA DUARTE

DEPICTING RESIDUAL FEED INTAKE IN NELLORE CATTLE THROUGH GENE EXPRESSION, LIPIDOMIC PROFILING AND PATHWAY-BASED META-ANALYSIS

Thesis presented to the Animal Science Graduate Program of the Universidade Federal de Viçosa, in partial fulfillment of the requirements for degree of *Doctor Scientiae.*

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SUMMARY

ABSTRACT

DUARTE, Darlene Ana Souza, D.Sc., Universidade Federal de Viçosa, February, 2019. **Depicting residual feed intake in Nellore cattle through gene expression, lipidomic profiling and pathway-based meta-analysis.** Adviser: Fabyano Fonseca e Silva.

Increase in feed efficiency result in decrease in feed costs. Thus, to improve profitability in beef cattle production system is necessary to select animals more efficient in the use of feed sources. The residual feed intake (RFI) has become the preferable measure for feed efficiency, once that RFI is not correlated with growth rate or other production trait. Researchers have demonstrated that many physiological mechanisms contribute to variation in RFI, among them are protein turnover, tissue metabolism, synthesis and transportation of fatty acid, inflammatory response, glucose absorption and fatty acid oxidation. Even so, the molecular bases controlling this trait are not well elucidate yet. Thus, we aimed to investigate the biological bases that could be responsible for differences in animals classified for RFI. For that, we performed a pathway meta-analysis approach using several genome association studies results to search for significant pathways that may explain the genetic mechanism underlying this trait. We used an efficient permutation hypothesis test that takes into account the linkage disequilibrium patterns between SNPs. One significant pathway (valine, leucine and isoleucine degradation) related to RFI was found. This pathway is related to protein turnover and gluconeogenic process. In addition, we evaluated samples from muscle and liver tissue of 27 Nellore steers, classified as high, medium and low RFI. The samples were used to evaluate the mRNA expression of genes involved in the lipid metabolism and to carry out lipidomic analysis. None significant differences in mRNA expression were found. In the lipidomic analysis, it was found that animals from the high RFI group exhibited higher levels of fatty acids and triglycerides compared to medium and low RFI animals, indicating a possible increase in the requirement of lipid energy sources in the low RFI animals and also an increase in fat deposition in high RFI animals. In addition, it was observed differences in the lipid main components of cell membrane, which are related with cell signaling and

molecules transportation. These results suggest that difference in RFI is associated with changes in lipid metabolism, but these changes are not caused by differences in the mRNA expression of the genes involved in this metabolism.

RESUMO

DUARTE, Darlene Ana Souza, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Caracterização do consumo alimentar residual em bovinos Nelore através de expressão gênica, do perfil lipodômico e meta-análises para vias metabólicas**. Orientador: Fabyano Fonseca e Silva.

O aumento na eficiência alimentar resulta em diminuição no custo de alimentação. Assim, para melhorar a rentabilidade do sistema de produção de bovinos é necessário selecionar animais com maior eficiência alimentar. O consumo alimentar residual (CAR) tornou-se a medida preferencial para eficiência alimentar, uma vez que o CAR não está correlacionado com a taxa de crescimento. Pesquisadores demonstraram que muitos mecanismos fisiológicos contribuem para a variação do CAR, entre eles a o *turnover* proteico, metabolismo dos tecidos, síntese e transporte de ácidos graxos, resposta inflamatória, absorção de glicose e oxidação de ácidos graxos. Mesmo assim, as bases moleculares que controlam essa característica ainda não estão bem elucidadas. Assim, objetivamos investigar as bases biológicas que poderiam ser responsáveis por diferenças na classificação de animais quanto ao CAR. Para isso, realizamos uma meta-análise para vias metabólicas usando vários resultados de estudos de associação genômica com o intuito de procurar vias que possam explicar o mecanismo de controle dessa característica. Uma via significativa (degradação de valina, leucina e isoleucina) relacionada ao CAR foi encontrada. Esta via está relacionada ao *turnover* proteico e a gliconeogênese. Além disso, foram avaliadas amostras de tecido muscular e hepático de 27 novilhos da raça Nelore, classificadas como alto, médio e baixo CAR. As amostras foram utilizadas para avaliar a expressão de mRNA de genes envolvidos no metabolismo lipídico e para realizar análises lipidômicas. Não foram encontradas diferenças significativas na expressão de mRNA. Na análise lipidômica, verificou-se que os animais do grupo alto CAR apresentaram maiores níveis de ácidos graxos e triglicerídeos em comparação aos animais de médio e baixo CAR, indicando um possível aumento no uso de fontes de energia lipídica nos animais de baixo CAR e um aumento na deposição de lipídeos nos animais de alto CAR. Além disso, foram observadas diferenças nos principais componentes lipídicos da membrana

celular, que estão relacionados com a sinalização celular e com o transporte de moléculas. Estes resultados sugerem que a diferença no CAR está associada a alterações no metabolismo lipídico, porém essas alterações não são causadas por diferenças na expressão de mRNA dos genes envolvidos neste metabolismo.

Chapter 1

1.1 General Introduction

1.1.1 Feed efficiency

In the current beef production system, efficiency is a key factor and it is under influence of several factors such animal production cost, genetics, environmental conditions and animal health and welfare. Feed cost is the most economically important item within beef cattle production systems (Ramsey et al., 2005). Feed intake depends on several components; among them are the physiological stage, birth weight, food quality and others.

Gibb and McAllister (1999) reported that improvement in feed efficiency could have a higher economic impact than improvement in the average daily gain rate. Thus, a way to improve profitability in beef production is the selection for animals more efficient in the use of feed. In other words, animals that are capable to produce more by consuming less.

To select animals more efficient, some measures were developed to compare individuals. There are several measures of feed efficiency; Archer et al. (1999) review some of them. In beef cattle, the measure most used is the feed conversation rate (FCR), which is obtained by the ratio of the total feed consumption and weight gain. The inverse of FCR, the ratio of weight gain and total feed consumption, have also been used and is denominated feed efficiency. However, some studies have shown that FCR is highly correlated to weight gain; and can lead to a substantial increase in maintenance energy and mature size if used as a selection criterion (Archer et al., 1999; Bishop et al., 1991, Herd and Bishop, 2000).

The residual feed intake (RFI) is a measure of feed efficiency that have become an alternative to FCR. RFI was proposed by Koch et al. (1963), it is defined as the difference between observed intake (of dry matter or energy) and the intake estimated by regression of the average metabolic live weight and the weight gain over the consumption. In other words, RFI is the difference between the observed and the predict intake considering the requirements for

maintenance and the production level. Animals that are more efficient have negative RFI values and a lower observed than predicted consumption, unlike, animals less efficient have positive RFI values and an observed consumption greater than predicted. According to Archer et al. (1999), RFI is by definition phenotypically independent of the production traits used to calculate expected feed intake and allows comparison between individuals from different production levels. These are advantages of RFI over the FCR.

Several studies have been demonstrated that RFI presents moderate to high values of heritability (Hoque et al. 2009; Arthur et al. 2001) and, therefore, can be explored in the identification and selection of animals genetically superior in relation to the use of feed.

The selection for residual feed intake is even more relevant when considering not only the economic impacts of decreasing feed intake but also the environmental impact of meat production. Nkrumah et al. (2006) reported significant differences in methane emissions among low and high RFI animals, with the first (more efficient) producing 28% less methane than the less ones (high CAR). The results reported by Hegarty et al. (2007) have confirmed that identification and selection of animals based on the genetic value for RFI provides a mechanism to reduce enteric methane emissions without compromising animal productivity.

1.1.2 Physiological basis for residual feed intake

According to Herd et al. (2004), variation in residual feed intake must be due to differences in biological process. These authors point out five major process affecting RFI variation: i) feed intake; ii) feed digestion; iii) metabolism; iv) activity and v) thermoregulation (Figure 1).

As feed intake increase, the energy expended to digest the feed increases. The amount of energy demand for maintenance of the digestive organs increases due to change in these organs size. It was demonstrated that animals less efficient spend more time ingesting food and go more often to the trough than animals more efficient (Mantanholi et al., 2010; Guimarães et al., 2017).

Besides that, increase in feed intake relative to maintenance causes decrease in digestion of feed (Herd et al., 2004). These authors affirm that animals more efficient have better capacity of digestion. Richardson et al., (2004) obtained a correlation between digestibility with RFI (r= - 0.44), which indicated that digestibility accounted for 19% of the phenotypic variation in RFI.

Activity also shows relation with RFI, some studies in chickens, mouse and pigs have been showing that animals more efficient are more active (Luiting et al., 1991; Hughes et al., 1997).

Lastly, Richardson et al., 2004 demonstrated that the proportion of lean and fat deposition differs between animals selected for RFI, their results shows that body composition is responsible for 5% variation in RFI. Differences in protein turnover, creatinine and calpastatin levels, and mitochondria proteins were observed in animals selected for high feed efficiency, suggesting several possible variation in metabolism affecting RFI (Herd et al., 2004, Kolath et al., 2006, Bottje and Carstens, 2009).

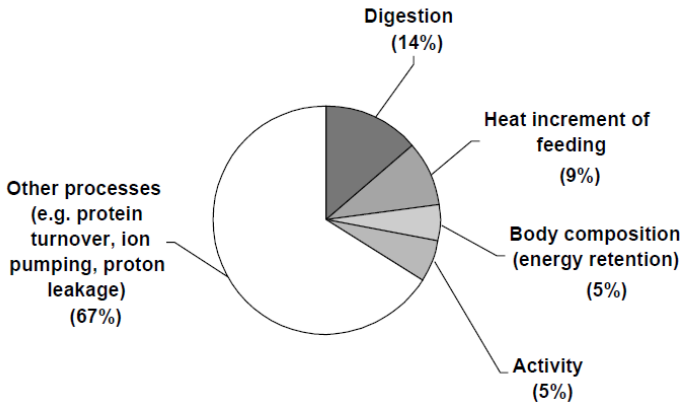

Figure 1 Percentage of mechanism contribution to residual feed intake variation (Herd at al., 2004)

1.1.3 Molecular basis for residual feed intake

Beyond the physiological mechanism underpinning variation in residual feed intake, it is important to know the genetic control of RFI variation. Many studies have been performed in order to understand the genes roles in residual feed intake. Nkrumah et al., (2007), performing a study in four beef cattle breeds, found out QTL in chromosomes 1, 5, 7, 8, 12, 16,17 e 26 for residual

feed intake. Rolf et al., (2011) carried out a genome wide association studies (GWAS) and found that 66 SNPs (single nucleotide polymorphism) explain about 62% of additive genetic variation for RFI. Santana et al. (2014) performed a GWAS in Nellore cattle and showed significant SNPs for RFI and dry matter intake near to genes related with appetite regulation and ion transportation (POMC, CART and ZNF804B for example).

In order to find genes associated with residual feed intake, Karisa el at. (2014) performed association studies and metabolomics analysis. The results showed that the genes and metabolites were associated with some biological process as lipid and steroid biosynthesis, protein and carbohydrate metabolism and with the AMPK pathway.

Alexandre et al. (2015) in a transcriptomic analysis of hepatic tissue carried out in Nellore animals, observed differences in gene expression between the high and low RFI animals. These genes (NR0B2, SOD3, RHOB, bta-mir-2904-3, ENSBTAG00000038430, CYP2E1, GADD45G, FASN) were especially related to fat metabolism, fatty acid synthesis and inflammatory response.

In other study evaluating the mRNA expression profiling of hepatic tissue of Nellore animals, Tizioto et al. (2015) showed that efficient animals have higher glucose uptake and less expression of genes responsible for intracellular transport of fatty acids.

Animals divergently selected for RFI of Angus breed, were also evaluated by global gene expression by Chen et al. (2011). The genes found in this study were involved in cellular growth and proliferation, cell signaling, drug metabolism, protein synthesis, lipid metabolism, and carbohydrate metabolism.

Altogether, this studies shows that several genes are responsible for variation in residual feed intake. Furthermore, these genes are related with protein and lipid metabolism, intracellular transportation and carbohydrate metabolism. Thus, it becomes important to understand the role of the metabolism of lipid and energy in animals with low and high RFI.

1.1.4 AMPK signaling pathway

The adenosine monophosphate-activated protein kinase (AMPK) is a key protein complex for cellular energy control. The AMPK pathway has been implicated in regulation of diverse metabolic pathways by acting on both inhibition and activation of genes and enzymes (Carling et al., 2011). Among the processes that genes from the AMPK pathway regulate are the absorption of glucose, glycolysis and absorption and oxidation of fatty acids.

The AMPK is formed by three subunits, one catalytic subunit (α) and two regulatory subunits (β) and (γ). Different genes encode these subunits, with the possibility of different combinations. AMPK is sensitive to differences in cellular energy balance, changes in the AMP / ATP ratio are responsible for the activation of AMPK.

Once activated, AMPK interferes in metabolism through two main ways, increasing catabolism and inhibiting anabolism through phosphorylation of key proteins on different pathways (Herzig and Shaw, 2017). In low energy situations, AMPK increases glucose uptake by increasing the presence of GLUT4 and GLUT1 (Glucose Transporter Type 1, Insulin-Responsive) proteins in the cell membrane. AMPK induces this effect in different ways: through the phosphorylation of TBC1D1 (TBC1 domain family member 1), which has inhibitory action in the vesicle transport system containing GLUT-4 (Chavez et al., 2008). As well as, through phosphorylation of TXNIP (Thioredoxin Interacting Protein), which leads to its degradation and consequently increases the presence of GLUT1 in the cell membrane (Wu et al., 2013). Jäger et al. (2007) showed that PGC1α is required for the action of AMPK on GLUT4, whereas Michael et al. (2001) showed that PGC1α increases GLUT4 expression through binding to MEF2C (Myocyte Enhancer Factor 2 C).

Kim et al. (2010) reported that AMPK could increase cellular uptake of glucose by activation of phospholipase D1 (PLD1), which stimulates ERK (Extracellular Signal-Regulated Kinase 2). Activation of ERK stimulates glucose transport via increased GLUT4 in the cell membrane (Chen et al., 2002). According to these studies, it is possible to conclude that AMPK acts on the cell uptake of glucose through several pathways, with main effect in increasing the GLUT4 translocation to cell membrane.

AMPK can also increases the ATP production by increasing the absorption and oxidation of fatty acids by the cell in different ways. AMPK phosphorylates acetyl-CoA carboxylase protein, ACC (inhibiting its activities), which are key to lipid synthesis. In addition to inhibiting the action of these proteins, AMPK acts on other proteins like the lipase ATGL (Patatin Like Phospholipase Domain Containing 2), responsible for fatty acids stored release in the form of triglycerides. AMPK also activates CPT1 (Carnitine Palmitoyltransferase 1), responsible for the transport of fatty acids to the mitochondria.

In addition, CPT1 is inhibited by malonyl-CoA produced from acetyl-CoA, reaction catalyzed for ACC. Thus, inhibition of ACC may be the way by which AMPK activates CPT1. Besides CPT1, which is a protein responsible for the transport of fatty acids into mitochondria, other proteins such as FAT / CD36 (Fatty Acid Translocase) and FATP (Solute Carrier Family 27 Member 1), that carry long chain fatty acids into the cells, have also been found in the mitochondrial membrane (Sebastián et al., 2009; Smith et al., 2011). Some studies suggest that FAT / CD36 can be regulated by AMPK, but the mechanism is not well elucidated yet (O'Neill et al., 2013). In addition to these mechanisms, AMPK also acts on adipose tissue by activating lipolysis through the activation of ATGL that plays a role in lipid breakage in this tissue (O'Neill et al., 2013).

Some studies have investigate the AMP role in RFI. Faure et al. (2013) found that the presence of phosphorylated AMPK (Thr 172) in *Longissimus dorsi* of pigs is higher in low-efficiency animals. In addition, the glycogen content is lower in these animals. Since AMPK increase glucose uptake through increasing glycolysis, this suggests that less efficient animals use more glucose as a source of energy compared to animals that are more efficient.

Fonseca et al. (2015) demonstrated that animals more efficient have higher liver expression of TFCC (Transcription Factor A, Mitochondrial) and UCP2 (Uncoupling Protein 2) compared with less efficient animals, whereas TFAM had greater muscle expression in less efficient animals. These genes are involved in mitochondrial function and are controlled by AMPK through PGC1α (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1

Alpha). PGC1α had higher expression in muscle of high efficiency beef cattle in the study performed by Kelly et al. (2011), but these authors did not find differences in expression of the gene encoding AMPK α-subunit (PRKAA1).

However, Fitzsimons et al. (2014) found no differences in the expression of genes encoding the AMPK subunits in beef cattle, nor differences in genes related to glucose metabolism such as GLUT4 (Glucose Transporter Type 4, Insulin-Responsive) and no differences in insulin levels. These authors have suggested that fatty acid oxidation and glucose metabolism do not influence feed efficiency variation.

From what have been presented here, we can conclude that energetic metabolism could influence the variation in RFI and additionally, AMPK pathway genes, which are involved in the energetic metabolism, could be key factors to understand how this variation occurs.

1.2 Objectives

The main objective was to investigate the molecular bases underlying residual feed intake in animals of Nellore breed. For, that, the specifics objectives were to find pathway associate with RFI and evaluate gene expression of genes with key roles in the lipid metabolism. In addition, we aimed to find whether there are differences in the lipid profile of animals low, medium and high RFI.

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Chapter 2

Genome-wide association studies pathway-based meta-analysis for residual feed intake in beef cattle[1]

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Abstract: Genome-wide association studies (GWASes) have been performed to search for genomic regions associated with residual feed intake (RFI); however inconsistent results have been obtained. A meta-analysis may improve these results by decreasing the false-positive rate. Additionally, pathway analysis is a powerful tool that complements GWASes, as it enables identification of gene sets involved in the same pathway that explain the studied phenotype. Because there are no reports on GWAS pathways-based meta-analyses for RFI in beef cattle, we used several GWAS results to search for significant pathways that may explain the genetic mechanism underlying this trait. We used an efficient permutation hypothesis test that takes into account the linkage disequilibrium patterns between SNPs and the functional feasibility of the identified genes over the whole genome. One significant pathway (valine, leucine and isoleucine degradation) related to RFI was found. The three genes in this pathway—methylcrotonoyl-CoA carboxylase 1 (MCCC1), aldehyde oxidase 1 (AOX1) and propionyl-CoA carboxylase alpha subunit (PCCA)—were found in three different studies. This same pathway was also reported in a transcriptome analysis from two cattle populations divergently selected for high and low RFI. We conclude that a GWAS pathway-based meta-analysis can be an appropriate method to uncover biological insights into RFI by combining useful information from different studies.

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2.1 Main text

Residual feed intake (RFI) is a widely used measure to evaluate animal feed efficiency. RFI is defined as the difference between observed and predicted feed intake (Koch et al. 1963) based on an animal's production and/or body weight. In beef cattle, Herd & Arthur (2009) demonstrated that differences between more efficient and less efficient animals are due to factors related to energy expenditure and protein turnover. Thus, a number of genome-wide association studies (GWASes) have been performed to search for genomic regions associated with variation in RFI.

The GWASes have generated inconsistent results in relation to RFI, with a lack of overlap between studies (Appendix 1 - Table S1). A meta-analysis may provide additional insight into this area, as it combines GWAS results from different studies to decrease the false-positive rate (Evangelou & Ioannidis 2013). Pathway analysis is a powerful tool that complements GWASes (Manoli et al. 2006), because different genes may be involved in the same pathway and may help detect associations missed by standard single-marker approaches (Cabrera et al. 2012). Thus, through a pathways-based meta-analysis it is possible to evaluate the biological consistency across GWAS results.

Pathway analysis aims to identify significant gene sets that together may explain the studied phenotype (Wang et al. 2007). In general, this significance has been assessed through SNP (single nucleotide polymorphism) permutation tests. However, this procedure does not consider the linkage disequilibrium (LD) between SNPs, which can lead to reduced power for hypothesis tests, as the markers are considered uncorrelated (Cabrera et al. 2012). To overcome this problem, Cabrera et al. (2012) proposed a rapid and simple permutation approach that uses SNP association results to establish the significance of pathways considering the LD structure of SNPs and the clustering of functionally related genes over the whole genome. Although Cabrera et al. (2012) used this approach in a single GWAS, here we proposed to use this methodology to combine GWAS results from different studies.

To the best of our knowledge, there are no reports on GWAS pathways-based meta-analysis for RFI in beef cattle. Thus, we used several GWAS results to search for significant pathways that may explain the genetic mechanism underlying feed efficiency in beef cattle, taking into account the LD patterns between SNPs and the functional feasibility of the identified genes over the genome.

To investigate the pathways associated with RFI, we used 201 significant SNP markers identified in 10 GWASes investigating RFI (Table S1). The SNPs were ordered according to their genomic position (chromosome and location on the chromosome). The methodology (Cabrera et al. 2012) assumes the genome to be circular and ordered from chromosome 1 to chromosome X and restarting at chromosome 1 again. Thus, the complete sets of P-values were permuted by rotation with respect to their genomic locations, which makes it possible to consider the LD between adjacent markers. Under this approach, SNPs retain the same position with respect to each other but, at each permutation, gain new random values of association with adjacent P-values.

For each permutation, the joint gene P-values were calculated using Fisher's combination test followed by the hypergeometric test to identify significant pathways (Cabrera et al. 2012). In the present study, this process was repeated 10 000 times to obtain the test statistic distribution under the null hypothesis that the pathway genes are no more associated with the phenotype than are the non-pathway genes. A threshold of 0.0002 (0.05/225) was used based on a Bonferroni correction considering a database of 225 pathways. This pathways database was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al. 2012). In addition, we considered a window size of 14 kb to search for genes close to the used markers. This window size was estimated based in the LD studies for different beef cattle breeds (Porto-Neto et al. 2014). All analyses were implemented in the GENOMICPER package (Cabrera et al. 2016) of R software (R Development Core Team 2018), which uses the KEGG.db package from Bioconductor (Carlson 2016) as the database for pathways. The complete dataset and all computational code are available in Tables S2 and S3 on Appendix 2 e 3.

One pathway was found to be significant for RFI, namely valine, leucine and isoleucine degradation (bta00280; Fig. 1), and the three genes in this pathway were found in three different studies (Table 1). Valine, leucine and isoleucine are branched chain amino acids (BCAAs), which have diverse metabolic roles, such as in protein synthesis and energy production (Monirujjaman & Ferdouse 2014).

The degradation of valine, leucine and isoleucine leads to the production of acetyl-CoA and propionyl-CoA. The propionyl-CoA is converted to the tricarboxylic acid cycle intermediate succinyl-CoA; thus these amino acids can be used to synthesize glucose. Ruminant animals normally have low blood glucose levels, as rumen bacteria use most of the dietary carbohydrates as a substrate for fermentation. Rumen fermentation results in the production of short chain fatty acids, including propionic acid, which together with amino acids represents the main source of gluconeogenesis in ruminants (Fassah et al. 2018). Thus, the pathway found in the current study suggests that genes that encode key enzymes in the gluconeogenic process may cause differences in feed efficiency.

Table 1. Significant markers from GWAS results and genes involved in the valine, leucine and isoleucine degradation pathway.

Marker	Chromosome	Position	Gene Symbol	Gene Name	Study
rs42548511	1	84580772	*MCCC1*	*methylcrotonoyl-CoA carboxylase 1*	Rolf et al. (2012)
rs110994776	2	89545687	*AOX1*	*aldehyde oxidase 1*	Karisa et al. (2013)
rs29012348	12	80892258	*PCCA*	*propionyl-CoA carboxylase alpha subunit*	Sherman et al. (2010)

The BCAAs also influence protein synthesis and turnover. Studies have found that when supplemented with these amino acids, rats showed more protein synthesis and less degradation of muscle protein in skeletal muscle (Freund & Hanani 2002; Kimball & Jefferson 2006). In addition, leucine

activates mTOR (mammalian target of rapamycin) signaling (Monirujjaman & Ferdouse 2014), which is a central regulator of cell growth and metabolism, and leucine also increases fatty acid oxidation (Sun & Zemel 2007). These studies suggest that BCAAs have an important role in cellular metabolism, increasing synthesis of protein and fatty acids degradation. In this sense, BCAAs could have a meaningful role in the variation of RFI, as energy metabolism and protein turnover are key factors influencing this phenotype (Herd & Arthur 2009) as well as body composition (Richardson et al. 2001).

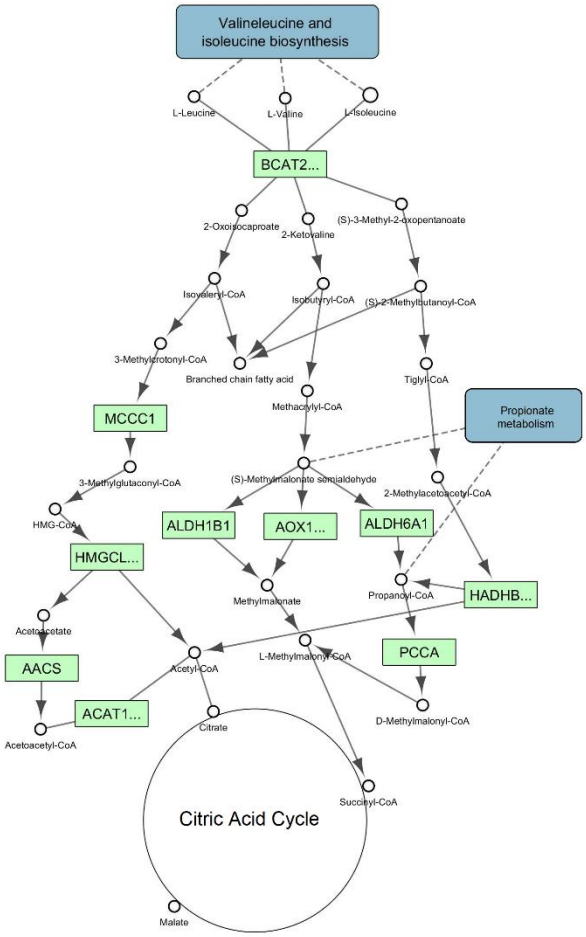

Figure 2 Valine, leucine and isoleucine degradation pathway with genes in green rectangles and compounds in blank circles. This figure was made based on the KEGG database.

Furthermore, this same pathway has already been found to be overrepresented in a transcriptome study in two cattle populations (Angus and Holstein) divergently selected for high and low RFI (Khansefid et al. 2017). Taking all this into account, we can consider that this pathway could be a key

factor underlying variation in RFI. Therefore, the methodology used here seems adequate for the identification of consistent mechanisms influencing RFI.

The genes that have a role in this pathway—methylcrotonoyl-CoA carboxylase 1 (MCCC1), aldehyde oxidase 1 (AOX1) and propionyl-CoA carboxylase alpha subunit (PCCA)—were found in three different studies. Considering the studies separately, there is no overlap among them, because they did not find the same significant genes—each gene was found in a different study (Table 1). However, the pathway-based meta-analysis showed that these genes collaborate in the same pathway (Fig. 1). Thus, apparently non-reproducible GWAS results (different genes found in different studies) could show consistent biological roles. This result shows that meta-analysis under a pathway analysis approach can be an appropriate method to combine GWAS results and uncover biological insights that are otherwise subtle when focusing only on genes in each study independently.

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2.4 Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 List of SNPs and QTL from the 10 studies used in the analysis.

Table S2 SNP information.

Table S3 SNP P-values.

Appendix S1 Script.

Chapter 3

High residual feed intake Nellore beef cattle exhibited higher levels of fatty acids and triglycerides in skeletal muscle and liver

Abstract: Several researches have been demonstrated that some biological process contribute to variation in residual feed intake (RFI). Among them are protein turnover, synthesis and transportation of fatty acids, inflammatory response, glucose absorption and fatty acid oxidation. In this study, we aimed to investigate the role of genes related to lipid metabolism in RFI. Besides, we aimed to find whether there are differences in the lipid profile among animals with high, medium and low RFI. Samples from muscle and liver tissue of 27 Nellore steers, classified as high, medium and low RFI, were collect. The samples were used to evaluate the mRNA expression of genes related to lipid metabolism and to carry out lipidomic analysis. None significant differences in mRNA expression were found here. In lipidomic analysis, it was found that high RFI animals exhibited higher levels of fatty acids and triglycerides compared to medium and low animals, indicating a possible increase in the requirement of lipid energy sources in these animals. In addition, it was observed differences in the lipids main components of cell membranes, glycerophospholipids and sphingolipids, which are related with cell signaling and molecules transportation. This is the first study using lipodomic tools to search for differences in animals divergently classified for RFI. Further research is need to explore the role of these lipids in feed efficiency.

3.1 Introduction

The residual feed intake (RFI) has become the measure of feed efficiency of most interest for livestock. Proposed by Koch et al. (1963), RFI was defined as the difference between the actual feed intake of an animal and the predicted intake based on growth and maintenance requirements. Unlike other feed efficiency measures, as feed conversion rate, the RFI is not correlated with growth rate or mature body weight (Moore et al., 2009). Additionally, its independence of production level allows comparison between individuals in different production level (Herd and Arthur, 2009). Besides, increasing the feed efficiency result in decreasing in feed cost and decreasing in emission of methane (Hegarty et al., 2007). Because of all these factors, RFI has been largely studied. However, there is yet a lack of knowledge in various aspects regarding the control of this trait.

One of these aspects is the molecular mechanism, which enables some animal be more efficient than other. Until now, the researchers were able to demonstrate that many physiological mechanisms contribute to variation in RFI. Richardson and Herd (2004) showed that differences in protein turnover, tissue metabolism and stress accounted for at least 37% of the variation in RFI. Furthermore, many studies have been shown differences in lipid metabolism among the animals classified as high and low RFI (Alexandre et al., 2014; Weber et al., 2016; Mukiibi et al., 2018). Differences in expression of genes related to transportation of fatty acid (Chen et al., 2011), inflammatory response, fatty acids synthesis (Alexandre et al., 2015), glucose absorption and fatty acid oxidation (Tizioto et al., 2015) have been also reported.

Therefore, we aimed to evaluate whether genes known as key genes for lipid and carbohydrate metabolism could present different expression levels among Nellore steers ranked by RFI. In addition, we aimed to analyze the expression of potential transcription factors regulating these key genes. We also, aimed to use a lipidomic approach in order to find whether there are differences in the lipid profile of these animals.

3.2 Materials and methods

The experiment was conducted at Centro APTA Bovinos de Corte, Instituto de Zootecnia (IZ), Sertãozinho, State of São Paulo, Brazil. Procedures were approved by the Ethics Committee on Animal Use - CEUA/IZ, (approval number: 213-15).

3.2.1 Animals and phenotyping

A group of 129 Nellore steers (7 months of initial age and 239 ± 30.1 kg of initial body weight) were subjected to a growth period of 98 days (28 days for adaptation and 70 days of test) receiving a diet formulated to achieve 1 kg/day of body weight gain requirements. The animals were fed using a GrowSafe automated feeding system (GrowSafe Systems Ltd, Airdrie, Canada). After this test period, the residual feed intake was calculated using the following model:

$$FI = \beta_0 + \beta_P * BW^{0,75} + \beta_G * ADG + \varepsilon,$$

in which FI = estimated daily feed intake (kg/day), β_0= regression intercept, β_P= regression coefficient of FI on mid-test metabolic live weight (BW, kg), β_G = regression coefficient of FI on average daily gain (ADG, kg/day), and ε = residual error, which is the RFI (kg/day).

From the 129 animals used in the test period, 27 Nellore bulls (9 with the lowest (-1.18 ± 0.44), 9 with the medium (0.18 ± 0.30), and 9 with the highest (1.20 ± 0.46) calculated RFI) were selected. The animals were subject to a finishing period of 125 days receiving a diet formulated to achieve 1.3 kg/day gain with a target finish weight of 550 kg.

The animals were slaughtered in experimental slaughterhouse, according to Brazilian government inspection procedures. Liver and skeletal muscle tissue samples were collected immediately after exsanguination of the animals and stored in liquid nitrogen.

3.2.3 Genes from the AMP-activated protein kinase (AMPK) signaling pathway

The mRNA expression of genes part of the AMPK signaling pathway were evaluated. These genes were chose considering its action in lipid and carbohydrate metabolism. The primers were designed using PrimerQuest software (http://www.idtdna.com/Scitools/Aplications/PrimerQuest) from IDT Scitools (Integrated DNA Technologies, Inc., Iowa City, IO, USA) (Table 3.1).

Table 3.1 AMPK pathway genes and its primers.

Gene	Symbol	Forward sequence	Reverse sequence
Acetyl-CoA carboxylase 1	ACC1	GCACGCCAGGTTCTTATT	CATGTCAATGGCGGATAGG
Adiponectin receptor 1	ADIPOR1	AGGACAACGACTACCTACTG	GTGTGGATGCGGAAGATG
CD36 molecule	CD36	GAGGCAGACACAACAAGAG	CAGTGGTAACCAGTTGGAAG
carnitine palmitoyltransferase 1B	CPT1	GTCCCTTCCCTTGCTCTA	GGACAGCAGAGACCCATA
Fatty acid synthase	FASN	ATCGCTGGCTACTCCTAC	GCCGTCAAACAGGAAGAG
Lipase, hormone-sensitive	HSL	GAGGGTGATGAGAGGGTAAT	GATGGCAGGTGTGAACTG
Leptin receptor	LEPR	TCCTGGGTCTTCGTATGG	GTAAGAAGGGCACTCCAATC
Lipoprotein lipase	LPL	CAGACAGGATTACAGGAGGA	GGAATGAGGTGGCAAGTG
Malonyl-CoA decarboxylase	MLYCD	CACTTCCACCTGCAGAAC	CCTCTAGGAAGTAGCGGTAG
Myocyte enhancer factor 2A	MEF2A	CCACCTCAAGCCACATTAC	CTGAAGTGCTCAACATCCC
myocyte enhancer factor 2D	MEF2D	ACGCCGTCCTCACTAAT	GTAGCTCTCGCCCATAGT
nuclear respiratory factor 1	NRF1	GGTGACTCTGTCCCTGTAT	GTGAGGGCTGATTACAAGAC
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	PFKFB2	GTCCCTTCCCTTGCTCTA	GGACAGCAGAGACCCATA
peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	PPARGC1A	ACCTCCACCATCCAAGAA	CTGTGCGTACAACTCAGAC
Sterol regulatory element binding transcription factor 1	SREBP1	CTCGTCTTCCTCTGTCTCTC	GTTGATGCTGGTGGTGTC
TBC1 domain family member 1	TBC1D1	GTTTCGCCCTCTGGATTG	GTGAACTCGTTGAGGCTTAC

3.2.4 Transcription Factors Analysis

In order to investigated potential transcription factors regulating key genes of AMPK signaling pathway, an analysis of transcription factor was carried out. The analysis was performed using TFMExplorer, a freely available software (Defrance and Touzet, 2006). This software takes a set of gene sequences and searches for locally overrepresented transcription factor

binding sites (TFBS) in the promoter region. After, it uses the weight matrices from JASPAR database (Mathelier et al., 2014) to detect all potential TFBS, and extracts significant clusters (region of the input sequences associated with a factor) by calculating a score function.

The analysis found out 22 transcription factors (TF) related with these genes (Figure 3.1).

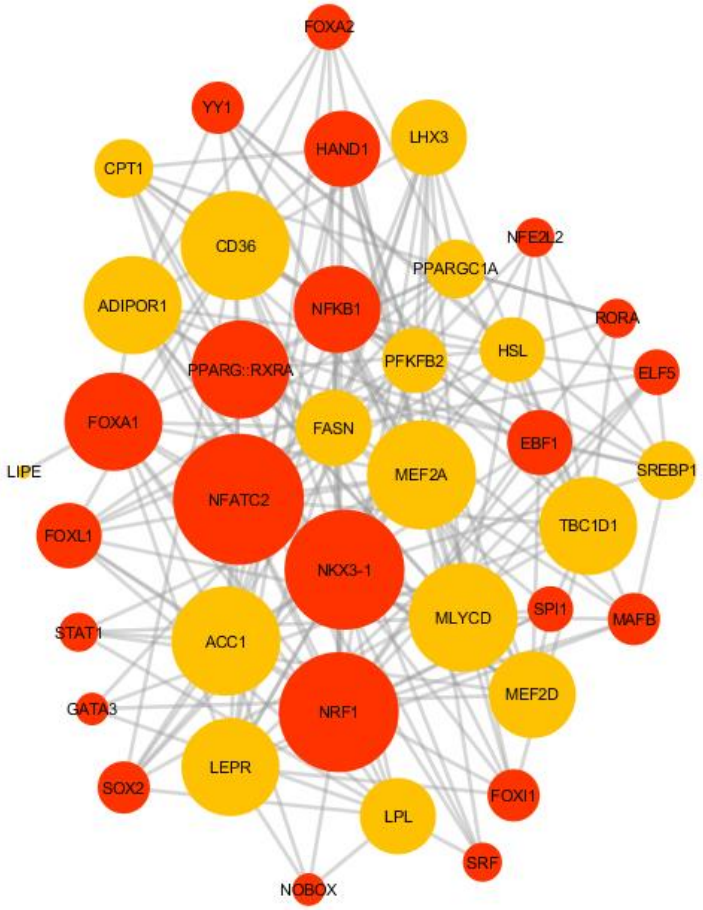


Figure 3.1 Transcription factors (red) and their target genes (yellow)

The mRNA expression of these TF found in this analysis were also evaluated. Table 3.2 shows the transcription factors and its primers sets designed as mentioned before.

Table 3.2 Transcription factors and its primers.

Gene	Symbol	Forward sequence	Reverse sequence
early B-cell factor 1	EBF1	AACTAAGGGTCGGCTTCT	GCTAAAGGGTGGCATGTT
E74 like ETS transcription factor 5	ELF5	GCCTCCAGAGTTCTCATCTA	TCCCTATCTTCCCATTCCAG
forkhead box A1	FOXA1	GACCCTCACTACTCCTTCAA	ACTGTAGTGCCTGCTCAT
forkhead box A2	FOXA2	CAGACACCTCCTACTACCAG	ACTTCCTGTCTCTCCCATC
forkhead box I1	FOXI1	CCAGAGTCATGCAAACTAGG	CTGCTCAACCAATCCTTCC
forkhead box L1	FOXL1	CACGAGGGTTAGGGAATCT	CCCTACTTCCACTTGCTTTC
GATA binding protein 3	GATA3	CGGGCATTACCTGTGTAAC	ACGTACCTGCCCTTCTT
heart and neural crest derivatives expressed 1	HAND1	GCGTTCTCTCCTCCCTAAT	AGGCAGCCCTTTCTTCT
LIM homeobox 3	LHX3	CCAGTCCAGACTAGGTGTT	GGGATGTTTGGCAATGGA
nuclear factor of activated T-cells 2	NFATC2	CCTTCGCCTTCACTTTCTC	GCCACGTTCCTTTGGTT
nuclear factor, erythroid 2 like 2	NFE2L2	ACAAGCTGGCTGAGACTA	TCCAGTGAGGGCATTGA
nuclear factor kappa B subunit 1	NFKB1	CTTTCCTTCCAGACAGCAC	GCTGTCCTGTCCATTCTTAC
NK3 homeobox 1	NKX3-1	CCAAGAACCTCAAGCTCAC	GCTTCCGCTTGGTCTTATAG
NOBOX oogenesis homeobox	NOBOX	GTTCTTCCAACACCCACAG	CACAGGACAAGGCAAAGAG
peroxisome proliferator activated receptor gamma	PPARG	TGGAGACCGCCCAGGTTTGC	AGCTGGGAGGACTCGGGGTG
RAR related orphan receptor A	RORA	GCAGAGAGACAGCTTGTATG	CGTTGGCCGAGATGTTATAG
retinoid X receptor alpha	RXRA	CCGGGTTCGAATGAATACC	GGCTAAGGCATTGCTAAGG
SRY-box 2	SOX2	CAAGATGGCCCAAGAGAAC	TCCGGGTGTTCCTTCAT
serum response factor	SRF	CGGCTTTGAAGAGACAGAC	CAGGTTGGTGACGGTAAAC
signal transducer and activator of transcription 1	STAT1	TTTCCGTTCTGGCTTTGG	CCACGATACACCCATCATTC
YY1 transcription factor	YY1	AATCCACACCGGAGACA	GGAGGGTCTTCTCTCTTCTT
Spi-1 proto-oncogene	SPI1	GACAGGCAGCAAGAAGAAG	TTGTCCACCCACCAGAT
MAF BZIP Transcription Factor B	MAFB	ACACCACCTGGAGAATGA	ACCTTGTAGGCGTCTCTT

The TF related with genes, their biological process and pathways, in which they are involved, are shown in the Figure 3.2. We can see that the TF founded here are involved in many biological processes regarding lipid and carbohydrate metabolism.

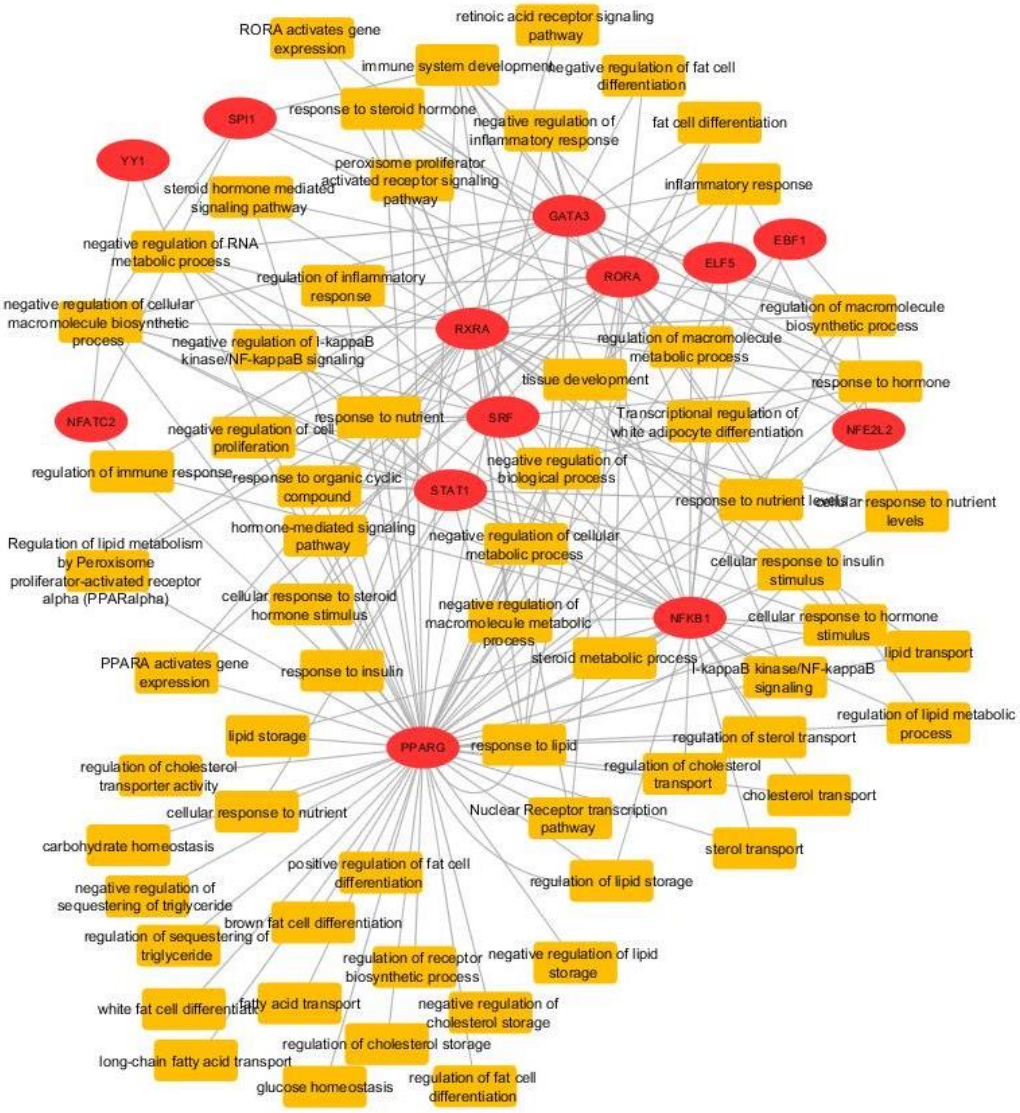


Figure 3.2 Transcription factor (red) and biological processes and pathways (yellow), in which they are involved.

3.2.5 RNA isolation and cDNA synthesis

The total RNA was isolated from individual samples of muscle and liver using RNeasy® Micro Kit (50) (QIAGEN, Valencia, CA, USA) following the

manufacturer's instructions. The cDNA was synthetized using the GoScript Reverse Transcription (RT) System (Promega, Madison, WI) according to the manufacturer's instructions.

3.2.6 RT-qPCR

The RT-qPCR reactions were performed in an ABI Prism 7300 Detection System thermocycler (Applied Biosystems, Foster City, CA) using GoTaq® qPCR Master Mix (Promega, Madison, USA), according to the manufacturer's instructions. Gene expression values were calculated and expressed relative to 18S, as described by Livak and Schmittgen (2001).

3.2.7 Statistical analysis of RT-qPCR data

Gene expression data were analyzed through a model including the fixed effects of group classification (high and low RFI), as follows:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij},$$

in which T_i is the i-th level of the treatment effect of RFI group, ε_{ij} is the random error associated with Y_{ij}, the $2^{-\Delta\Delta Ct}$ values for each samples. The residuals from the analysis of each variable were assessed for normality using Shapiro-Wilk's test and for homogeneity of variance using Levene's test. The data's residuals that not achieve normality and homogeneity of variance was transformed using ln ($2^{-\Delta\Delta Ct}$ +1) (Voge et al., 2004).

All statistical procedures were performed using the *aov* function from R software (R Development Core Team 2018).

3.2.8 Lipidomics analysis

3.2.8.1 Lipid extraction and mass spectrometric analyses

The lipids were extracted from liver and muscle tissues samples (25 mg) using the Folch Method (Folch et al., 1957). The extract was analyzed on

an Q-Exactive Plus (Thermo Fisher Scientific) mass spectrometer, which was coupled to a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca NY, USA).

The data were analyzed using the FIEmspro workflow (R-package available at http://users.aber.ac.uk/jhd/). Principal Component Analysis (PCA) was followed by PC-Linear Discriminant Analysis (PC-LDA). To test for the effects of treatment on lipid compounds one-way Analysis of Variance (ANOVA) was used.

3.2.8.2 Lipids Identification

For metabolite identification, accurate mass to charge ratio (m/z) values were used queried using MZedDB, a mass annotation tool (Draper et al. 2009). To further analysis of lipid ontologies, we used the LIPID MAPS database (Fahy et al. 2007) and PubChem (https://pubchem.ncbi.nlm.nih.gov/).

3.3 Results

3.3.1 RT-qPCR

Seeking to better understand the mechanisms that may influence the differentiated metabolism of the animals from divergent groups for RFI, we evaluated the mRNA expression of the genes of AMPK signaling pathway and their transcription factors. The abundance of mRNA of these genes and TF did not differ ($P>0.05$) between the RFI groups (H-RFI and L-RFI) in skeletal muscle (Table 3.3).

Table 3.3 Muscle RT-qPCR results for high and low RFI animals.

Gene	High	Low	F value	p-value
ACC1	5.212452	5.854824	0.395	0.538
ADIPOR	1.5621	1.8312	0.235	0.634
CD36	1.692	2.3416	0.752	0.399
CPT1	2.573727	2.579159	0	0.995
FASN	1.5758	2.12	1.081	0.314
HSL	3.9882	4.3522	0.285	0.601
LEPR	1.949	2.5844	1.072	0.316
LPL	1.7534	2.3089	0.74	0.402
MEF2D	1.77012	1.74591	0.002	0.968
NRF1	1.7761	2.0644	0.255	0.62
PGC1	1.702	1.8927	0.1138	0.7402
SREBP1	2.4751	2.1187	0.233	0.636
EBF1	2.0401	2.856	1.295	0.272
GATA3	2.3439	3.09	1.509	0.237
NFACT2	2.947	3.6029	0.619	0.443
NFE2L2	2.0729	2.7704	0.75	0.399
NFKB1	2.1298	3.3391	1.972	0.179
PPARG	2.1319	2.7725	0.734	0.404
SPI1	2.5307	2.8462	0.176	0.68
RXRH	2.2885	0.9291	1.247	0.281
SRF	1.894	2.6569	1.136	0.302
ELF	2.3241	3.0874	1.149	0.3
RORA	2.1903	2.8193	1.079	0.314
STAT1	2.0884	2.7882	1.003	0.331
YYI	2.2125	3.808	2.034	0.173

Likewise, no differences (P>0.05) were observed between the RFI groups (H-RFI and L-RFI) for the abundance of mRNA in liver tissue (Table 3.4). The medium RFI treatment showed problems in the reactions during the RT-qPCR and therefore was remove from the mRNA expression analysis.

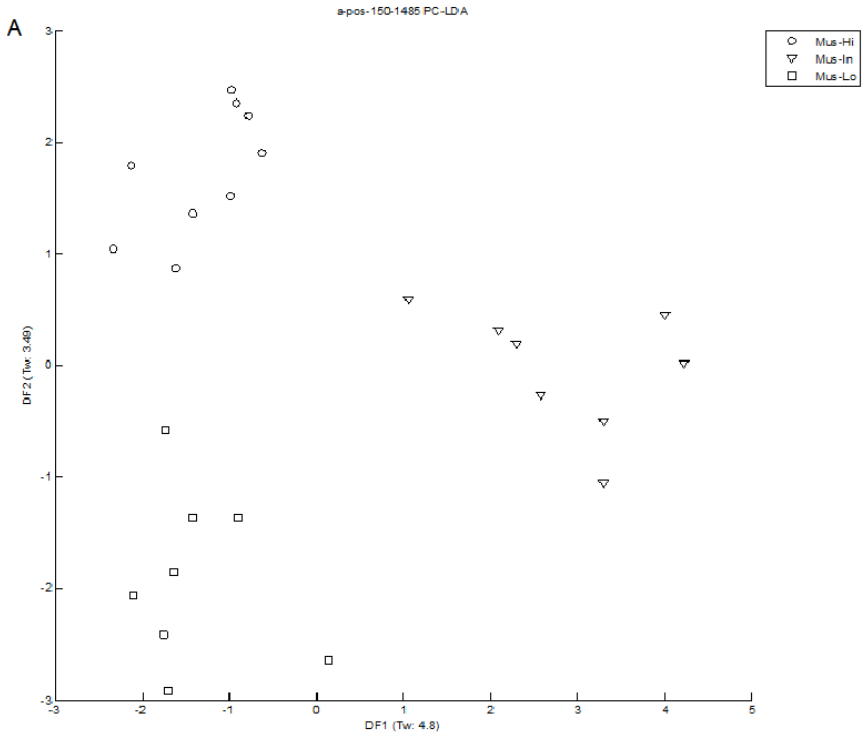
Table 3.4 Liver RT-qPCR results for high and low RFI animals.

Gene	High	Low	F value	p-value
ACC1	3.4067	2.5174	2.107	0.169
ADIPOR	1.7229	1.104	4.547	0.0512
CD36	3.04641	2.95689	0.01	0.92
CPT1	3.9127	4.9533	1.829	0.198
FASN	2.05637	2.01092	0.013	0.911
INSR	3.2668	2.7747	0.546	0.472
LEPR	4.1088	3.2757	1.244	0.283
LPL	3.5047	3.8126	0.175	0.682
MCD	3.6153	2.9552	2.032	0.176
MEF2A	2.7793	2.0619	1.327	0.269
MEF2D	3.2149	2.9736	0.145	0.709
NRF1	4.1974	5.322	0.929	0.352
PFK-2	2.2171	2.5274	0.183	0.675
PGC-1	2.7843	2.1398	1.206	0.291
SREBP1	2.8974	2.4839	0.787	0.39
TBC1D1	2.0909	1.944	0.11	0.745
EBF1	2.2186	1.8884	0.582	0.457
GATA3	1.94709	2.02155	0.049	0.828
ELF5	2.8672	2.5726	0.293	0.596
FOXA2	2.2825	1.8711	1.385	0.258
FOXL1	2.4944	2.246	0.214	0.65
HAND1	2.5562	2.1608	0.493	0.493
LHX3	2.9882	2.8074	0.121	0.733
MAFB	2.8933	2.5495	0.226	0.641
NFE2L2	2.4782	2.2758	0.195	0.665
NFKB1	1.5779	1.7691	0.52	0.482
NKX31	3.235	3.028	0.117	0.737
NOBOX	2.6987	2.2182	1.082	0.315
PPARG	2.4658	2.1264	0.573	0.461
SPI1	3.0623	2.8107	0.156	0.699
RXRA	2.0593	1.8032	0.804	0.384
SOX2	2.6522	2.3879	0.307	0.588
SRF	1.8199	1.3317	1.871	0.192
RORA	2.5513	1.948	1.702	0.212
STAT1	1.23588	1.28809	0.0456	0.8338
YYI	3.0917	2.8178	0.202	0.659

3.3.2 Lipidomics analysis

3.3.2.1 Discrimination analysis to detect metabolites associated with RFI

Class discrimination was visualized in PC-LDA score plots (Figure 3.3). In muscle tissue, discrimination was accentuated for *m/z* ranges 150–1485 in positive and 150–1495 in negative ionization mode. In liver tissue, discrimination was accentuated for *m/z* ranges 150–1485 in positive ionization mode. The further analysis of identification were performed in these ranges. We did not reach good results in negative ionization mode for liver, thus we do not show the results here.



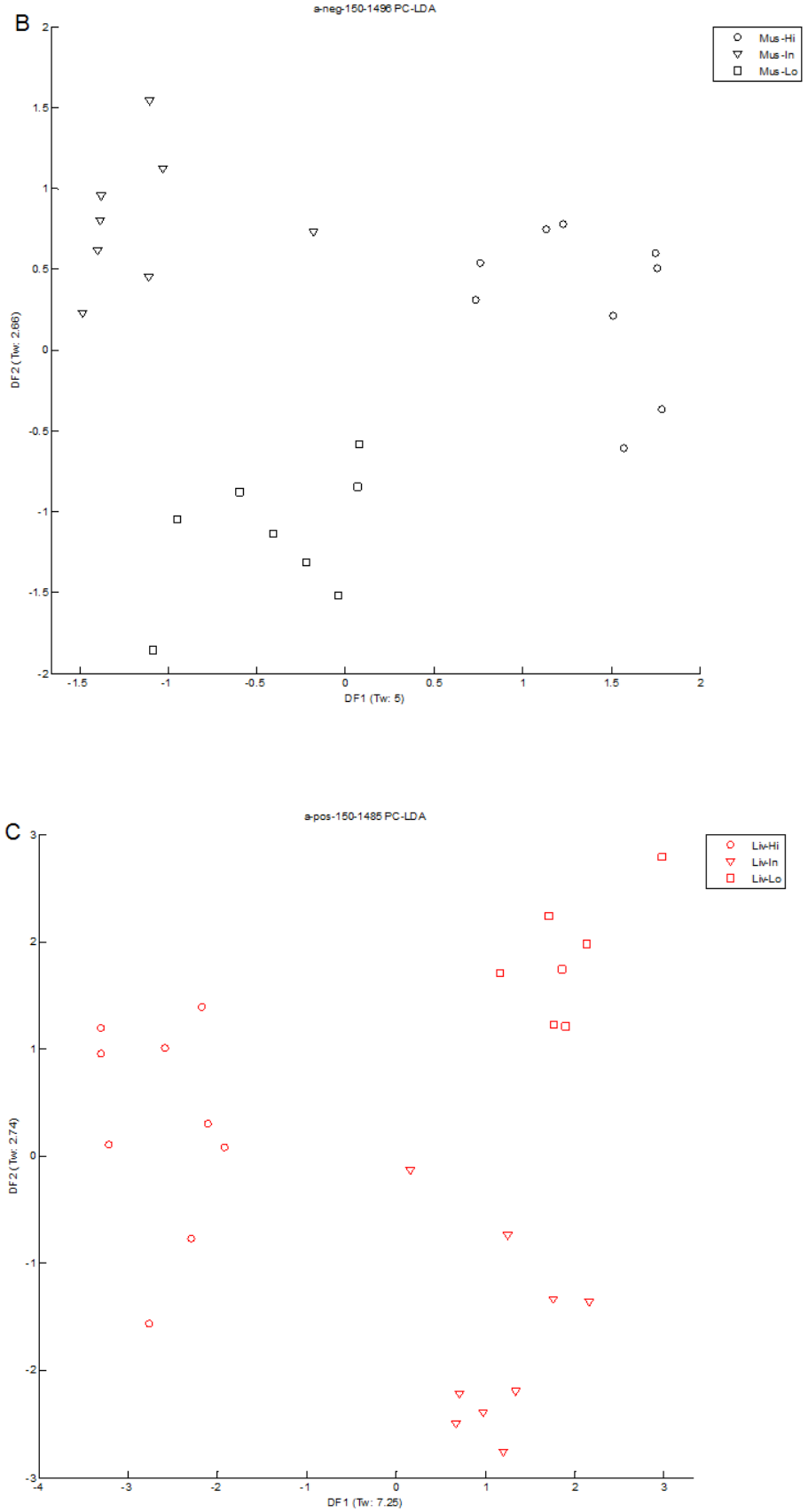


Figure 3.3 PC-LDA scores plot of A) positive ionization mode for muscle tissue, B) negative ionization mode for muscle tissue and C) positive ionization mode for liver tissue.

3.3.2.2 Selection of features explaining the differences in RFI

The results showed 70 and 65 important m/z peaks in muscle and liver tissues, respectively, that the abundance differed significantly abundance among the RFI groups. Each m/z value can represent more than one lipid species (isobaric); thus, we identified 42 lipid classes in muscle (Table 3.5) and 47 lipid classes in liver (Table 3.6). From this, the most relevant results were chose for further discussion.

3.3.2.3 Muscle tissue

In muscle tissue, the high RFI animals showed significantly higher abundance of lipids from the fatty acyls and prenol categories, then the medium and low RFI animals. Within these two categories are lipids from subclasses of unsaturated fatty acids, branched fatty acids and straight chain fatty acids. The high RFI animals and the medium also shows highest levels of triacylglycerols than animals from the low RFI group (Table 3.5, more information in Table S4 in Appendix 5).

Table 3.5 Lipids class identified in muscle (in positive and negative ionization mode) with significantly different concentrations for high, medium and low RFI.

m/z	High	Inter	Low	Classification	p-Value
253.0574	4.100254	0.934374		Fatty alcohols	0.000295
441.253	4.534123	4.17648		Other Fatty Acyls Ansamycins and related polyketides	0.000419
521.3114	3.159176	4.130984		Cholesterol and derivatives	0.000838
523.3424		4.268323	4.926786	Vitamin D3 and derivatives	0.000495
570.3725	0.895016	3.714802		Vitamin D3 and derivatives	0.000108
686.6228		2.227493	2.613938	N-acylsphinganines (dihydroceramides)	0.000309
741.5093		0.459289	3.750237	Vitamin D3 and derivatives	0.000717829
177.007	5.469236	5.292363		Fatty alcohols Coumestan flavonoids	0.00102
206.0812	4.354165	0.96339		Unsaturated fatty acids Clavulones C10 isoprenoids (monoterpenes) Prostaglandins	0.000164

213.0522	3.876415	0.487031		Dicarboxylic acids	0.000367
218.1176	4.331876	2.961719		Fatty alcohols Wax diesters	0.000331
265.1564	4.15199		1.01747	Unsaturated fatty acids Branched fatty acids Oxygenated hydrocarbons	0.000486
365.3172	3.969535	0.927659		Fatty alcohols N-acyl ethanolamines (endocannabinoids)	7.54E-05
397.2334	4.472833		3.305784	Other Fatty Acyls Methoxy fatty acids Hydroxy fatty acids Vitamin D3 and derivatives Cardanolides and derivatives Unsaturated fatty acids C24 bile acids, alcohols, and derivatives	0.001096
408.2515	4.427529	2.725102		Monoacylglycero-phosphocholines Monoacylglycerophospho-ethanolamines Fatty acyl carnitines Prostaglandins Other Docosanoids Vitamin D3 and derivatives C21 steroids (gluco/mineralocorticoids,progest ogins) and derivatives C19 steroids (androgens) and derivatives Glycosyldiacylglycerols	7.33E-06
418.2186	4.161167	1.307911		Hydroperoxy fatty acids	0.00106
434.3471	4.490634		3.018067	Triacylglycerols	0.000252
540.4252	4.255119	2.982334		Triacylglycerols	0.000498
542.3835		4.97041	5.628018	Monoacylglycero-phosphocholines Monoacylglycerophospho-ethanolamines Diacylglycerophospho-monoradylglycerols Vitamin D3 and derivatives	0.001356
544.5082	3.427096		0	Straight chain fatty acids Wax monoesters	0.00044
560.4092		0.951133	4.003176	Monoacylglycero-phosphocholines	0.000904
592.3457	3.737285	1.043712		Cholesterol and derivatives	0.00106
607.4166	4.457127	0.967555		Fatty alcohols	0.000134
685.4645	3.988374	0.523726		Fatty acyl glycosides of mono- and disaccharides Diacylglycerophosphoglycerols Oxo fatty acids Hydroxy/hydroperoxyeicosatrienoi c acids	0.000479

731.5046		0	3.362896	Diacylglycerophosphates Macrolides and lactone polyketides 1-(1Z-alkenyl),2-acylglycerophospho-ethanolamines Diacylglycerols C24 bile acids, alcohols, and derivatives	0.000202
824.6145		2.245731	3.849867	Diacylglycerophospho-ethanolamines Diacylglycerophosphates	0.001215
896.7689	4.632408	5.071874		Triacylglycerols	0.001197
978.9414	3.082747	0		Triacylglycerols	0.00021
1005.96	4.457963		2.827003	Triacylglycerols	0.0003
1065.806	4.513784	1.014673		Triacylglycerols	0.000193

3.3.2.4 Liver tissue

In the liver tissue, the high RFI animals showed significantly higher abundance of triacylglycerols then the low RFI animals. The same result found in the muscle tissue. Interestingly, the low RFI animals showed significantly higher abundance of lipids within the categories glycerophospholipids and sphingolipids regarding the other two groups, both categories largely known to be part of cell membrane (Table 3.6, more information in Table S5 in Appendix 6).

Table 3.6 Lipids class identified in liver (in positive ionization mode only) with significantly different concentrations.

m/z	High	Inter	Low	Classification	p-Value
182.0909		1.495907	3.955426	C10 isoprenoids (monoterpenes) Amino fatty acids	0.001103
194.1516		0.986388	4.43726	Fatty alcohols	1.91E-05
200.1645		5.187419	5.268909	Unsaturated fatty acids Wax monoesters Prostaglandins N-acyl ethanolamines (endocannabinoids) Straight chain fatty acids Branched fatty acids Wax monoesters	0.001707
236.1623	3.812638		1.086119	Monoacylglycerophospho-ethanolamines	2.58E-05

				Monoacylglycero-phosphocholines Wax monoesters C10 isoprenoids (monoterpenes) Branched fatty acids Straight chain fatty acids	
244.1544		5.132836	5.264277	Fatty acyl carnitines Jasmonic acids Dicarboxylic acids Cholesterol and derivatives C27 bile acids, alcohols, and derivatives	0.000493
255.1591		4.898884	5.016283	Lysosphingomyelins and lysoglycosphingolipids C15 isoprenoids (sesquiterpenes) C20 isoprenoids (diterpenes) Retinoids Unsaturated fatty acids	0.000756
291.2681		4.689786	4.582808	C20 isoprenoids (diterpenes) Fatty alcohols Unsaturated fatty acids	0.000601
308.2946	4.635227		4.730191	N-acyl ethanolamines (endocannabinoids) C20 isoprenoids (diterpenes) Fatty alcohols Fatty aldehydes	0.001762
357.1667	4.166651		2.459675	C10 isoprenoids (monoterpenes) Prostaglandins Clavulones C18 steroids (estrogens) and derivatives C20 isoprenoids (diterpenes) Retinoids	4.08E-05
370.1281		0.988249	4.066769	Unsaturated fatty acids Isoflavonoids Flavones and Flavonols Flavanones	0.001619
383.1673	4.122324	2.664524		Thromboxanes Prostaglandins	0.001075
395.1673		2.25415	3.868201	Hydroperoxy fatty acids	0.000983
435.4195		0.483709	4.080804	Ergosterols and C24-methyl derivatives Cholesterol and derivatives Ergosterols and C24-methyl derivatives	0.001324
450.2036		0.49999	3.435587	1-alkyl,2-acylglycerophosphocholines Monoacylglycero-phosphocholines Monoacylglycerophospho-ethanolamines	0.00112
473.2353	4.232148		2.559709	Dicarboxylic acids	0.000267

491.2891		0	4.044748	Vitamin D2 and derivatives	0
515.2824	4.604957	1.510894		Dicarboxylic acids Fatty acyl glycosides of mono- and disaccharides Vitamin D3 and derivatives	0.000409
522.5242	4.564269	4.788297		N-acylsphinganines (dihydroceramides) Wax monoesters	0.001168
539.2822	4.044365	0.994487		Macrolides and lactone polyketides	0.000761
543.3643	4.554457		2.941478	Macrolides and lactone polyketides Vitamin D3 and derivatives	0.00071
570.4873	3.96388	1.53284		Hopanoids	0.000937
579.3851	4.516756	1.515093		C40 isoprenoids (tetraterpenes)	0.000516
587.318	4.028923	1.007974		Dicarboxylic acids Vitamin D3 and derivatives	0.001007
589.5173	4.415474		1.128912	Diacylglycerols Diacylglycerols Unsaturated fatty acids Carbocyclic fatty acids Hopanoids	0.001569
636.4776	4.431892	4.682726		C40 isoprenoids (tetraterpenes)	0.001031
792.6275	4.517126	4.872572		Hopanoids	0.001631
810.5251	4.585221	4.767805		Diacylglycerophosphoserines Diacylglycerophosphoserines Diacylglycerophosphoglycerols Diacylglycerophospho-ethanolamines	0.001352
817.5685	1.096579		3.515376	Bactoprenol monophosphates	0.000272
874.5439	1.540522		4.636876	Sulfoglycosphingolipids (sulfatides) Diacylglycerophosphoinositols	0.000751
929.7897	2.867483		0	Triacylglycerols	0.001043
932.6212	1.053852		4.139408	Diacylglycerophosphoinositols	0.000949

3.4 Discussion

According with recent studies, differences in animals ranked as high and low RFI could be due to different response regarding lipid metabolism, xenobiotics and oxidative metabolisms (Chen et al., 2011, Alexandre et al, 2015). In order to assess whether these differences could happen in liver or/and skeletal muscle tissue, we assessed the mRNA expression of keys

genes to lipid and carbohydrate metabolism. For this purpose, we chose the genes involved in the AMPK signaling pathway, largely known by their role in in the energy homeostasis. Moreover, an in silico transcription factor (TF) analysis was carried out in order to find TFs that could be controlling the genes in the AMPK signaling pathway simultaneously in an upstream level and accordingly could help explain differences in the RFI phenotypic groups. The Figure 3.2 shows the TFs found here and their AMPK pathway target genes. Based on the literature, we could confirm the role in energy metabolism of those TFs founded here (Figure 3.3).

An mRNA expression analysis of the AMPK signaling pathway genes and their TFs were performed. However, we did not observe differences in the expression of these genes in this present study in both tissues. In spite of that, the differences in the lipid profile founded here shows that lipid metabolism could differ among the animals classified for RFI. Thus, we can hypothesize that differences in these genes expression could happen in post-transcriptional process, which should be further investigated.Lipidomics aims at the quantitative characterization of the full lipid complement produced by cells. Investigations of tissue lipidome profile is interesting approach, once enables the study of cellular metabolism by quantifying the changes of individual lipid classes, subclasses and that reflect metabolic differences (Han, 2016).

In order to investigate differences in the lipid profiling among animals classified as high, medium and low RFI, we performed a shotgun lipidomics analysis of liver and muscle tissues samples. We found out 70 m/z significant for the muscle tissue and 65 m/z for the liver tissue. Considering the larger number of compounds found here, we chose the most relevant ones to further discussion.

Our results show that the muscle tissue of high RFI animals have a higher abundance of many classes of fatty acids, among them unsaturated fatty acids, branched fatty acids and straight chain fatty acids. Fat acids are involved in important biological actions and are used in body as a means of energy storage (Lunn and Theobald, 2006). This lipid class can affect meat quality traits, such as shelf life and flavor. According to Wood et al. (2003) the rapidly oxidize of unsaturated fatty acids can lead to the development of rancidity in meat, but oxidation of unsaturated fatty acids is important in flavor

development during cooking. However, it is not clear whether RFI could be associated with meat quality (Ribeiro et al., 2012; Fidelis et al., 2017).

Furthermore, this higher abundance of fatty acids in high RFI animals suggest that animals less efficiently could have increased fat deposition on the carcass. In fact, some studies have stated that there is a positive genetic correlation between measure of fatness and RFI (Herd et al., 2003) and also, a positive phenotypic correlations between RFI and carcass fat deposition (Nkrumah et al., 2007).

Likewise, the high-RFI and medium-RFI animals also shows higher abundance of triacylglycerol than low-RFI. Triacylglycerol is the most common fatty source of energy, comprise three fatty acids esterified with a glycerol, and is storage most in the fat tissue. Herd and Bishop (2000) demonstrated that selection for low-RFI could increase carcass leanness and, as mentioned before, there is a positive phenotypic correlation between RFI and carcass fat deposition (Nkrumah et al., 2007). Thus, we suggest that the less abundance of triacylglycerol in low RFI animals reflects higher expenditure of this energy source for lean meat deposition, once that lean meat deposition expend more energy than fat deposition (Owens et al., 1995). In other hand, high RFI animals have more energy storages, since they have more carcass fat deposition, which is less energy demanding.

In this way, the lower levels of triacylglycerol in the more efficient animals could indicate increase in mobilization of this lipid in muscle tissue, to be used as energy source, since the energy requirement for lean meat deposition is greater in these animals.

On the other hand, increased levels of intramuscular triacylglycerol content is associated with insulin resistance (Stannard and Johnson, 2003). Richardson et al. (2004) have shown that high-RFI animals tended to have higher insulin concentration and it have decrease insulin sensitivity of muscle. Therefore, these high levels of triacylglycerol content in high-RFI animals could be due to defective response to insulin. However, we cannot declare that the high level of triacylglycerol showed for the high-RFI animals is abnormal, such as in cases of insulin resistance condition.

In the liver tissue, we could not find a distinct difference in fatty acids abundance among the divergent RFI groups as we found in muscle. Still,

analogously to muscle tissue, the high-RFI animals exhibited higher triacylglycerol abundance. Triacylglycerols are formed in the liver and exported to other tissues by very low-density lipoprotein (VLDL) particles. Higher triacylglycerol in liver is also associated with insulin resistance (Nagle et al., 2009). An interesting fact is that insulin resistance can be affected by cell membrane composition (Perona et al., 2017) this could be related to the differences found here in the lipid components of cell membrane.

Considering the liver tissue, all classes of lipids founded in this study within the categories glycerophospholipids and sphingolipids have higher abundance in the low-RFI regarding the other two groups. These two lipids category are involved in many signaling and regulation mechanism, as they are the main components of cell membranes (van Meer et al., 2008). It is not clear how discrepancies in glycerophospholipids and sphingolipids levels could influence animal efficiency. As these lipids are related with cell signaling and molecules transportation, we could hypothesize that this biological process could happen in a more efficient way in low-RFI animals and that is due to differences in the lipids constituents of the cell membrane.

Some studies have already demonstrated that membrane composition is affected by diet, obesity state and age (Bonzón-Kulichenko et al., 2018; Pietilainen et al., 2011; Pollock et al., 2016). In addition, the kind of lipid in the membrane could altered its fluidity (Pietilainen et al., 2011). Here, however, we observed that, regardless of composition, low-RFI has higher abundance of all lipids species within glycerophospholipids and sphingolipids identified in this analysis. Therefore, more studies focusing on these lipid classes can help elucidate the relation between RFI phenotype and the cell membrane.

3.5 Conclusion

To the best of our knowledge, this is the first study using lipodomic tools to search for differences in animals divergently classified for RFI. Our results here shows discrepancies in RFI animals regarding abundance of lipid classes. Some of them corroborate with the literature, as they show increased levels of fatty acids and triglycerides in high- RFI animals, indicating different mechanism in the use of this compound. Other results, the differences in

glycerophospholipids and sphingolipids levels, bring a new factor associated with RFI, which could be investigated in further studies. However, none differences was found here regarding mRNA expression of genes influencing the lipid metabolism, which could suggest that differences regarding the lipid and carbohydrate metabolism could happen in post-transcriptional process.

3.6 References

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Chapter 4

4.1 General conclusions

The main objective of this thesis was to uncover biological mechanisms underpinning feed efficiency in beef cattle. Herd et al., (2004) and Richardson et al., (2004) estimated that the major part of variation in RFI is due to wide range of biological process within them protein turnover and tissue metabolism. Other many studies have been showed the lipid metabolism, glucose uptake, ion transportation and protein synthesis (Chen et al., 2011; Karisa el at., 2014, Santana et al., 2014; Alexandre et al., 2015).

To investigate the bases for RFI we, firstly, performed a meta-analysis pathway approach, in which we could combine results from several GWAS in a meta-analysis in order to find out, pathways that could be related to RFI. As GWAS results sometimes find out different regions and consequently different genes affecting the same trait, we aimed to combined the GWAS results and investigate if this significant genes found in different GWAS could have convergent roles, such as being part of the same pathway.

From this study, we found one significant pathway relate too RFI, the valine, leucine and isoleucine degradation pathway. This pathway are linked to protein synthesis and energy production, process largely related to RFI. Besides, the genes which participate of this pathway were found in three different GWA studies (as show in Table 1 of the chapter 2), which shows that even though genes were found in different GWAS, they collaborate in the same pathway. This shows the relevancy of this approach to combine GWAS results and help elucidate the mechanism affecting RFI.

In a second step, we aimed to investigate the influences of lipid and carbohydrate metabolism on RFI, for that we evaluate samples from muscle and liver tissues of animals classified for high, medium and low RFI. The samples from the high and low RFI group were used to analyze mRNA expression of some key genes for energy metabolism and their transcription factors; and the samples from the three groups were used for a lipidomic analysis. The genes choose are part of AMPK signaling pathway and have a known role in lipid metabolism. Transcription factors (TF) influencing this

genes expression could also affect lipid metabolism by inhibition or activation of this genes. Thus, we carried out an *in silico* analysis in order to find TF that could bind to these genes and, for that, influence its expression. By doing that, we could search for others genes, which may have a role in RFI. Therefore, we performed an mRNA expression analysis of these TF and their target genes from AMPK signaling pathway. However, we did not find any significant differences in mRNA expression of these genes, suggesting that differences regarding the lipid and carbohydrate metabolism could happen in post-transcriptional process, which is supported by the differences found here in the lipid profile.

In the lipidomic analysis, we could find differences in the level of fatty acids and triglycerides among the animals selected for RFI. The animals from the low RFI group exhibited lower levels of lipids compounds of these classes, indicating a possible increase in the requirement of lipid energy sources in these animals. We could also observed, differences in the lipids main components of cell membranes, which are related with cell signaling and molecules transportation.

These two studies present here (chapter 2 and 3) shows that feed efficiency are likely to be associated with gluconeogenic process, protein synthesis and fatty acids and triacylglycerol content. Therefore, we could hypothesized that animals from RFI could diverge in the way they use its energy sources.

5. Appendices

5.1 Appendix 1

Table S1 List of SNPs and QTL from the 10 studies used in the analysis.

Marker/QTL	Chromossome	Position	P-values
Abo-Ismail et al., 2014			
rs136892391	8	1456250	0.0017
rs41755948	15	32674699	0.007
rs208805443	15	32681447	0.007
rs41821600	16	68614446	0.0033
rs41820824	16	68690299	0.0064
rs134420976	18	18193043	0.0028
rs41914675	19	36734474	0.004
rs43020736	21	29654483	0.016
rs43020769	21	29660419	0.009
rs109561809	28	9255542	0.005
rs209765899	28	14993619	0.009
rs42670352	15	47385604	0.002
rs42670351	15	47386394	0.028
rs42670353	15	47385334	0.0135
Karisa et al., 2013			
rs43242284	1	67635587	0.028
rs208270150	1	138045479	0.004
rs137393885	1	137963324	0.048
rs210348685	2	5397909	0.026
rs135748641	2	73815258	0.036
rs110994776	2	89545687	0.0001
rs109065702	2	105138600	0.034
rs209148339	2	133935523	0.023
rs43330759	2	136257492	0.013
rs109314460	4	117907734	0.006
rs109727850	7	98485261	0.026
rs210072660	7	98535683	0.007
rs133193054	15	36171715	0.04
rs29010201	18	50581375	0.014
rs109638814	20	10186470	0.008
rs209676814	20	31891107	0.026
rs41580312	20	5544340	0.006
rs41943134	20	35942763	0.005
rs133951891	20	38205059	0.023
rs110293711	20	56539092	0.0001
rs42190891	29	46550309	0.018
Lindholm-Perry et al., 2012			
BTB-00210449	4	117035128	0.067

77244_529	4	117035643	0.011
BFGL-NGS-19480	4	117049903	0.108
Lindholm-Perry et al., 2012			
rs41722889	14	25092333	0.0004
rs41722844	14	25243108	0.02
rs41722854	14	25254418	0.009
rs41722036	14	25267155	0.009
rs41722043	14	25272251	0.003
rs41722045	14	25272477	0.007
Nkrumah et al., 2007			
QTL 1	2	6900000	0.01295
QTL 2	5	33700000	0.00519
QTL 3	7	34000000	0.0132
QTL 4	7	36900000	0.01184
QTL 5	7	71800000	0.00308
QTL 6	7	115500000	0.01419
QTL 7	8	12700000	0.00935
QTL 8	8	30400000	0.00382
QTL 9	12	90900000	0.00596
QTL 10	12	92900000	0.00684
QTL 11	14	41000000	0.0003
QTL 12	17	4000000	0.00365
QTL 13	17	4900000	0.00067
QTL 14	17	70200000	0.00407
QTL 15	18	4800000	0.00427
QTL 16	18	4900000	0.00384
QTL 17	19	34600000	0.00165
QTL 18	19	36500000	0.00119
QTL 19	19	64400000	0.00049
QTL 20	19	65400000	0.00144
QTL 21	20	23800000	0.00213
QTL 22	20	59400000	0.00935
QTL 23	20	73300000	0.01265
QTL 24	21	38600000	0.00208
QTL 25	21	73300000	0.00568
QTL 26	24	7600000	0.01249
QTL 27	24	56200000	0.00155
QTL 28	24	63100000	0.00176
QTL 29	26	31500000	0.00654
QTL 30	29	42700000	0.00266
QTL 31	29	43700000	0.00214
Rolf et al., 2012			
rs109384003	1	132555764	7.43E-11
rs42548511	1	84580772	6.96E-07
rs43266121	1	129101492	9.48E-07
rs109095895	2	73234975	1.12E-06
rs109949037	2	30131000	4.57E-11

rs110073925	2	30102137	8.68E-11
rs110211659	2	43436458	1.07E-07
rs41585097	3	7052779	1.88E-07
rs42936243	3	65848664	2.57E-08
rs109406059	4	60324045	1.16E-10
rs42824767	4	74006260	7E-07
rs110188299	5	32861996	4.15E-07
rs109201532	6	89251522	3.41E-09
rs109373082	6	103133828	1.11E-06
rs43455987	6	42155077	8.65E-07
rs110202648	7	17046540	6.99E-08
rs109380245	8	54507754	7E-07
rs110937563	8	6687056	1.06E-06
rs41661176	8	107272311	3.02E-07
rs110608668	9	33321976	1.5E-07
rs41610951	9	101785435	7.88E-07
rs42797639	9	20734430	3.27E-11
rs109635380	10	68789694	6.65E-09
rs41597140	10	78608571	5.08E-07
rs109280551	11	102153173	7.28E-07
rs109589152	11	68687376	3.92E-08
rs41624451	11	38733905	4.44E-07
rs110643661	12	78694446	4.19E-07
rs41614805	12	48794617	2.65E-07
rs41626249	12	53822508	7.11E-11
rs109272278	14	5245003	4.84E-12
rs110371924	14	81584369	4.04E-07
rs110442376	14	11621252	1.27E-06
rs109918426	15	62428148	1.01E-06
rs41759150	15	43779412	2.1E-08
rs41662390	16	16244657	1.78E-07
rs109762011	17	3766546	2.27E-08
rs109762073	17	27950124	4.53E-11
rs41636597	17	27808938	7.67E-12
rs41639029	18	52971233	1.05E-06
rs108970074	19	29325678	1.93E-07
rs109672208	19	35619269	1.16E-07
rs41907619	19	35286010	5.39E-09
rs110275805	20	35557902	3.24E-09
rs41591215	20	38920878	8.88E-07
rs41613438	20	47776960	4.33E-07
rs43461171	20	6505111	5.51E-07
rs41973640	21	31443165	5.52E-07
rs41980271	21	34243875	1.01E-11
rs110978254	22	55257755	1.92E-09
rs29013532	22	56526462	1.83E-08
rs41620834	22	30769143	2.55E-08
rs109404118	23	32331699	7.66E-10

rs41617133	23	32876929	5.18E-07
rs109899690	25	13749103	4.66E-07
rs41587267	25	22197501	1.01E-06
rs110763390	27	37357125	5.16E-09
rs109417884	28	35247970	2.75E-08
rs110842770	28	15721558	2.34E-07
rs110252499	29	20601858	4.63E-07
rs110710999	29	18647026	3.82E-08
rs29027034	29	37472079	6.92E-07
	Santana et al., 2014		
rs41660853	8	458919	1.13E-07
rs135777172	21	71091607	5.37E-08
	Sherman et al., 2009		
QTL 1	1	10000000	0.0005
QTL 2	1	6000000	1.16E-05
QTL 3	3	82000000	7.60E-05
QTL 4	4	31000000	0.011
QTL 5	6	103000000	0.0049
QTL 6	7	23000000	0.0025
QTL 7	9	18000000	0.0188
QTL 8	9	26000000	0.0189
QTL 9	11	31000000	0.0086
QTL 10	11	58000000	0.0009
QTL 11	12	107000000	0.0285
QTL 12	13	68000000	0.0471
QTL 13	13	69000000	0.0179
QTL 14	14	87000000	0.0207
QTL 15	17	55000000	0.0279
QTL 16	18	29000000	0.0028
QTL 17	19	41000000	0.0007
QTL 18	19	105000000	0.0007
QTL 19	19	97000000	0.0045
QTL 20	21	4000000	0.0055
QTL 21	22	25000000	0.0014
QTL 22	23	66000000	0.0008
QTL 23	23	67000000	0.0013
QTL 24	24	2000000	0.0102
QTL 25	25	13000000	0.031
QTL 26	25	12000000	0.0232
QTL 27	25	66000000	0.0232
QTL 28	25	67000000	0.0122
QTL 29	26	64000000	0.0014
QTL 30	26	49000000	0.0018
	Sherman et al., 2010		
rs43703977	1	393248	0.001
rs29025433	1	95997539	0.02
rs29014082	2	134281005	0.003
rs29017229	4	30202937	0.044

rs43710085	6	87878904	0.003
rs41793507	8	53334416	0.02
rs43705534	8	90553133	0.001
rs29013479	10	15477566	8.68E-07
rs43705629	10	16562010	0.032
rs29011971	11	52491236	0.000316
rs29013423	11	58208950	0.006
rs29012348	12	80892258	0.000172
rs29015791	15	48068911	0.024
rs43708449	18	82260685	2.76E-05
rs29019628	18	17380202	0.000208
rs42536809	18	21509622	0.008
rs29009652	18	54470798	0.001
rs29015137	18	56121920	0.008
rs29015011	19	54287604	0.001
rs29014055	23	7810956	0.009
rs29027245	24	18730719	0.002
rs29013464	25	37958335	0.009
rs29013727	26	8221270	0.004
rs43708521	26	33605534	0.007
rs29011694	28	19731619	0.011
rs17872022	29	43205758	0.03
	Sherman et al., 2008		
rs29019569	2	127176061	0.0051
rs43706834	5	78776781	0.0056
rs29021916	10	94339279	0.053
rs29021101	20	42598095	0.013
rs29014641	20	50407500	0.069
rs17872022	29	43205758	0.0013

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5.2 Apendix 2

Table S2 SNP information

name	CHR	POS	ID	SYMBOL	STRING
rs109393868	1	2211567	535482	C1H21ORF59	+
rs110953962	1	69753034	281568	UMPS	+
rs137393885	1	1.38E+08	507522	NPHP3	+
rs137393885	1	1.38E+08	509292	UBA5	-
rs208270150	1	1.38E+08	526956	ACAD11	+
rs29025433	1	95997539	615534	FNDC3B	-
rs42548511	1	84580772	513504	MCCC1	+
rs42548511	1	84580772	531327	LAMP3	+
rs43242284	1	67635587	540789	PARP14	+
rs43243495	1	61026239	1E+08	LOC100336456	-
rs43266121	1	1.29E+08	1E+08	LOC100295095	-
rs43703977	1	393248	528014	KCNE1	+
1b	1	86500000	0	0	0
2a	1	6.00E+06	0	0	0
rs109384003	1	1.33E+08	0	0	0
rs42745627	1	1.57E+08	0	0	0
1c	2	1.26E+08	518699	SMPDL3B	-
2b	2	6900000	521240	WDR75	-
3b	2	85300000	531691	HECW2	-
rs109065702	2	1.05E+08	338072	SMARCAL1	+
rs109949037	2	30131000	533065	SCN9A	+
rs110073925	2	30102137	533065	SCN9A	+
rs110073925	2	30102137	780963	LOC780963	+
rs110994776	2	89545687	338074	AOX1	+
rs135748641	2	73815258	509598	NIFK	-
rs135748641	2	73815258	509943	TSN	+
rs17870910	2	6611059	539672	ASNSD1	-
rs209148339	2	1.34E+08	338052	CAPZB	+
rs209148339	2	1.34E+08	512930	PQLC2	-
rs210348685	2	5397909	614576	BIN1	+
rs210348685	2	5397909	1E+08	MIR2350	+
rs29014082	2	1.34E+08	788579	IFFO2	+
rs29019569	2	1.27E+08	533908	RPS6KA1	-
rs43330759	2	1.36E+08	509439	NECAP2	-
4b	2	90200000	0	0	0
rs109095895	2	73234975	0	0	0
rs110211659	2	43436458	0	0	0
rs43712960	3	1.03E+08	512622	JMJD2A	-
3a	3	8.20E+07	0	0	0
rs41585097	3	7052779	0	0	0
rs42936243	3	65848664	0	0	0
4a	4	3.10E+07	514631	CDCA7L	-

4a	4	3.10E+07	519566	RAPGEF5	-
77244_529	4	1.17E+08	281123	DPP6	+
BFGL-NGS-19480	4	1.17E+08	281123	DPP6	+
BTB-00210449	4	1.17E+08	281123	DPP6	+
rs109314460	4	1.18E+08	511899	INSIG1	+
rs41256901	4	1.07E+08	282603	PRSS2	+
rs41256901	4	1.07E+08	509513	LOC509513	+
rs41256901	4	1.07E+08	786254	LOC786254	+
rs42233645	4	83121400	614722	AMPH	+
rs109406059	4	60324045	0	0	0
rs29017229	4	30202937	0	0	0
rs42824767	4	74006260	0	0	0
rs43706834	5	78776781	516544	DENND5B	+
5b	5	33700000	0	0	0
6b	5	1.23E+08	0	0	0
rs110188299	5	32861996	0	0	0
5a	6	1.03E+08	537631	MAPK10	-
rs109201532	6	89251522	534082	ADAMTS3	-
rs41577868	6	37983812	536203	ABCG2	+
rs43455987	6	42155077	614299	KCNIP4	-
rs43710085	6	87878904	514798	RUFY3	+
2c	6	5.50E+07	0	0	0
rs109373082	6	1.03E+08	0	0	0
10b	7	71800000	785561	LOC785561	-
6a	7	2.30E+07	280824	IL4	-
6a	7	2.30E+07	541246	KIF3A	-
7b	7	21400000	618380	ZFR2	+
rs109727850	7	98485261	281039	CAST	+
rs110202648	7	17046540	338067	PRKCSH	+
rs110202648	7	17046540	517994	CCDC151	-
rs110202648	7	17046540	520376	ELAVL3	-
rs210072660	7	98535683	281039	CAST	+
rs41591260	7	86291041	538062	EDIL3	-
rs42857364	7	27413672	1E+08	LOC100138814	-
11b	7	1.16E+08	0	0	0
3c	7	9.30E+07	0	0	0
8b	7	3.40E+07	0	0	0
9b	7	36900000	0	0	0
14b	8	78400000	613960	GKAP1	-
rs109380245	8	54507754	533044	PSAT1	+
rs110937563	8	6687056	512259	HPGD	-
rs136892391	8	1456250	522719	CLCN3	+
rs41660853	8	458919	505322	ANXA10	+
rs41660853	8	458919	787280	LOC787280	-
rs41661176	8	1.07E+08	282647	PAPPA	+

rs41793507	8	53334416	613814	FOXB2	+
rs42248819	8	64593005	282382	TGFBR1	+
rs42248819	8	64593005	785629	NDUFA2	-
rs43705534	8	90553133	512696	SHC3	+
12b	8	12700000	0	0	0
13b	8	30400000	0	0	0
7a	9	1.80E+07	0	0	0
8a	9	2.60E+07	0	0	0
rs110608668	9	33321976	0	0	0
rs41610951	9	1.02E+08	0	0	0
rs42797639	9	20734430	0	0	0
4c	10	3.10E+07	767944	ATPBD4	-
rs109635380	10	68789694	1E+08	LOC100337503	+
rs110205401	10	91955798	614412	NRXN3	+
rs110594909	10	92127288	614412	NRXN3	+
rs29013479	10	15477566	615274	LOC615274	+
rs29021916	10	94339279	0	0	0
rs29025348	10	95198461	0	0	0
rs41597140	10	78608571	0	0	0
rs41649229	10	31622218	0	0	0
rs43705629	10	16562010	0	0	0
5c	11	2.90E+07	514773	SOCS5	+
rs109280551	11	1.02E+08	525389	MED27	-
rs109589152	11	68687376	515741	CAPN14	+
rs41624451	11	38733905	525800	CCDC85A	+
10a	11	5.80E+07	0	0	0
9a	11	3.10E+07	0	0	0
rs29011971	11	52491236	0	0	0
rs29013423	11	58208950	0	0	0
15b	12	88900000	282191	COL4A1	-
16b	12	90900000	767974	C12H13orf46	-
16b	12	90900000	282532	RASA3	-
rs29012348	12	80892258	614302	PCCA	+
11a	12	1.07E+08	0	0	0
17b	12	92900000	0	0	0
rs110643661	12	78694446	0	0	0
rs29015935	12	17723687	0	0	0
rs41614805	12	48794617	0	0	0
rs41626249	12	53822508	0	0	0
rs41678448	12	85500588	0	0	0
12a	13	6.80E+07	1E+08	LOC100140857	+
6c	13	1.80E+07	505118	YME1L1	-
rs109232165	13	39569134	616407	SLC24A3	+
rs41566172	13	40001727	786312	LOC786312	+
13a	13	6.90E+07	0	0	0
rs109272278	14	5245003	521058	COL22A1	+

rs110371924	14	81584369	1E+08	LOC100138329	-
rs110442376	14	11621252	327705	ASAP1	+
rs41722889	14	25092333	526726	SDR16C5	-
14a	14	8.70E+07	0	0	0
18b	14	4.10E+07	0	0	0
19b	14	1.07E+08	0	0	0
rs41722036	14	25267155	0	0	0
rs41722043	14	25272251	0	0	0
rs41722045	14	25272477	0	0	0
rs41722844	14	25243108	0	0	0
rs41722854	14	25254418	0	0	0
rs133193054	15	36171715	528261	PLEKHA7	+
rs208805443	15	32681447	533166	SORL1	+
rs29015791	15	48068911	515027	LOC515027	-
rs29015791	15	48068911	787843	OR56B4	-
rs41755948	15	32674699	533166	SORL1	+
rs41759150	15	43779412	513707	IPO7	-
rs41759150	15	43779412	533243	ZNF143	-
rs42670351	15	47386394	281665	CCKBR	-
rs42670352	15	47385604	281665	CCKBR	-
rs42670353	15	47385334	281665	CCKBR	-
rs43705159	15	66208534	508345	APIP	-
rs109589739	15	26532213	0	0	0
rs109918426	15	62428148	0	0	0
20b	16	40400000	506315	LOC506315	+
rs109316815	16	27692042	617138	MGC151839	-
rs110913390	16	27720210	617138	MGC151839	-
rs41662390	16	16244657	531710	FAM5C	-
rs41820824	16	68690299	521326	HMCN1	+
rs41821600	16	68614446	521326	HMCN1	+
21b	16	41300000	0	0	0
7c	16	4.30E+07	0	0	0
15a	17	5.50E+07	515631	CCDC62	+
15a	17	5.50E+07	614238	DENR	-
22b	17	4.00E+06	505156	LOC505156	-
25b	17	18800000	613439	ELF2	+
26b	17	70200000	537659	TTC28	+
23b	17	4900000	0	0	0
24b	17	17800000	0	0	0
rs109762011	17	3766546	0	0	0
rs109762073	17	27950124	0	0	0
rs41636597	17	27808938	0	0	0
16a	18	2.90E+07	1E+08	LOC100138373	-
29b	18	61500000	528568	LOC528568	-
29b	18	61500000	618873	LOC618873	+
rs134420976	18	18193043	508025	ZNF423	-

rs29009652	18	54470798	540310	GRLF1	+
rs29010201	18	50581375	504769	CYP2B	-
rs29010201	18	50581375	789588	LOC789588	-
rs29015137	18	56121920	505162	PPFIA3	-
rs29015137	18	56121920	617610	HRC	+
rs29015137	18	56121920	1E+08	TRPM4	-
rs41639029	18	52971233	282862	BCAM	+
rs41639029	18	52971233	505580	PVRL2	+
rs41639029	18	52971233	615866	CBLC	+
27b	18	4800000	0	0	0
28b	18	4900000	0	0	0
rs29019628	18	17380202	0	0	0
rs41869104	18	20890089	0	0	0
rs42536809	18	21509622	0	0	0
rs43708449	18	82260685	0	0	0
17a	19	4.10E+07	504613	MED24	-
30b	19	34600000	616474	SLC47A1	-
31b	19	36500000	768016	TOB1	-
rs108970074	19	29325678	613567	STX8	+
rs109290793	19	7875822	538147	DGKE	+
rs109290793	19	7875822	1E+08	LOC100295656	+
rs109672208	19	35619269	533979	MPRIP	-
rs41914675	19	36734474	282411	CACNA1G	-
rs41914675	19	36734474	533151	ABCC3	-
18a	19	1.05E+08	0	0	0
19a	19	9.70E+07	0	0	0
32b	19	64400000	0	0	0
33b	19	65400000	0	0	0
34b	19	96200000	0	0	0
35b	19	1.00E+08	0	0	0
rs29015011	19	54287604	0	0	0
rs41907619	19	35286010	0	0	0
36b	20	23800000	520256	SKIV2L2	+
36b	20	23800000	617172	PPAP2A	+
36b	20	23800000	1.05E+08	LOC104968431	+
38b	20	59400000	1E+08	LOC100298754	+
rs109638814	20	10186470	512405	OCLN	-
rs110275805	20	35557902	514720	OSMR	+
rs110293711	20	56539092	281935	MYO10	+
rs133951891	20	38205059	537188	UGT3A2	+
rs209676814	20	31891107	280805	GHR	-
rs41580312	20	5544340	538794	CPEB4	-
rs41580312	20	5544340	613880	LOC613880	-
rs41943134	20	35942763	539504	LIFR	+
rs41946086	20	39867446	538746	SLC45A2	-
rs41946086	20	39867446	539509	RXFP3	-

37b	20	54500000	0	0	0
39b	20	73300000	0	0	0
rs109772819	20	44188479	0	0	0
rs29014641	20	50407500	0	0	0
rs29021101	20	42598095	0	0	0
rs41591215	20	38920878	0	0	0
rs41613438	20	47776960	0	0	0
rs43461171	20	6505111	0	0	0
20a	21	4.00E+06	529593	GABRB3	-
40b	21	2.00E+06	1E+08	LOC100336577	-
rs135777172	21	71091607	539146	GPR132	+
rs41973640	21	31443165	510423	PSMA4	+
rs41973640	21	31443165	530270	AGPHD1	+
rs41980271	21	34243875	506063	ULK3	+
rs41980271	21	34243875	509248	CPLX3	-
rs41980271	21	34243875	534312	SCAMP2	-
rs41980271	21	34243875	617152	LMAN1L	-
rs43020736	21	29654483	524684	PCSK6	+
rs43020769	21	29660419	524684	PCSK6	+
41b	21	38600000	0	0	0
42b	21	73300000	0	0	0
rs110688989	22	38080562	613433	SYNPR	-
rs110978254	22	55257755	613800	ATP2B4	-
rs111018193	22	58099542	539766	FGD5	-
rs29013532	22	56526462	613414	TRH	-
rs41620834	22	30769143	515903	FOXP1	-
21a	22	2.50E+07	0	0	0
rs109404118	23	32331699	537314	LRRC16A	-
rs109404118	23	32331699	537017	LOC537017	+
rs110861313	23	27444064	513252	BAT5	+
rs110861313	23	27444064	539235	CSNK2B	-
rs110861313	23	27444064	539236	LY6G5B	+
rs110861313	23	27444064	617546	C23H6ORF47	+
rs110861313	23	27444064	617553	BAT4	-
rs29014055	23	7810956	521960	IP6K3	-
22a	23	6.60E+07	0	0	0
23a	23	6.70E+07	0	0	0
rs41617133	23	32876929	0	0	0
44b	24	7600000	615496	CD226	+
rs41584268	24	37732967	538404	MYOM1	-
24a	24	2.00E+06	0	0	0
43b	24	5700000	0	0	0
45b	24	56200000	0	0	0
46b	24	63100000	0	0	0
rs29027245	24	18730719	0	0	0
rs41594485	24	29301773	0	0	0

rs41601284	24	29405619	0	0	0
26a	25	1.20E+07	1E+08	LOC100139490	+
rs109899690	25	13749103	1E+08	LOC100295754	+
rs41587267	25	22197501	538496	CACNG3	+
rs42064706	25	14110867	617444	MGC140681	+
rs42064706	25	14110867	1E+08	LOC100295204	+
25a	25	1.30E+07	0	0	0
27a	25	6.60E+07	0	0	0
28a	25	6.70E+07	0	0	0
rs29013464	25	37958335	0	0	0
47b	26	31500000	525419	RBM20	-
49b	26	51500000	614810	JAKMIP3	+
rs29013727	26	8221270	282004	PRKG1	-
29a	26	6.40E+07	0	0	0
30a	26	4.90E+07	0	0	0
48b	26	47500000	0	0	+
rs43708521	26	33605534	0	0	0
rs110763390	27	37357125	524648	HOOK3	+
rs109615803	27	20757482	0	0	0
50b	28	24100000	780777	CTNNA3	-
rs109417884	28	35247970	616669	LOC616669	+
rs109417884	28	35247970	1E+08	LOC100296133	+
rs209765899	28	14993619	540918	FAM13C	+
rs209765899	28	14993619	780878	PHYHIPL	+
rs29011694	28	19731619	512704	REEP3	-
rs110842770	28	15721558	0	0	0
51b	29	42700000	359721	RTN3	+
52b	29	43700000	511908	EHD1	-
52b	29	43700000	518121	CDC42BPG	-
52b	29	43700000	529808	ATG2A	+
53b	29	48500000	618649	SHANK2	+
rs110252499	29	20601858	785165	MGC157332	+
rs17872022	29	43205758	505015	GPR137	+
rs17872022	29	43205758	615013	BAD	-
rs17872022	29	43205758	617184	KCNK4	+
rs17872022	29	43205758	1.07E+08	CATSPERZ	+
rs42190891	29	46550309	534450	LRP5	-
rs110710999	29	18647026	0	0	0
rs29027034	29	37472079	0	0	0

5.3 Appendix 3

Table S3 SNP P-values

name	rfi
rs43242284	0.028
rs208270150	0.004
rs137393885	0.048
rs210348685	0.026
rs135748641	0.036
rs110994776	0.0001
rs109065702	0.034
rs209148339	0.023
rs43330759	0.013
rs109314460	0.006
rs109727850	0.026
rs210072660	0.007
rs133193054	0.04
rs29010201	0.014
rs109638814	0.008
rs209676814	0.026
rs41580312	0.006
rs41943134	0.005
rs133951891	0.023
rs110293711	0.0001
rs42190891	0.018
rs109384003	7E-11
rs42548511	7E-07
rs43266121	9E-07
rs109095895	1E-06
rs109949037	5E-11
rs110073925	9E-11
rs110211659	1E-07
rs41585097	2E-07
rs42936243	3E-08
rs109406059	1E-10
rs42824767	7E-07
rs110188299	4E-07
rs109201532	3E-09
rs109373082	1E-06
rs43455987	9E-07
rs110202648	7E-08
rs109380245	7E-07
rs110937563	1E-06

rs41661176	3E-07
rs110608668	2E-07
rs41610951	8E-07
rs42797639	3E-11
rs109635380	7E-09
rs41597140	5E-07
rs109280551	7E-07
rs109589152	4E-08
rs41624451	4E-07
rs110643661	4E-07
rs41614805	3E-07
rs41626249	7E-11
rs109272278	5E-12
rs110371924	4E-07
rs110442376	1E-06
rs109918426	1E-06
rs41759150	2E-08
rs41662390	2E-07
rs109762011	2E-08
rs109762073	5E-11
rs41636597	8E-12
rs41639029	1E-06
rs108970074	2E-07
rs109672208	1E-07
rs41907619	5E-09
rs110275805	3E-09
rs41591215	9E-07
rs41613438	4E-07
rs43461171	6E-07
rs41973640	6E-07
rs41980271	1E-11
rs110978254	2E-09
rs29013532	2E-08
rs41620834	3E-08
rs109404118	8E-10
rs41617133	5E-07
rs109899690	5E-07
rs41587267	1E-06
rs110763390	5E-09
rs109417884	3E-08
rs110842770	2E-07
rs110252499	5E-07
rs110710999	4E-08
rs29027034	7E-07

rs43703977	0.001
rs29025433	0.02
rs29014082	0.003
rs29017229	0.044
rs43710085	0.003
rs41793507	0.02
rs43705534	0.001
rs29013479	9E-07
rs43705629	0.032
rs29011971	0.0003
rs29013423	0.006
rs29012348	0.0002
rs29015791	0.024
rs43708449	3E-05
rs29019628	0.0002
rs42536809	0.008
rs29009652	0.001
rs29015137	0.008
rs29015011	0.001
rs29014055	0.009
rs29027245	0.002
rs29013464	0.009
rs29013727	0.004
rs43708521	0.007
rs29011694	0.011
rs17872022	0.03
rs41660853	1E-07
rs135777172	5E-08
rs136892391	0.0017
rs41755948	0.007
rs208805443	0.007
rs41821600	0.0033
rs41820824	0.0064
rs134420976	0.0028
rs41914675	0.004
rs43020736	0.016
rs43020769	0.009
rs109561809	0.005
rs209765899	0.009
rs42670352	0.002
rs42670351	0.028
rs42670353	0.0135
rs29019569	0.0051
rs43706834	0.0056

rs29021916	0.053
rs29021101	0.013
rs29014641	0.069
rs17872022	0.0013
1a	0.0005
2a	1E-05
3a	8E-05
4a	0.011
5a	0.0049
6a	0.0025
7a	0.0188
8a	0.0189
9a	0.0086
10a	0.0009
11a	0.0285
12a	0.0471
13a	0.0179
14a	0.0207
15a	0.0279
16a	0.0028
17a	0.0007
18a	0.0007
19a	0.0045
20a	0.0055
21a	0.0014
22a	0.0008
23a	0.0013
24a	0.0102
25a	0.031
26a	0.0232
27a	0.0232
28a	0.0122
29a	0.0014
30a	0.0018
2b	0.013
5b	0.0052
8b	0.0132
9b	0.0118
10b	0.0031
11b	0.0142
12b	0.0094
13b	0.0038
16b	0.006
17b	0.0068

18b	0.0003
22b	0.0037
23b	0.0007
26b	0.0041
27b	0.0043
28b	0.0038
30b	0.0017
31b	0.0012
32b	0.0005
33b	0.0014
36b	0.0021
38b	0.0094
39b	0.0127
41b	0.0021
42b	0.0057
44b	0.0125
45b	0.0016
46b	0.0018
47b	0.0065
51b	0.0027
52b	0.0021
BTB-00210449	0.067
77244_529	0.011
BFGL-NGS-19480	0.108
rs41722889	0.0004
rs41722844	0.02
rs41722854	0.009
rs41722036	0.009
rs41722043	0.003
rs41722045	0.007

5.4 Appendix 4

Script

```
library(genomicper)
#Reading files
pva<-read.table("SNP-pvalo.txt", h=T, sep="\t")
gen<-read.table("gene-SNP.txt", h=T, sep="\t")

all_data <- read_pvals(data_name=pva,snps_ann=gen)
head(all_data)
#genome_order=Orders the SNPs according to their genomic location, it
creates a list
genome_results <-genome_order(all_data=all_data)
names(genome_results)
# Results from genome_order
ordered_alldata <- genome_results$ordered_alldata
head(ordered_alldata)
gs_locs <- genome_results$gs_locs
head(gs_locs)
library(KEGG.db)
gper.env <- new.env()
paths <- get_pathways(source="kegg",all_paths=TRUE,envir = gper.env)
"bta"

###genes permutation
paths_res <- read2_paths(ordered_alldata=ordered_alldata,gs_locs=gs_locs,
sets_from="workspace",sets_prefix="bta",level="gene",envir=gper.env)

pers_ids <- paths_res$per_ors #A list of identifiers mapped to each pathway
pathways<- paths_res$pathways # Pathway Id, Description, Number of
Genes in the pathway, Number of genes found in the dataset, Number of
SNPs found in the dataset

# Create new environment to save the permutations to:
gper.env <- new.env()

genes_permutation(ordered_alldata=ordered_alldata,
pers_ids=pers_ids,pathways=pathways,ntraits=c(7),
nper=10000,threshold=0.0002222222, saveto="workspace",
gs_locs=gs_locs,envir = gper.env)

results <- get_results(res_pattern="Permus",level="gene",
from="workspace",threshold=0.05,envir = gper.env)
results
```

5.5 Appendix 5

Table S4 Lipids compounds identified in muscle with significantly different concentrations for RFI (positive and negative ionization mode).

m/z	High	Inter	Low	Lipids	Classification	p-Value
253.0574	4.100254	0.934374		D-tagaturonic acid	Fatty alcohols [FA05]	0.000295
441.253	4.534123	4.17648		N-linolenoyl-glutamine	Other Fatty Acyls [FA00]	0.000419
				Spiramycin II	Ansamycins and related polyketides [PK05]	
521.3114	3.159176	4.130984		3-dehydroecdysone	Cholesterol and derivatives [ST0101]	0.000838
523.3424		4.268323	4.926786	1α-hydroxy-22-[3-(1-hydroxy-1-methylethyl)phenyl]-23,24,25,26,27-pentanorvitamin D3 / 1α-hydroxy-22-[3-(1-hydroxy-1-methylethyl)phenyl]-23,24,25,26,27-pentanorcholecalciferol	Vitamin D3 and derivatives [ST0302]	0.000495
570.3725	0.895016	3.714802		11α-(4-dimethylaminophenyl)-1α,25-dihydroxyvitamin D3 / 11α-(4-dimethylaminophenyl)-1α,25-dihydroxycholecalcifero	Vitamin D3 and derivatives [ST0302]	0.000108
686.6228		2.227493	2.613938	Cer(d18:0/24:0)	N-acylsphinganines (dihydroceramides) [SP0202]	0.000309
741.5093		0.459289	3.750237	1α,23-dihydroxy-24,25,26,27-tetranorvitamin D3 / 1α,23-dihydroxy-24,25,26,27-tetranorcholecalciferol	Vitamin D3 and derivatives [ST0302]	0.000718
177.007	5.469236	5.292363		2E-hexeno	Fatty alcohols [FA05]	0.00102
				3Z-hexenol	Fatty alcohols [FA05]	
				3Z-hexenol	Fatty alcohols [FA05]	
				2E-hexenol	Fatty alcohols [FA05]	
				Wedelolactone	Coumestan flavonoids [PK1209]	

206.0812	4.354165	0.96339		9-hydroxy-7Z-Nonene-3,5-diynoic acid	Unsaturated fatty acids [FA0103]	0.000164
				9-hydroxy-7E-Nonene-3,5-diynoic acid	Unsaturated fatty acids [FA0103]	
				clavulolactone II	Clavulones [FA0312]	
				clavulolactone I	Clavulones [FA0312]	
				clavulolactone III	Clavulones [FA0312]	
				pyrethrin II	C10 isoprenoids (monoterpenes) [PR0102]	
				16-phenyl-tetranor-PGE2	Prostaglandins [FA0301]	
213.0522	3.876415	0.487031		Ethyladipic acid	Dicarboxylic acids [FA0117]	0.000367
218.1176	4.331876	2.961719		Safynol	Fatty alcohols [FA05]	0.000331
				2-Decene-4,6-diynoic acid, methyl ester, (E)-	Wax diesters [FA0702]	
265.1564	4.15199		1.01747	2-tetradecenoic acid	Unsaturated fatty acids [FA0103]	0.000486
				3E-tetradecenoic acid	Unsaturated fatty acids [FA0103]	
				Tsuzuic acid	Unsaturated fatty acids [FA0103]	
				Physeteric acid	Unsaturated fatty acids [FA0103]	
				7Z-tetradecenoic acid	Unsaturated fatty acids [FA0103]	
				8Z-tetradecenoic acid	Unsaturated fatty acids [FA0103]	
				Myristelaidic acid	Unsaturated fatty acids [FA0103]	
				5-methyl-2E-tridecenoic acid	Branched fatty acids [FA0102]	
				2,4-dimethyl-2E-dodecenoic acid	Branched fatty acids [FA0102]	
				Falcarinone	Oxygenated hydrocarbons [FA12]	
365.3172	3.969535	0.927659		Behenyl alcohol	Fatty alcohols [FA05]	7.54E-05

				Anandamide (20:4, n-6)	N-acyl ethanolamines (endocannabinoids)[FA0804]	
				Anandamide (18:2, n-6)	N-acyl ethanolamines (endocannabinoids)[FA0804]	
397.2334	4.472833		3.305784	(9S,10S)-10-hydroxy-9-(phosphonooxy)octadecanoic acid	Other Fatty Acyls [FA00]	0.001096
				9,12-dimethoxy-13-hydroxy-10-octadecenoic acid	Methoxy fatty acids [FA0108]	
				9,13-dihydroxy-12-ethoxy-10-octadecenoic acid	Hydroxy fatty acids [FA0105]	
				9,10-dimethoxy-13-hydroxy-11-octadecenoic acid	Methoxy fatty acids [FA0108]	
				9,13-dihydroxy-10-ethoxy-11-octadecenoic acid	Hydroxy fatty acids [FA0105]	
				11,13-dimethoxy-12-hydroxy-9-octadecenoic acid	Methoxy fatty acids [FA0108]	
				1α-hydroxy-24,25,26,27-tetranorvitamin D3 23-carboxylic acid / calcitroic acid	Vitamin D3 and derivatives [ST0302]	
				Digitoxigenin	Cardanolides and derivatives [ST0112]	
				4,8,12,15,19,21-tetracosahexaenoic acid	Unsaturated fatty acids [FA0103]	
				5β-Chola-8(14),11-dien-24-oic Acid	C24 bile acids, alcohols, and derivatives [ST0401]	
				5β-Chola-7,9(11)-dien-24-oic Acid	C24 bile acids, alcohols, and derivatives [ST0401]	
				5β-Chola-3,11-dien-24-oic Acid	C24 bile acids, alcohols, and derivatives [ST0401]	
				tetracosahexaenoic acid	Unsaturated fatty acids [FA0103]	
408.2515	4.427529	2.725102		PC(11:0/0:0)	Monoacylglycero-phosphocholines [GP0105]	7.33E-06
				PE(14:0/0:0)	Monoacylglycerophospho-ethanolamines [GP0205]	
				cis-5-Tetradecenoylcarnitine	Fatty acyl carnitines [FA0707]	
				1a,1b-dihomo-15-deoxy-δ-12,14-PGJ2	Prostaglandins [FA0301]	
				(+/-)-16-HDoHE	Other Docosanoids [FA0400]	

(+/-)-17-HDoHE	Other Docosanoids [FA0400]
16(17)-EpDPE	Other Docosanoids [FA0400]
17S-HDHA	Other Docosanoids [FA0400]
(+/-)-13-HDoHE	Other Docosanoids [FA0400]
(+/-)-14-HDoHE	Other Docosanoids [FA0400]
13(14)-EpDPE	Other Docosanoids [FA0400]
(+/-)-10-HDoHE	Other Docosanoids [FA0400]
(+/-)-11-HDoHE	Other Docosanoids [FA0400]
10(11)-EpDPE	Other Docosanoids [FA0400]
(+/-)-7-HDoHE	Other Docosanoids [FA0400]
(+/-)-8-HDoHE	Other Docosanoids [FA0400]
7(8)-EpDPE	Other Docosanoids [FA0400]
(+/-)-4-HDoHE	Other Docosanoids [FA0400]
1α-hydroxy-22-oxo-23,24,25,26,27-pentanorvitamin D3 / 1α-hydroxy-22-oxo-23,24,25,26,27-pentanorcholecalciferol	Vitamin D3 and derivatives [ST0302]
medroxyprogesterone	C21 steroids (gluco/mineralocorticoids,progestogins) and derivatives [ST0203]
Testosterone propionate	C19 steroids (androgens) and derivatives [ST0202]
(+/-)-20-HDoHE	Other Docosanoids [FA0400]
19(20)-EpDPE	Other Docosanoids [FA0400]
(+/-)-17-HDoHE	Other Docosanoids [FA0400]
SQDG(16:0/16:1(13Z))	Glycosyldiacylglycerols [GL0501]
SQDG(16:0/16:1(11Z))	Glycosyldiacylglycerols [GL0501]

				SQDG(16:0/16:1(9Z))	Glycosyldiacylglycerols [GL0501]	
418.2186	4.161167	1.307911		5-hydroperoxy-7-[3,5-epidioxy-2-(2-octenyl)-cyclopentyl]-6-heptenoic acid	Hydroperoxy fatty acids [FA0104]	0.00106
434.3471	4.490634		3.018067	TG(16:1(9Z)/16:1(9Z)/18:1(9Z))[iso3]	Triacylglycerols [GL0301]	0.000252
				TG(16:0/16:0/18:3(9Z,12Z,15Z))[iso3]	Triacylglycerols [GL0301]	
				TG(16:0/16:1(9Z)/18:2(9Z,12Z))[iso6]	Triacylglycerols [GL0301]	
				TG(16:0/17:1(9Z)/17:2(9Z,12Z))[iso6]	Triacylglycerols [GL0301]	
				TG(16:1(9Z)/17:0/17:2(9Z,12Z))[iso6]	Triacylglycerols [GL0301]	
				TG(16:1(9Z)/17:1(9Z)/17:1(9Z))[iso3]	Triacylglycerols [GL0301]	
540.4252	4.255119	2.982334		TG(22:0/22:4(7Z,10Z,13Z,16Z)/22:5(7Z,10Z,13Z,16Z,19Z))[iso6]	Triacylglycerols [GL0301]	0.000498
				TG(22:0/22:3(10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso6]	Triacylglycerols [GL0301]	
				TG(22:1(13Z)/22:4(7Z,10Z,13Z,16Z)/22:4(7Z,10Z,13Z,16Z))[iso3]	Triacylglycerols [GL0301]	
				TG(22:1(13Z)/22:3(10Z,13Z,16Z)/22:5(7Z,10Z,13Z,16Z,19Z))[iso6]	Triacylglycerols [GL0301]	
				TG(22:1(13Z)/22:2(13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso6]	Triacylglycerols [GL0301]	
				TG(22:2(13Z,16Z)/22:2(13Z,16Z)/22:5(7Z,10Z,13Z,16Z,19Z))[iso3]	Triacylglycerols [GL0301]	
				TG(22:3(10Z,13Z,16Z)/22:2(13Z,16Z)/22:4(7Z,10Z,13Z,16Z))[iso6]	Triacylglycerols [GL0301]	
				TG(22:3(10Z,13Z,16Z)/22:3(10Z,13Z,16Z)/22:3(10Z,13Z,16Z))	Triacylglycerols [GL0301]	
542.3835		4.97041	5.628018	PC(15:0/0:0)	Monoacylglycero-phosphocholines [GP0105]	0.001356
				PE(18:0/0:0)	Monoacylglycerophospho-ethanolamines [GP0205]	
				SLBPA(54:3)	Diacylglycerophospho-monoradylglycerols [GP0409]	

				1α,25-dihydroxy-2α-(3-hydroxypropoxy)-19-norvitamin D3 / 1α,25-dihydroxy-2α-(3-hydroxypropoxy)-19-norcholecalciferol	Vitamin D3 and derivatives [ST0302]	
				PC(17:0/0:0)	Monoacylglycero-phosphocholines [GP0105]	
544.5082	3.427096		0	Lacceroic acid	Straight chain fatty acids [FA0101]	0.00044
				Palmityl palmitate	Wax monoesters [FA0701]	
560.4092		0.951133	4.003176	PC(22:1(13Z)/0:0)	Monoacylglycero-phosphocholines [GP0105]	0.000904
592.3457	3.737285	1.043712		Cucurbitacin A	Cholesterol and derivatives [ST0101]	0.00106
607.4166	4.457127	0.967555		PM-Toxin A	Fatty alcohols [FA05]	0.000134
685.4645	3.988374	0.523726		1-(O-α-D-glucopyranosyl)-27-keto-(1,3R,29R)-triacontanetriol	Fatty acyl glycosides of mono- and disaccharides [FA1301]	0.000479
				1-(O-α-D-glucopyranosyl)-3-keto-(1,27R,29R)-triacontanetriol	Fatty acyl glycosides of mono- and disaccharides [FA1301]	
				1-(O-α-D-galactopyranosyl)-3-keto-(1,27R,29R)-triacontanetriol	Fatty acyl glycosides of mono- and disaccharides [FA1301]	
				PG(12:0/13:0)	Diacylglycerophosphoglycerols [GP0401]	
				15-oxo-11Z,13E-eicosadienoic acid	Oxo fatty acids [FA0106]	
				15-HETrE	Hydroxy/hydroperoxyeicosatrienoic acids [FA0305]	
				5-HETrE	Hydroxy/hydroperoxyeicosatrienoic acids [FA0305]	
				15S-HETrE	Hydroxy/hydroperoxyeicosatrienoic acids [FA0305]	
				12R-HETrE	Hydroxy/hydroperoxyeicosatrienoic acids [FA0305]	
				8-HETrE	Hydroxy/hydroperoxyeicosatrienoic acids [FA0305]	
731.5046		0	3.362896	PA(18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Diacylglycerophosphates [GP1001]	0.000202

			Azithromycin	Macrolides and lactone polyketides [PK04]	
			PE(P-16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	1-(1Z-alkenyl),2-acylglycerophospho-ethanolamines [GP0203]	
			DG(20:2(11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)[iso2]	Diacylglycerols [GL0201]	
			DG(20:3(8Z,11Z,14Z)/22:5(7Z,10Z,13Z,16Z,19Z)/0:0)[iso2]	Diacylglycerols [GL0201]	
			DG(20:5(5Z,8Z,11Z,14Z,17Z)/22:3(10Z,13Z,16Z)/0:0)[iso2]	Diacylglycerols [GL0201]	
			DG(20:4(5Z,8Z,11Z,14Z)/22:4(7Z,10Z,13Z,16Z)/0:0)[iso2]	Diacylglycerols [GL0201]	
			5β-Chola-3,8(14),11-trien-24-oic Acid	C24 bile acids, alcohols, and derivatives [ST0401]	
824.6145	2.245731	3.849867	PE(18:0/22:1(13Z))	Diacylglycerophospho-ethanolamines [GP0201]	0.001215
			PE(20:0/20:1(11Z))	Diacylglycerophospho-ethanolamines [GP0201]	
			PE(20:1(13Z)/20:0)	Diacylglycerophospho-ethanolamines [GP0201]	
			PE(20:1(13E)/20:0)	Diacylglycerophospho-ethanolamines [GP0201]	
			PE(18:1(9Z)/22:0)	Diacylglycerophospho-ethanolamines [GP0201]	
			PE(22:0/18:1(9Z))	Diacylglycerophospho-ethanolamines [GP0201]	
			PE(22:0/18:1(7Z))	Diacylglycerophospho-ethanolamines [GP0201]	
			PE(18:1(11Z)/22:0)	Diacylglycerophospho-ethanolamines [GP0201]	
			PE(18:1(11E)/22:0)	Diacylglycerophospho-ethanolamines [GP0201]	
			PE(18:1(9Z)/22:0)	Diacylglycerophospho-ethanolamines [GP0201]	

			PA(20:0/20:0)	Diacylglycerophosphates [GP1001]	
			PA(18:0/22:0)	Diacylglycerophosphates [GP1001]	
			PA(18:0/22:0)	Diacylglycerophosphates [GP1001]	
896.7689	4.632408	5.071874	TG(16:0/16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso3]	Triacylglycerols [GL0301]	0.001197
			TG(16:0/18:2(9Z,12Z)/20:4(5Z,8Z,11Z,14Z))[iso6]	Triacylglycerols [GL0301]	
			TG(16:0/18:3(9Z,12Z,15Z)/20:3(8Z,11Z,14Z))[iso6]	Triacylglycerols [GL0301]	
			TG(16:0/16:1(9Z)/22:5(7Z,10Z,13Z,16Z,19Z))[iso6]	Triacylglycerols [GL0301]	
			TG(16:0/18:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))[iso6]	Triacylglycerols [GL0301]	
			TG(17:0/17:2(9Z,12Z)/20:4(5Z,8Z,11Z,14Z))[iso6]	Triacylglycerols [GL0301]	
			TG(17:0/17:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))[iso6]	Triacylglycerols [GL0301]	
			TG(18:0/18:3(9Z,12Z,15Z)/18:3(9Z,12Z,15Z))[iso3]	Triacylglycerols [GL0301]	
			TG(16:1(9Z)/18:0/20:5(5Z,8Z,11Z,14Z,17Z))[iso6]	Triacylglycerols [GL0301]	
			TG(18:1(9Z)/18:2(9Z,12Z)/18:3(9Z,12Z,15Z))[iso6]	Triacylglycerols [GL0301]	
			TG(17:1(9Z)/17:2(9Z,12Z)/20:3(8Z,11Z,14Z))[iso6]	Triacylglycerols [GL0301]	
			TG(17:1(9Z)/17:1(9Z)/20:4(5Z,8Z,11Z,14Z))[iso3]	Triacylglycerols [GL0301]	
			TG(16:1(9Z)/18:3(9Z,12Z,15Z)/20:2(11Z,14Z))[iso6]	Triacylglycerols [GL0301]	
			TG(16:1(9Z)/18:2(9Z,12Z)/20:3(8Z,11Z,14Z))[iso6]	Triacylglycerols [GL0301]	
			TG(16:1(9Z)/16:1(9Z)/22:4(7Z,10Z,13Z,16Z))[iso3]	Triacylglycerols [GL0301]	
			TG(17:2(9Z,12Z)/17:2(9Z,12Z)/20:2(11Z, 14Z))[iso3]	Triacylglycerols [GL0301]	
			TG(18:2(9Z,12Z)/18:2(9Z,12Z)/18:2(9Z,12Z))	Triacylglycerols [GL0301]	
			TG(16:1(9Z)/18:1(9Z)/20:4(5Z,8Z,11Z,14Z))[iso6]	Triacylglycerols [GL0301]	
			TG(16:0/16:0/20:4(5Z,8Z,11Z,14Z))[iso3]	Triacylglycerols [GL0301]	
			TG(16:0/18:2(9Z,12Z)/18:2(9Z,12Z))[iso3]	Triacylglycerols [GL0301]	
			TG(16:0/18:1(9Z)/18:3(9Z,12Z,15Z))[iso6]	Triacylglycerols [GL0301]	
			TG(16:0/16:1(9Z)/20:3(8Z,11Z,14Z))[iso6]	Triacylglycerols [GL0301]	

				TG(17:0/17:2(9Z,12Z)/18:2(9Z,12Z))[iso6]	Triacylglycerols [GL0301]	
				TG(17:0/17:1(9Z)/18:3(9Z,12Z,15Z))[iso6]	Triacylglycerols [GL0301]	
				TG(17:2(9Z,12Z)/17:2(9Z,12Z)/18:0)[iso3]	Triacylglycerols [GL0301]	
				TG(16:1(9Z)/18:0/18:3(9Z,12Z,15Z))[iso6]	Triacylglycerols [GL0301]	
				TG(17:1(9Z)/17:2(9Z,12Z)/18:1(9Z))[iso6]	Triacylglycerols [GL0301]	
				TG(17:1(9Z)/17:1(9Z)/18:2(9Z,12Z))[iso3]	Triacylglycerols [GL0301]	
				TG(16:1(9Z)/16:1(9Z)/20:2(11Z,14Z))[iso3]	Triacylglycerols [GL0301]	
				TG(16:1(9Z)/18:1(9Z)/18:2(9Z,12Z))[iso6]	Triacylglycerols [GL0301]	
				TG(16:0/16:0/18:1(9Z))	Triacylglycerols [GL0301]	
				TG(16:0/16:0/18:1(11E))	Triacylglycerols [GL0301]	
				TG(16:1(9Z)/17:0/17:0)[iso3]	Triacylglycerols [GL0301]	
				TG(16:0/17:0/17:1(9Z))[iso6]	Triacylglycerols [GL0301]	
				TG(16:0/16:1(9Z)/18:0)[iso6]	Triacylglycerols [GL0301]	
978.9414	3.082747	0		TG(19:0/20:0/20:0)[iso3]	Triacylglycerols [GL0301]	0.00021
				TG(17:0/21:0/21:0)[iso3]	Triacylglycerols [GL0301]	
				TG(18:0/20:0/21:0)[iso6]	Triacylglycerols [GL0301]	
				TG(19:0/19:0/21:0)[iso3]	Triacylglycerols [GL0301]	
				TG(16:0/21:0/22:0)[iso6]	Triacylglycerols [GL0301]	
				TG(17:0/20:0/22:0)[iso6]	Triacylglycerols [GL0301]	
				TG(18:0/19:0/22:0)[iso6]	Triacylglycerols [GL0301]	
1005.96	4.457963		2.827003	TG(21:0/22:0/22:1(13Z))[iso6]	Triacylglycerols [GL0301]	0.0003
1065.806	4.513784	1.014673		TG(21:0/22:5(7Z,10Z,13Z,16Z,19Z)/22:5(7Z,10Z,13Z,16Z,19Z))[iso3]	Triacylglycerols [GL0301]	0.000193
				TG(21:0/22:4(7Z,10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso6]	Triacylglycerols [GL0301]	

5.6 Appendix 6

Table S5 Lipids compounds identified in liver with significantly different concentrations for RFI (positive ionization mode).

m/z	High	Inter	Low	Lipids	Classification	*p*-Value
182.0909		1.495907	3.955426	(-)-menthyl β-D-glucoside	C10 isoprenoids (monoterpenes) [PR0102]	0.001103
				2,4-diamino-butyric acid	Amino fatty acids [FA0110]	
				2,4-diamino-butyric acid	Amino fatty acids [FA0110]	
194.1516		0.986388	4.43726	n-octanol	Fatty alcohols [FA05]	1.91E-05
				xi-2-Ethyl-1-hexanol	Fatty alcohols [FA05]	
200.1645		5.187419	5.268909	2E,4E-undecadienoic acid	Unsaturated fatty acids [FA0103]	0.001707
				(E)-3,7-Dimethyl-2,6-octadienyl formate	Wax monoesters [FA0701]	
				2-undecynoic acid	Unsaturated fatty acids [FA0103]	
				3-undecynoic acid	Unsaturated fatty acids [FA0103]	
				4-undecynoic acid	Unsaturated fatty acids [FA0103]	
				5-undecynoic acid	Unsaturated fatty acids [FA0103]	
				6-undecynoic acid	Unsaturated fatty acids [FA0103]	
				7-undecynoic acid	Unsaturated fatty acids [FA0103]	
				8-undecynoic acid	Unsaturated fatty acids [FA0103]	
				9-undecynoic acid	Unsaturated fatty acids [FA0103]	
				10-undecynoic acid	Unsaturated fatty acids [FA0103]	
				PGF2α dimethyl amide	Prostaglandins [FA0301]	

				11,12-DiHETrE-EA	N-acyl ethanolamines (endocannabinoids)[FA0804]	
				8,9-DiHETrE-EA	N-acyl ethanolamines (endocannabinoids)[FA0804]	
				5,6-DiHETrE-EA	N-acyl ethanolamines (endocannabinoids)[FA0804]	
				14,15-DiHETrE-EA	N-acyl ethanolamines (endocannabinoids)[FA0804]	
				Tricosylic acid	Straight chain fatty acids [FA0101	
				Isotricosanoic acid	Branched fatty acids [FA0102]	
				(+)-20-methyl-docosanoic acid	Branched fatty acids [FA0102]	
				5-methyl-octanoic acid	Branched fatty acids [FA0102]	
				Isopropyl hexanoate	Branched fatty acids [FA0102]	
				4-methyl-octanoic acid	Branched fatty acids [FA0102]	
				3-methyl-octanoic acid	Branched fatty acids [FA0102]	
				Oenanthic ether	Wax monoesters [FA0701]	
				2-methyl-octanoic acid	Branched fatty acids [FA0102]	
236.1623	3.812638		1.086119	PE(16:0/0:0)	Monoacylglycerophospho-ethanolamines [GP0205]	2.58E-05
				PC(13:0/0:0)	Monoacylglycero-phosphocholines [GP0105]	
				Isopentyl isopentanoate	Wax monoesters [FA0701]	
				1α,3β,4β-p-menthane-3,8-diol	C10 isoprenoids (monoterpenes) [PR0102]	
				7-methyl-nonanoic acid	Branched fatty acids [FA0102]	
				3-methyl-nonanoic acid	Branched fatty acids [FA0102]	
				ethyl octanoate	Wax monoesters [FA0701]	
				2-methyl nonaoic acid	Branched fatty acids [FA0102]	
				Capric acid	Straight chain fatty acids [FA0101]	
244.1544		5.132836	5.264277	Tiglylcarnitine	Fatty acyl carnitines [FA0707]	0.000493

			Tuberonic acid	Jasmonic acids [FA0202]	
			epi-4'-hydroxyjasmonic acid	Jasmonic acids [FA0202]	
			Sebacic acid	Dicarboxylic acids [FA0117]	
			Ecdysone	Cholesterol and derivatives [ST0101]	
			ponasterone A	Cholesterol and derivatives [ST0101]	
			3α,6α,7α,12α-Tetrahydroxy-5β-cholest-24-en-26-oic acid	C27 bile acids, alcohols, and derivatives [ST0403]	
255.1591	4.898884	5.016283	Sphingosine-1-phosphocholine	Lysosphingomyelins and lysoglycosphingolipids [SP0106]	0.000756
			(6S)-dehydrovomifoliol	C15 isoprenoids (sesquiterpenes) [PR0103]	
			Sphingosine-1-phosphocholine	Lysosphingomyelins and lysoglycosphingolipids [SP0106]	
			(6S)-dehydrovomifoliol	C15 isoprenoids (sesquiterpenes) [PR0103]	
			Dehydroabietic acid	C20 isoprenoids (diterpenes) [PR0104]	
			Retinoic Acid	Retinoids [PR0109]	
			9-cis-retinoic acid	Retinoids [PR0109]	
			20,14-retro-retinoic acid	Retinoids [PR0109]	
			13-cis-retinoic acid,Isotretinoin	Retinoids [PR0109]	
			11-cis-Retinoic acid	Retinoids [PR0109]	
			9,13-di-cis-retinoic acid	Retinoids [PR0109]	
			9,12,15-Eicosatriynoic acid	Unsaturated fatty acids [FA0103]	
			8,11,14,18-Eicosatetraynoic acid	Unsaturated fatty acids [FA0103]	
			7,10,13-Eicosatriynoic acid	Unsaturated fatty acids [FA0103]	
			6,9,12-Eicosatriynoic acid	Unsaturated fatty acids [FA0103]	
			5,8,11-Eicosatriynoic acid	Unsaturated fatty acids [FA0103]	
291.2681	4.689786	4.582808	Geranylgeraniol	C20 isoprenoids (diterpenes) [PR0104]	0.000601

			Verrucosan-2b-ol	C20 isoprenoids (diterpenes) [PR0104]	
			8-[3]-ladderane-1-octanol	Fatty alcohols [FA05]	
			aphidicolan-16β-ol	C20 isoprenoids (diterpenes) [PR0104]	
			tuberculosinol	C20 isoprenoids (diterpenes) [PR0104]	
			8Z,11Z-eicosadienoic acid	Unsaturated fatty acids [FA0103]	
			11Z,14Z-eicosadienoic acid	Unsaturated fatty acids [FA0103]	
			11Z,15Z-eicosadienoic acid	Unsaturated fatty acids [FA0103]	
			sclareol	C20 isoprenoids (diterpenes) [PR0104]	
308.2946	4.635227	4.730191	N-oleoyl ethanolamine	N-acyl ethanolamines (endocannabinoids)[FA0804]	0.001762
			Geranylgeraniol	C20 isoprenoids (diterpenes) [PR0104]	
			Verrucosan-2b-ol	C20 isoprenoids (diterpenes) [PR0104]	
			8-[3]-ladderane-1-octanol	Fatty alcohols [FA05]	
			aphidicolan-16β-ol	C20 isoprenoids (diterpenes) [PR0104]	
			tuberculosinol	C20 isoprenoids (diterpenes) [PR0104]	
			9-octadecenal	Fatty aldehydes [FA06]	
			11-octadecenal	Fatty aldehydes [FA06]	
			2E,13Z-octadecadien-1-ol	Fatty alcohols [FA05]	
			3Z,13Z-octadecadien-1-ol	Fatty alcohols [FA05]	
			3E,13Z-octadecadien-1-ol	Fatty alcohols [FA05]	
357.1667	4.166651	2.459675	(-)-menthyl β-D-glucoside	C10 isoprenoids (monoterpenes) [PR0102]	4.08E-05
			PGA2 1,15-lactone	Prostaglandins [FA0301]	

				preclavulone lactone I	Clavulones [FA0312]	
				preclavulone lactone II	Clavulones [FA0312]	
				15-deoxy-δ-12,14-PGA2	Prostaglandins [FA0301]	
				2-Methoxyestradiol-3-methylether	C18 steroids (estrogens) and derivatives [ST0201]	
				gibberellin A12 aldehyde	C20 isoprenoids (diterpenes) [PR0104]	
				15-deoxy-δ-12,14-PGJ2	Prostaglandins [FA0301]	
				all-trans-5,6-Epoxyretinoic acid	Retinoids [PR0109]	
				all-trans-4-hydroxyretinoic acid	Retinoids [PR0109]	
				all-trans-18-Hydroxyretinoic acid	Retinoids [PR0109]	
				19-Hydroxy-all-trans-retinoic acid	Retinoids [PR0109]	
				19-Hydroxy-13-cis-retinoic acid	Retinoids [PR0109]	
				3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide	C20 isoprenoids (diterpenes) [PR0104]	
370.1281		0.988249	4.066769	10-hydroxy-8E-Decene-2,4,6-triynoic acid	Unsaturated fatty acids [FA0103]	0.001619
				7-Hydroxy-2',4',5'-trimethoxyisoflavone	Isoflavonoids [PK1205]	
				6-Hydroxy-5,7,4'-trimethoxyflavone	Flavones and Flavonols [PK1211]	
				βgarin	Flavanones [PK1214]	
383.1673	4.122324	2.664524		2,3-Dinor-TXB2	Thromboxanes [FA0303]	0.001075
				2,3-dinor, 6-keto-PGF1α	Prostaglandins [FA0301]	
395.1673		2.25415	3.868201	5-hydroperoxy-7-[3,5-epidioxy-2-(2-octenyl)-cyclopentyl]-6-heptenoic acid	Hydroperoxy fatty acids [FA0104]	0.000983
435.4195		0.483709	4.080804	Campestanol	Ergosterols and C24-methyl derivatives [ST0103]	0.001324
				4α-methyl-5β-cholestan-3β-ol	Cholesterol and derivatives [ST0101]	
				4α-methyl-5α-cholestan-3β-ol	Cholesterol and derivatives [ST0101]	
				Ergostanol	Ergosterols and C24-methyl derivatives [ST0103]	

450.2036		0.49999	3.435587	PC(O-8:0/2:0) PC(10:0/0:0) PE(13:0/0:0)	1-alkyl,2-acylglycerophosphocholines [GP0102] Monoacylglycero-phosphocholines [GP0105] Monoacylglycerophospho-ethanolamines [GP0205]	0.00112
473.2353	4.232148		2.559709	Undecanedioic acid	Dicarboxylic acids [FA0117]	0.000267
491.2891		0	4.044748	(24R)-24-fluoro-1α,25-dihydroxyvitamin D2 / (24R)-24-fluoro-1α,25-dihydroxyergocalciferol (24S)-24-fluoro-1α,25-dihydroxyvitamin D2 / (24S)-24-fluoro-1α,25-dihydroxyergocalciferol	Vitamin D2 and derivatives [ST0301] Vitamin D2 and derivatives [ST0301]	0
515.2824	4.604957	1.510894		3-hydroxy-dodecanedioic acid 1-O-α-D-glucopyranosyl-1,2-eicosandiol 1α,25-Dihydroxy-2α-(3-hydroxypropyl)vitamin D3 1α,25-dihydroxy-2β-(3-hydroxypropyl)vitamin D3 / 1α,25-dihydroxy-2β-(3-hydroxypropyl)cholecalciferol 1α-hydroxy-2β-(3-hydroxypropoxy)vitamin D3 / 1α-hydroxy-2β-(3-hydroxypropoxy)cholecalciferol 1α,25-dihydroxy-26,27-dimethyl-24a,24b-dihomo-22-oxa-20-epivitamin D3 / 1α,25-dihydroxy-26,27-dimethyl-24a,24b-dihomo-22-oxa-20-epicholecalciferol 1α-hydroxy-18-(4-hydroxy-4-ethylhexyloxy)-23,24,25,26,27-pentanorvitamin D3 / 1α-hydroxy-18-(4-hydroxy-4-ethylhexyloxy)-23,24,25,26,27-pentanorcholecalciferol (22R)-1α,22,25-trihydroxy-26,27-dimethyl-24a-homo-20-epivitamin D3 / (22R)-	Dicarboxylic acids [FA0117] Fatty acyl glycosides of mono- and disaccharides [FA1301] Vitamin D3 and derivatives [ST0302] Vitamin D3 and derivatives [ST0302] Vitamin D3 and derivatives [ST0302] Vitamin D3 and derivatives [ST0302] Vitamin D3 and derivatives [ST0302] Vitamin D3 and derivatives [ST0302]	0.000409

				1α,22,25-trihydroxy-26,27-dimethyl-24a-homo-20-epicholecalciferol	Vitamin D3 and derivatives [ST0302]	
				(22S)-1α,22,25-trihydroxy-26,27-dimethyl-24a-homovitamin D3 / (22S)-1α,22,25-trihydroxy-26,27-dimethyl-24a-homocholecalciferol	Vitamin D3 and derivatives [ST0302]	
				(20S)-1α,25-dihydroxy-20-methoxy-26,27-dimethylvitamin D3 / (20S)-1α,25-dihydroxy-20-methoxy-26,27-dimethylcholecalciferol	Vitamin D3 and derivatives [ST0302]	
				26,27-diethyl-1α,25-dihydroxy-22-oxavitamin D3 / 26,27-diethyl-1α,25-dihydroxy-22-oxacholecalciferol	Vitamin D3 and derivatives [ST0302]	
				26,27-diethyl-1α,25-dihydroxy-23-oxavitamin D3 / 26,27-diethyl-1α,25-dihydroxy-23-oxacholecalciferol	Vitamin D3 and derivatives [ST0302]	
522.5242	4.564269	4.788297		Cer(d18:0/16:0)	N-acylsphinganines (dihydroceramides) [SP0202]	0.001168
				Palmityl palmitate	Wax monoesters [FA0701]	
539.2822	4.044365	0.994487		L-Olivosyl-oleandolide	Macrolides and lactone polyketides [PK04]	0.000761
543.3643	4.554457		2.941478	Pikromycin	Macrolides and lactone polyketides [PK04]	0.00071
				1α,25-Dihydroxy-21-(3-hydroxy-3-methylbutyl)vitamin D(3)	Vitamin D3 and derivatives [ST0302]	
				(20S)-1α,25-dihydroxy-20-ethoxy-26,27-dimethyl-24a-homovitamin D3 / (20S)-1α,25-dihydroxy-20-ethoxy-26,27-dimethyl-24a-homocholecalcifero	Vitamin D3 and derivatives [ST0302]	
				1α,25-dihydroxy-2β-(5-hydroxypentyl)vitamin D3 / 1α,25-dihydroxy-2β-(5-hydroxypentyl)cholecalciferol	Vitamin D3 and derivatives [ST0302]	
				1α-hydroxy-2β-(5-hydroxypentoxy)vitamin D3 / 1α-hydroxy-2β-(5-hydroxypentoxy)cholecalciferol	Vitamin D3 and derivatives [ST0302]	

				26,27-diethyl-1α,25-dihydroxy-24a,24b-dihomo-23-oxa-20-epivitamin D3 / 26,27-diethyl-1α,25-dihydroxy-24a,24b-dihomo-23-oxa-20-epicholecalciferol	Vitamin D3 and derivatives [ST0302]	
				(20S)-1α,25-dihydroxy-20-methoxy-26,27-diethylvitamin D3 / (20S)-1α,25-dihydroxy-20-methoxy-26,27-diethylcholecalciferol	Vitamin D3 and derivatives [ST0302]	
570.4873	3.96388	1.53284		32,35-anhydrobacteriohopaneterol	Hopanoids [PR04]	0.000937
579.3851	4.516756	1.515093		4-Ketoalloxanthin	C40 isoprenoids (tetraterpenes) [PR0107]	0.000516
				Astaxanthin	C40 isoprenoids (tetraterpenes) [PR0107]	
				Mytiloxanthinone	C40 isoprenoids (tetraterpenes) [PR0107]	
				Amarouciaxanthin B/ Sidnyaxanthin	C40 isoprenoids (tetraterpenes) [PR0107]	
				4,4'-Dihydroxyalloxanthin	C40 isoprenoids (tetraterpenes) [PR0107]	
				4-Ketocapsanthin	C40 isoprenoids (tetraterpenes) [PR0107]	
				meso-Astaxanthin/ (3S,3'R)-Astaxanthin	C40 isoprenoids (tetraterpenes) [PR0107]	
				4-Keto-4'-hydroxydiatoxanthin	C40 isoprenoids (tetraterpenes) [PR0107]	
				Phillipsiaxanthin/ 1,1'-(OH)2-2,2'-diketo-3,4,3',4'-tetradehydrolycopene	C40 isoprenoids (tetraterpenes) [PR0107]	
587.318	4.028923	1.007974		3-hydroxy-tetradecanedioic acid	Dicarboxylic acids [FA0117]	0.001007
				16-Glutaryloxy-1α,25-dihydroxy-20-epivitamin D3	Vitamin D3 and derivatives [ST0302]	
				16-Glutaryloxy-1α,25-dihydroxyvitamin D3	Vitamin D3 and derivatives [ST0302]	
589.5173	4.415474		1.128912	DG(17:0/18:2(9Z,12Z)/0:0)[iso2]	Diacylglycerols [GL0201]	0.001569
				DG(17:2(9Z,12Z)/18:0/0:0)[iso2]	Diacylglycerols [GL0201]	
				DG(15:0/20:2(11Z,14Z)/0:0)[iso2]	Diacylglycerols [GL0201]	

			18-nonadecynoic acid	Unsaturated fatty acids [FA0103]	
			Sterculic acid	Carbocyclic fatty acids [FA0114]	
			32,35-anhydrobacteriohopaneterol	Hopanoids [PR04]	
636.4776	4.431892	4.682726	Dihydroparasiloxanthin/ 7,8,7',8'-Tetrahydrozeaxanthin	C40 isoprenoids (tetraterpenes) [PR0107]	0.001031
			Dihydroxylycopene/ OH-Rhodopin	C40 isoprenoids (tetraterpenes) [PR0107]	
			OH-Demethylspheroidene	C40 isoprenoids (tetraterpenes) [PR0107]	
792.6275	4.517126	4.872572	N-tryptophanyl-35-aminobacteriohopane-32,33,34-triol	Hopanoids [PR04]	0.001631
810.5251	4.585221	4.767805	PS(18:0/18:2(9Z,12Z))	Diacylglycerophosphoserines [GP0301]	0.001352
			PS(18:0/18:2(9Z,12Z))	Diacylglycerophosphoserines [GP0301]	
			PS(18:1(9Z)/18:1(9Z))	Diacylglycerophosphoserines [GP0301]	
			PG(18:3(9Z,12Z,15Z)/20:4(5Z,8Z,11Z,14Z))	Diacylglycerophosphoglycerols [GP0401]	
			PG(18:3(6Z,9Z,12Z)/20:4(5Z,8Z,11Z,14Z))	Diacylglycerophosphoglycerols [GP0401]	
			PG(16:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Diacylglycerophosphoglycerols [GP0401]	
			PG(18:2(9Z,12Z)/18:3(6Z,9Z,12Z))	Diacylglycerophosphoglycerols [GP0401]	
			PG(18:2(9Z,12Z)/18:3(9Z,12Z,15Z))	Diacylglycerophosphoglycerols [GP0401]	
			PG(18:3(6Z,9Z,12Z)/18:2(9Z,12Z))	Diacylglycerophosphoglycerols [GP0401]	
			PG(18:3(9Z,12Z,15Z)/18:2(9Z,12Z))	Diacylglycerophosphoglycerols [GP0401]	
			PG(16:1(9Z)/20:4(5Z,8Z,11Z,14Z))	Diacylglycerophosphoglycerols [GP0401]	

			PG(16:0/18:2(9Z,12Z))	Diacylglycerophosphoglycerols [GP0401]	
			PE(18:0/20:3(8Z,11Z,14Z))	Diacylglycerophospho-ethanolamines [GP0201]	
817.5685	1.096579	3.515376	Decaprenyl phosphate	Bactoprenol monophosphates [PR0302]	0.000272
874.5439	1.540522	4.636876	(3'-sulfo)Galβ-Cer(d18:1/20:0)	Sulfoglycosphingolipids (sulfatides) [SP0602]	0.000751
			PI(16:0/16:0)	Diacylglycerophosphoinositols [GP0601]	
929.7897	2.867483	0	TG(18:0/18:0/18:0)	Triacylglycerols [GL0301]	0.001043
			TG(16:0/19:0/19:0)[iso3]	Triacylglycerols [GL0301]	
			TG(17:0/18:0/19:0)[iso6]	Triacylglycerols [GL0301]	
			TG(16:0/18:0/20:0)[iso6]	Triacylglycerols [GL0301]	
			TG(17:0/17:0/20:0)[iso3]	Triacylglycerols [GL0301]	
			TG(16:0/17:0/21:0)[iso6]	Triacylglycerols [GL0301]	
			TG(16:0/16:0/22:0)[iso3]	Triacylglycerols [GL0301]	
932.6212	1.053852	4.139408	PI(18:0/22:4(7Z,10Z,13Z,16Z))	Diacylglycerophosphoinositols [GP0601]	0.000949
			PI(22:4(7z,10z,13z,16z)/18:0)	Diacylglycerophosphoinositols [GP0601]	
			PI(20:0/20:4(5Z,8Z,11Z,14Z))	Diacylglycerophosphoinositols [GP0601]	
			PI(20:4(5Z,8Z,11Z,14Z)/20:0)	Diacylglycerophosphoinositols [GP0601]	
			PI(20:2(11Z,14Z)/20:2(11Z,14Z))	Diacylglycerophosphoinositols [GP0601]	
			PI(22:2(13Z,16Z)/18:2(9Z,12Z))	Diacylglycerophosphoinositols [GP0601]	
			PI(18:2(9Z,12Z)/22:2(13Z,16Z))	Diacylglycerophosphoinositols [GP0601]	
			PI(16:0/22:2(13Z,16Z))	Diacylglycerophosphoinositols [GP0601]	

	PI(22:2(13Z,16Z)/16:0)	Diacylglycerophosphoinositols [GP0601]
	PI(18:0/20:2(11z,14z))	Diacylglycerophosphoinositols [GP0601]
	PI(20:2(11z,14z)/18:0)	Diacylglycerophosphoinositols [GP0601]
	PI(20:0/18:2(9z,12z))	Diacylglycerophosphoinositols [GP0601]
	PI(18:2(9z,12z)/20:0)	Diacylglycerophosphoinositols [GP0601]
	PI(20:1(11z)/18:1(9z))	Diacylglycerophosphoinositols [GP0601]
	PI(18:1(9z)/20:1(11z))	Diacylglycerophosphoinositols [GP0601]