UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Departamento de Análises Clínicas e Toxicológicas
Programa de Pós-Graduação em Farmácia
Área Fisiopatologia

Análise dos mecanismos regulatórios transcricionais mediados por microRNAs na Síndrome Metabólica

Thiago Dominguez Crespo Hirata

Tese apresentada para obtenção do título de Doutor em Ciências

Orientador: Prof. Dr. Helder Takashi Imoto Nakaya

São Paulo

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São Paulo, 13 de setembro de 2019

Dedication

This Thesis is dedicated to all people who are or have been affected by Metabolic Syndrome, the study volunteers, and the healthcare professionals who fight against it.

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HIRATA, T. D. C. **Análise dos mecanismos regulatórios transcricionais mediados por microRNAs na Síndrome Metabólica.** 2019. 110f. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2019.

Resumo

A Síndrome Metabólica (MetS) é um conjunto de doenças inter-relacionadas e associadas ao aumento de mortalidade e risco de eventos cardiovasculares. Entre os mecanismos moleculares elucidados da MetS, existem muitos genes regulados por miRNAs - RNAs pequenos não codificadores. O grande número de estudos transcriptômicos em banco dados públicos integrado a novos métodos de análise podem gerar novas descobertas. Deste modo, o objetivo deste estudo foi identificar miRNAs circulantes e genes alvos na MetS usando a abordagem de Biologia de Sistemas. Para isso, GEO-NCBI foi usado para obter e analisar 26 estudos de transcriptoma por microarray de MetS e obesidade. Após o pré-processamento, realizamos análises de expressão diferencial (método LIMMA), co-expressão gênica (CEMiTool), e enriquecimento (GSEA, Reactome). Identificamos uma assinatura de expressão gênica do tecido adiposo subcutâneo (SAT) de indivíduos obesos, composta por 291 genes consistentemente diferencialmente expressos (DEG). Essa assinatura teve um escore de enriquecimento normalizado (NES) positivo para ativação de respostas do sistema imune adaptativo, e NES negativo para vias de metabolismo. A rede consenso de co-expressão do SAT revelou 3 comunidades (CM) de genes densamente interconectadas. Essas CMs continham muitos genes regulados positivamente e com consistência de NES positivo entre os estudos. Os genes co-expressos dessas 3 comunidades pertenciam a vias de a degranulação de neutrófilos, infiltração de células do sistema imune e processos inflamatórios. Além disso, uma pequena coorte brasileira (6 indivíduos com MetS e 6 controles) foi submetida à dosagem sérica de miRNAs por PCR array. Dos 222 miRNAs detectados no soro, a análise de expressão diferencial identificou 4 miRNAs regulados positivamente (miR-30c-5p, miR-421, miR-542-5p e miR-574) nos pacientes com MetS ($p<0.01$). A análise integrativa miRNAs-mRNAs revelou que os miRNAs circulantes superexpressos tinham 12 alvos no SAT, 3 alvos no fígado; e

nenhum alvo no músculo e no sangue. Muitos desses alvos são moduladores de vias pró-inflamatórias. Em conclusão, a utilização da Biologia de Sistemas na análise de redes gênicas e miRNAs circulantes identificou alguns potenciais mecanismos moleculares e fisiopatológicos da Síndrome Metabólica. Os miRNAs circulantes identificados neste trabalho são potenciais biomarcadores e/ou alvos terapêuticos. Entretanto, mais estudos são necessários para validar esses miRNAs e seus mRNAs alvos.

Palavras-chave: Síndrome metabólica. Obesidade. MicroRNA. Assinatura gênica. Co-expressão. mRNA. Biologia de Sistemas. Bioinformática.

HIRATA, T. D. C. **Analysis of the transcriptional regulatory mechanisms mediated by microRNAs in Metabolic Syndrome.** 2019. 110f. Thesis (Doctoral) - School of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2019.

Abstract

Metabolic Syndrome (MetS) is a combination of diseases interrelated and associated with increased mortality and risk of cardiovascular events. Among the elucidated molecular mechanisms of MetS, there are several genes regulated by miRNAs - small non-coding RNAs. A large number of transcriptomic studies in public databases integrated with new analysis methods can generate new insights. Therefore, this study aimed to identify circulating miRNAs and their target genes in MetS using a Systems Biology approach. For this, we used GEO-NCBI to download and analyse 26 microarray transcriptome studies of MetS and obesity. After preprocessing, the data underwent differential expression (LIMMA method), gene co-expression (CEMiTool), and enrichment (GSEA, Reactome) analyses. We retrieved a gene expression signature for subcutaneous adipose tissue (SAT) for obese individuals that included 291 consistent differentially expressed genes (DEG). This signature had a positive normalized enrichment score (NES) for adaptive immune system activation responses, and negative NES for metabolic pathways. The consensus co-expression network of SAT revealed 3 communities (CM) of densely interconnected genes. These CMs had a high number of up regulated genes and a consistent positive NES among the studies. The co-expressed genes of these 3 CMs were related to neutrophil degranulation, infiltration of immune system cells, and inflammatory processes. Also, a small brazillian cohort (6 individuals with MetS and 6 controls) underwent a seric miRNA profiling using PCR array. From the 222 miRNAs detected in serum, the differential expression analysis identified 4 upregulated miRNAs (miR-30c-5p, miR-421, miR-542-5p and miR-574) in MetS patients ($p<0.01$). The integrative miRNAs-mRNAs analysis revealed that the circulating upregulated miRNAs had 12 targets in the SAT, 3 targets in the liver; and no targets in the muscle and blood. Many of these target genes are known modulators of proinflammatory pathways. In conclusion, the use of Systems Biology in the analysis of gene networks

and circulating miRNAs identified some potential molecular and pathophysiological mechanisms of the Metabolic Syndrome. The circulating miRNAs identified in this study are potential biomarkers and/or therapeutic targets. However, further studies are needed to validate these miRNAs and their target mRNA.

Keywords: Metabolic Syndrome. Obesity. MicroRNA. Gene signature. Co-expression. mRNA. Systems Biology. Bioinformatics.

List of Abbreviations

3'-UTR: 3' untranslated region

ABCA1: ATP-binding cassette subfamily A member 1

ABCG1: ATP-binding cassette subfamily G member 1

ATP: adenosine triphosphate

BMI: Body Mass Index

CAD: coronary arterial disease

Ct: cycle threshold

CVA: cerebrovascular accident

DAMPs: damage-associated molecular patterns

DEGs: differentially expressed genes

DL: dyslipidemia

DNA: deoxyribonucleic acid

EGFR: epidermal growth factor receptor

ES: Enrichment Score

FC: fold change

FFAs: free fatty acids

GEO: Gene Expression Omnibus

GSEA: Gene Set Enrichment Analysis

GTF3C3: General Transcription Factor IIIC Subunit 3

GWAS: Genome-Wide Association Study

HDL-c: high-density lipoprotein cholesterol

HT: hypertriglyceridemia

IDF: The International Diabetes Federation

IDPC: Dante Pazzanese Institute of Cardiology

IHD: ischemic heart disease

IPA: Ingenuity Pathway Analysis

LIMC: Laboratory of Molecular Investigation in Cardiology

MDP: Molecular Degree of Perturbation

MetS: Metabolic Syndrome

MHO: metabolically healthy

MIAME: Minimum Information About Microarray Experiment

miRNA: microRNA

mRNA: messenger RNA

MUO: metabolically unhealthy obese

NCBI: National Center for Biotechnology Information

NCD: non-communicable disease

NCEP ATP III: National Cholesterol Education Program's Adult Treatment Panel III

NES: Normalized Enrichment Score

NGS: next-generation sequencing

NIH: National Institutes of Health

ORA: Over Representation Analysis

PAMPs: Pathogen-Associated Molecular Patterns

PCA: Principal Component Analysis

PCC: Pearson’s Correlation Coefficient

PPARγ: peroxisome proliferator-activated receptor gamma

PRK1: protein related kinase 1

PRRs: pattern recognition receptors

PVCA: Principal Variance Component Analysis

RHOB: ras homolog gene family member B

RMA: Robust Multi-array Average

RNA: ribonucleic acid

SAH: systemic arterial hypertension

SAT: subcutaneous adipose tissue

NR3C1: nuclear receptor subfamily 3 group C member 1

SNP: single nucleotide polymorphism

TLR4: toll-like receptor 4

WC: waist circumference

WHO: World Health Organization

WHR: waist-to-hip ratio

WHTR: waist-to-height ratio

List of Tables

List of Figures

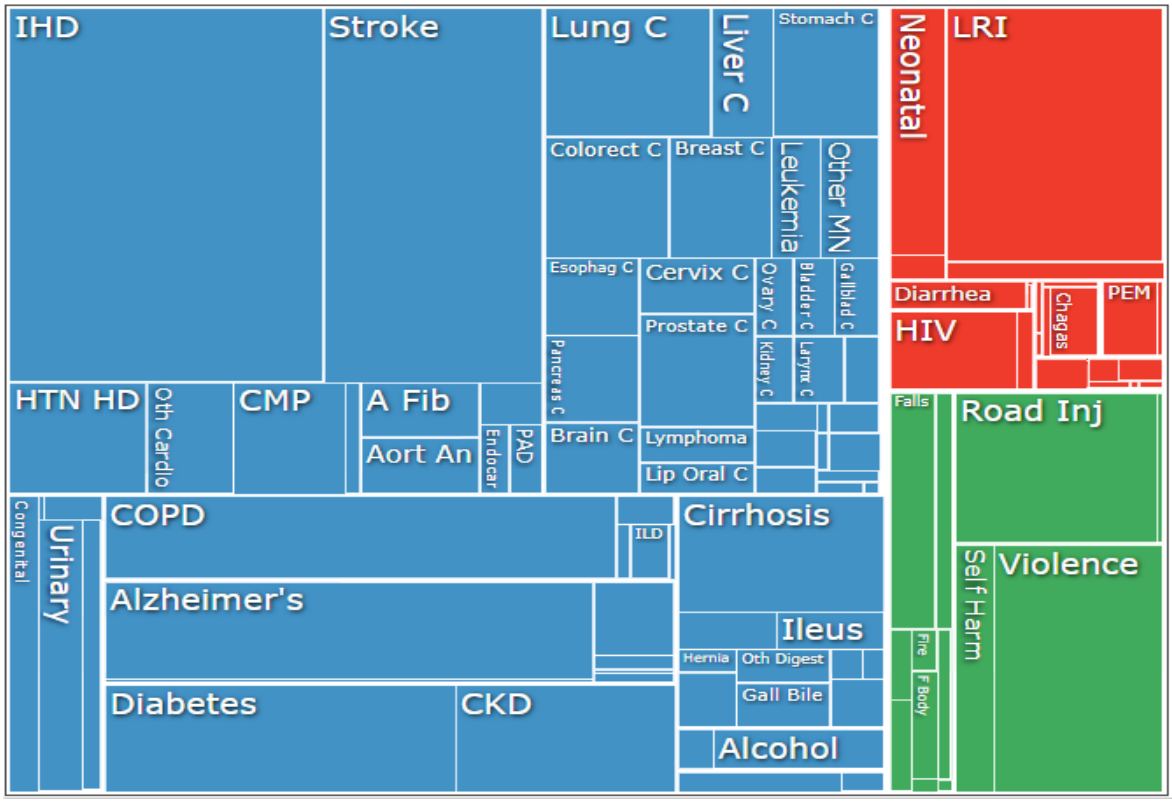
Summary (Table of Contents)

1. Introduction

1.1. Metabolic Syndrome

Cardiovascular disease is the leading cause of death worldwide (JOSEPH et al., 2017). In Brazil, ischemic heart disease (IHD) and stroke represent respectively, 13.0% and 9.1% of all deaths reported in 2017 (Figure 1). Both conditions cause a significant impact on public health due to the need for high complexity hospital procedures and associated high costs (MOZAFFARIAN et al., 2016). Metabolic Syndrome (MetS) encompasses many pathologic conditions that have shown to have a 50% increase in mortality and twice the risk of cardiovascular events (MOTTILLO et al., 2010).

Figure 1 - Main causes of death for all ages and genders in Brazil in 2017



The graph shows the proportion of deaths by chronic diseases in blue, death by infectious diseases in red and other causes of death in green. IHD (ischemic heart disease) represents 13.0 % of total deaths in the country and stroke 9.1 %. (Figure generated by the website: https://vizhub.healthdata.org/gbd-compare/).

Since the description of MetS (REAVEN, 1988), different clinical definitions were proposed by the World Health Organization (WHO), National Institutes of Health (NIH), and the International Diabetes Federation (IDF). Divergences between the formulated guidelines led to the harmonized proposal for the definition of MetS in 2009 (ALBERTI et al., 2009).

All the MetS diagnostic definitions take into consideration the presence of at least 3 of the following factors: body measurements (Body Mass Index - BMI, or abdominal circumference), lipids profile (low levels of HDL-c - High Density Lipoprotein cholesterol and high levels of triglycerides), blood pressure (Systemic Arterial Hypertension - SAH), and glycemic profile (Type 2 diabetes, altered fasting glycemia or glucose intolerance) (MOZAFFARIAN et al., 2016).

In this work, we utilized the latest guideline in Brazil for MetS diagnosis. The guideline is from the *IV Diretriz Brasileira de Dislipidemias e Prevenção da Aterosclerose* from the *Departamento de Aterosclerose da Sociedade Brasileira de Cardiologia* (SPOSITO et al., 2007). According to it, MetS diagnosis requires the presence of abdominal obesity, alongside two other factors described in Table 1. However, the most extensive epidemiologic study in Latin America, the *Estudo Longitudinal de Saúde do Adulto no Brasil* (ELSA-Brasil), utilized the guidelines from the National Cholesterol Education Program's Adult Treatment Panel III (NCEP ATP III). This multicenter cohort found a 15.2% MetS prevalence among 15.000 individuals (SCHMIDT et al., 2015).

The heterogeneity in diagnosis procedures makes it difficult to estimate the global prevalence of MetS, which varies from 10 to 30% of the adult population (GRUNDY, 2008). Brazilian epidemiological studies also found different prevalence values of MetS: 14.9% (PIMENTA; GAZZINELLI; VELÁSQUEZ-MELÉNDEZ, 2011); 19% (BARBOSA et al., 2006); 25.4% (MARQUEZINE et al., 2008); 29.8% (SALAROLI et al., 2007); 30% (DE OLIVEIRA; DE SOUZA; DE LIMA, 2006); 32% (DUTRA et al., 2012); 35.7% (DE OLIVEIRA et al., 2011); 35.9% (GRONNER et al.,

2011). Such discrepancy may be due to regional, methodological, and ethnic differences among the studies (DE CARVALHO VIDIGAL et al., 2013).

Table 1 - Diagnostic criteria for Metabolic Syndrome from the *IV Diretriz Brasileira Sobre Dislipidemias e Prevenção da Aterosclerose* (2007)*

Criteria	Definition
Abdominal obesity	
Men	
European Caucasian and afro descendent	≥ 94 cm
South-Asian, American e Chinese	≥ 90 cm
Japanese	≥ 85 cm
Women	
European Caucasian, Afro-descendent, South-Asian, American and Chinese	≥ 80 cm
Japanese	≥ 90 cm
Triglyceridemia	≥ 150 mg/dL or treating
HDL-cholesterol	
Men	< 40 mg/dL
Women	< 50 mg/dL
Systemic Arterial Blood Pressure	
Systolic or	≥ 130 mm Hg or treating
Diastolic	≥ 85 mm Hg or treating
Fasting Glycemia	≥ 100 mg/dL or treating

*Established by the *Departamento de Aterosclerose da Sociedade Brasileira de Cardiologia*. Metabolic Syndrome Diagnosis: abdominal obesity + 2 criteria from above. Source: Sposito et al., 2007.

Obesity is the most predominant risk factor (MANDVIWALA; KHALID; DESWAL, 2016) as well as a significant predictor of MetS (CARNETHON et al., 2004) (PALANIAPPAN et al., 2004). It reached a global epidemic status with a steadily increase in prevalence regardless of age, gender, and ethnicity (INOUE et

al., 2018). Also, obesity's association with decreased life expectancy and increased morbidity contributes to the overall burden of diseases (FONTAINE et al., 2003).

The cause for such an increase in obesity worldwide is still unclear (ROSS; FLYNN; PATE, 2016). Among the probable reasons, the main ones are the lack of physical exercise and a poor diet (VAN DER VALK et al., 2019). Indeed, countries with economic growth from industrialization, newly created technologies, and efficient means of transportation have boosted the sedentary lifestyle of its citizens (HRUBY; HU, 2015). Other factors have been suggested to contribute to obesity: chronic stress, medications (VAN DER VALK et al., 2019), increased consumption of highly processed foods, elevated maternal age, sleep deprivation, endocrine disruptors, pharmaceutical iatrogenesis, and intrauterine/intergenerational factors (MCALLISTER et al., 2009).

The imbalance of calorie intake and energy expenditure may result in excess of body adiposity. Although obesity can be defined subjectively, a precise diagnostic criterion is still missing. The Body Mass Index (BMI) is a commonly used anthropometric measure of obesity. It is calculated by dividing the weight by the height squared (kg/m^2). An adult is considered obese if the BMI is over 30 kg/m^2, though lower values are recommended for specific populations. For example, a BMI cut-off value of 25 kg/m^2 is suggested for Asians and South Asians due to associated health risks (NAM; PARK, 2018).

Other anthropometric measurements have been proposed to diagnose obesity. These include waist circumference (WC), waist-to-hip ratio (WHR) (DE KONING et al., 2007) and waist-to-height ratio (WHtR) (ASHWELL et al., 2014). Indices that use WC are more accurate when it comes to evaluating the health risks (CORRÊA et al., 2016) and predict chronic diseases (ASHWELL; GUNN; GIBSON, 2012).

Excess adipose tissue is associated with several diseases, including type 2 diabetes, cardiovascular disease, and some types of cancers. The expansion of adipose tissue can occur through cell multiplication, called hyperplasia, or due to the accumulation of lipids (hypertrophic expansion). Metabolic complications secondary

to obesity, such as insulin resistance, hypertension, and dyslipidemia have a more significant association to hypertrophy than hyperplasia, primarily when the accumulation of lipids occurs in the visceral region (SHERLING; PERUMAREDDI; HENNEKENS, 2017; TUNE et al., 2017) (ARNER, 1998).

1.2. Investigating Metabolic Syndrome with High-throughput Technologies

Omics technologies have been applied to better understand the genetic features of the MetS and related conditions. Genome-wide Association Studies (GWAS) have shown that complex trait diseases are highly polygenic and that each variant has very small contributions to the phenotype (FALL; INGELSSON, 2014). This can be partially explained by the lack of statistical power or the little influence that genetics alone plays in metabolic disorders (ZHU et al., 2017). Also, the genes found in GWAS studies are unable to explain metabolic changes without proper functional validation (VISSCHER et al., 2017). Still, several GWAS focused on MetS have been published to date (AVERY et al., 2011; KRAJA et al., 2011; ZABANEH; BALDING, 2010) (LEE; KIM; PARK, 2018; ZHU et al., 2017).

On the other hand, the number of MetS related diseases using transcriptome techniques is much larger. The literature is, however, very heterogeneous - most MetS studies focus on one of its components (DAO et al., 2018), in co-morbidities (HIRSCH et al., 2010), or involves some type of intervention or treatment (KOLEHMAINEN et al., 2012) (HULSMANS et al., 2012). The main MetS component found in these transcriptome studies was obesity. However, many of them focused on interventions (GRACE et al., 2019; TAKAHASHI et al., 2019), on associated conditions (SINNOTT et al., 2017), or on inflammatory processes (TAM et al., 2011).

Microarray transcriptome studies have been able to unravel various biological elements and complex pathways of MetS (BAKKER et al., 2018; DAO et al., 2018; D'AMORE et al., 2018). Most have tried to discover new molecular mechanisms (BADOUD et al., 2017) and different phenotypes (WRUCK et al., 2015). For example, a co-expression study identified IL-6 and IL1B as highly differentially

co-expressed in adipose tissue from obese individuals (KOGELMAN et al., 2016). The same group performed a transcription factor co-expression analysis and detected immune pathways, including the TGF-beta signaling pathway in adipose tissue from obese patients (SKINKYTE-JUSKIENE; KOGELMAN; KADARMIDEEN, 2018).

Another co-expression study, with discordant monozygotic twins, uncovered a co-expression module that had a positive correlation with BMI. This co-expression module was enriched with several lipid-related pathways, including regulation of phospholipase activity and cholesterol transporter activity. Also, the authors identified 32 DEGs from blood samples, and a possible association of *NAMPT*, *TLR9*, *PTGS2*, *HBD*, and *PCSK1N* and obesity (WANG et al., 2017).

Although informative, the genes reported being associated with a transcriptomic study overlap very poorly with those reported by another transcriptomic study. Such discrepancies are frequently observed (MIKLOS; MALESZKA, 2004), raising questions about the reproducibility of scientific work. These inconsistencies can be related to factors such as distinct gene expression platforms, sample collection (EIN-DOR; ZUK; DOMANY, 2006; RADICH et al., 2004), and even small sample sizes causing reduced statistical power (CHOI et al., 2003).

A very large number of samples is required to reach a decent level of marker stability (EIN-DOR; ZUK; DOMANY, 2006). The ideal solution to overcome this problem is to compare and integrate data from several studies into a meta-analysis (CAHAN et al., 2007), which improves the findings' reliability. Large datasets also allow for co-expression network analyses, where large sets of genes are positively correlated, leading to gene co-expression modules that increase the comprehension and predictive power over mechanisms underlying genetic diseases.

1.3. Microarray technology and GEO-NCBI

Microarray technology is based on hybridization between target DNA from samples and predefined DNA probes fixed on a platform. The technology is able to measure the expression levels of tens of thousands of transcripts simultaneously in a single sample. Despite its limitations compared with RNA-Seq technology (SULTAN et al., 2008), microarrays are well established in the scientific community and are still a widely used technology for transcriptome analysis.

In addition, public databases contain data from millions of microarray samples, allowing their use in large meta-analyses. For example, Kraja et al. performed a meta-analysis in which single nucleotide polymorphisms (SNPs) located near the genes *COBLL1*, *GRB14* and *LYPLAL1* were associated with high concentrations of fasting insulin, waist circumference, and risk for type 2 diabetes (KRAJA et al., 2014).

The GEO-NCBI (Gene Expression Omnibus - National Center for Biotechnology Information) has been the most comprehensive and curated database in the literature. The database only includes studies that follow strict content guidelines from the Minimum Information About a Microarray Experiment (MIAME) (BARRETT et al., 2007) and is accessible at (www.ncbi.nlm.nih.gov/geo/). This international public repository contains gene expression, DNA methylation, protein, SNP, and genomic variation studies (CLOUGH; BARRETT, 2016). The database also provides query tools to search and download raw and normalized data from arrays and sequencing-based studies. In 2018, there were over 2.8 million samples, and a whopping 106.000 studies (series) available in the database.

1.4. Gene Co-expression Analysis

Gene co-expression analysis aims to find genes with similar gene expression patterns in different biological conditions (ZHANG; HORVATH, 2005). Using this approach, we can construct a network by computing a similarity (correlation) score for each pair of genes. If the similarity score is higher than a threshold, then the

genes are connected in the graph in an undirected way (because the correlation is symmetric). Co-expression profiles can provide insight into cellular processes since they usually encode interacting proteins (BELLOT et al., 2015).

The Weighted Gene Co-expression Network Analysis (WGCNA) method considers the relationships between the transcripts by quantifying the correlations between gene pairs and evaluating the extent to which the genes share the same interaction neighbor (ZHANG; HORVATH, 2005). In summary, this method groups genes in modules according to the gene co-expression variation calculated by the Pearson correlation coefficient, which makes it possible to ascertain gene expression profiles between the different experimental conditions (LANGFELDER; HORVATH, 2008). These modules possibly contain genes belonging to the same biological processes (pathways) and regulation. The WGCNA method transforms thousands of probes from the microarray study into dozens of modules, reducing the high dimensionality of data and eliminating the need for multiple tests (LANGFELDER; HORVATH, 2012).

1.5. Systems Biology

Biological systems involve many types of components (e.g. genes, proteins, metabolites, etc.) that interact with each other in a complex manner. Analyzing this network can be challenging and overwhelming (WALPOLE; PAPIN; PEIRCE, 2013). Systems biology analysis strategy follows a holistic approach and seeks to understand, identify the patterns, and quantify the interactions of biological components by integrating various types of data using computational and statistical models (KRIETE et al., 2011).

The development of context-specific gene modules and gene networks of signaling pathways facilitate the visualization of systems biology results, in addition to keeping them within a biological context (STEVENS et al., 2014). Using this approach, we can apply systems biology tools to all health science areas, such as: immunology (PRADA-MEDINA et al., 2017), infectious diseases (KWISSA et al.,

2014), neurology (MORELLO et al., 2018; RUSSELL-BUCKLAND; BARNES; TACHTSIDIS, 2019), vaccinology (KAZMIN et al., 2017; NAKAYA; PULENDRAN, 2012), endocrinology (STEVENS et al., 2014), oncology (ARCHER et al., 2016), odontology (ADEOLA; PAPAGERAKIS; PAPAGERAKIS, 2019), and pharmacology (MA'AYAN et al., 2014; STÉPHANOU et al., 2018) as well as to biomarker discovery (LIN et al., 2018).

1.6. miRNAs

miRNAs (microRNAs) are small non-protein-coding RNA molecules that regulate the gene expression of thousands of mRNAs. They hybridize to complementary sequences from the 3' untranslated region (3'-UTR) of target messenger RNA (mRNA), leading to translation inhibition or destabilization and direct cleavage of the target transcript (BARTEL, 2004). In addition, miRNAs can compete for 5' CAP (PESTOVA et al., 2001), inhibit ribosome assembly (CHENDRIMADA et al., 2007), promote target mRNA deadenylation (WAKIYAMA; YOKOYAMA, 2010), prematurely disassemble the ribosome (PETERSEN et al., 2006), cleave target mRNA (LLAVE et al., 2002) (PALATNIK et al., 2003) or even promote the deadenylation followed by the removal of 5` CAP (BEHM-ANSMANT; REHWINKEL; IZAURRALDE, 2006).

Briefly, the canonical biogenesis of miRNAs begins when RNA polymerase II transcribes the miRNA gene into its primary miRNA (pri-miRNA). This precursor molecule has secondary structures called hairpins (SMALHEISER, 2003) that are cleaved by the RNase III DROSHA-DGCR8 complex (LEE et al., 2003). The resulting molecule is about 70 bases and is called a miRNA precursor (pre-miRNA). Subsequently, a nuclear export receptor-dependent on the Ran-GTP cofactor, Exportin-5, mediates the displacement of the pre-miRNA into the cytosol (LUND et al., 2004). Finally, the mature pre-miRNA is processed by cytoplasmic RNase III DICER to form the mature miRNA of approximately 18 to 24 nucleotides (BERNSTEIN et al., 2001).

miRNAs are non-canonical when their biogenesis bypasses the canonical biogenesis pathway (ABDELFATTAH; PARK; CHOI, 2014). Drosha or

Dicer-independent pathways can synthesize them. The absence of Drosha and Gdcr8 does not affect non-canonical miRNA production (RUBY; JAN; BARTEL, 2007). Although Dicer is required for most miRNA synthesis, there are miRNAs can mature independently of Dicer. For example, mir-451 matures without the microprocessor of the Dicer pathway because of its pri-miRNA small size after Drosha/Dgcr8 cleavage in the nucleus (GEBERT; MACRAE, 2019).

miRNAs are present in several human tissues (LIANG et al., 2007), as well as most biological fluids such as serum (GILAD et al., 2008), plasma (CHIM et al., 2008) and urine (MELKONYAN et al., 2008). Its extracellular stability can increase when associated with lipid or protein carriers. For instance, the bound with Ago2, a protein of the RNA-induced silencing complex, protects against endogenous RNAse (TURCHINOVICH et al., 2011) degradation. Furthermore, lipid carriers such as extracellular vesicles (exosomes, microparticles, microvesicles) (VICKERS; REMALEY, 2012) and lipoproteins (VICKERS et al., 2011) allow miRNAs to be transported throughout the body and exchanged between different cells.

The latest update of the miRBase database (version 22), has identified and cataloged 1982 precursors of miRNAs and 2694 mature miRNAs from humans (KOZOMARA; BIRGAOANU; GRIFFITHS-JONES, 2019; KOZOMARA; GRIFFITHS-JONES, 2014). Most of these miRNAs can regulate hundreds of mRNAs, and several miRNAs can target a single mRNA (KREK et al., 2005).

The immense influence and regulatory activity of miRNAs on the post-transcriptional mechanisms of mRNAs indicates a potential target in diseases. Analysis of the expression profile of miRNAs has already provided molecular markers for the detection of various diseases and may contribute to the discovery of new therapies (MCGREGOR; CHOI, 2011).

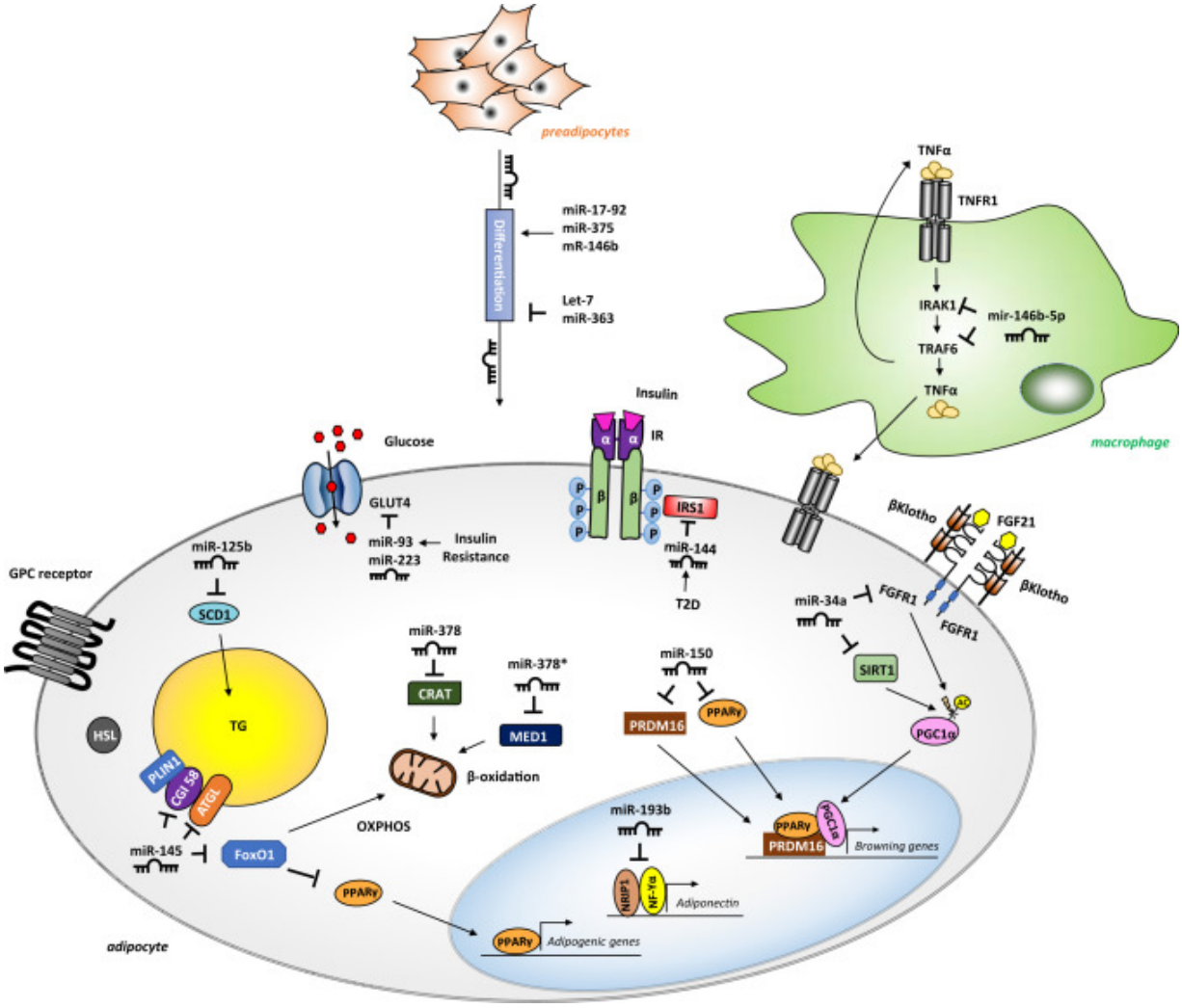
Associations between MetS and miRNAs have already been established by Karolina et al. (2012) in the analysis of circulating miRNAs in patients with MetS, hypercholesterolemia, type 2 diabetes, or systemic arterial hypertension. Groups of differentially expressed miRNAs in MetS were uncovered for each MetS associated disease (dyslipidemia, diabetes, and hypertension). They also uncovered miRNAs

expression positively correlated with BMI, high blood pressure, and fasting blood glucose (KAROLINA et al., 2012).

Increased expression of circulating let-7g and miR-221 was associated with hyperglycemia and other components of the MetS in women. Also, let-7g was associated with low HDL cholesterol and hypertension, while miR-221 was not associated with any risk (WANG et al., 2013b).

Many miRNAs identified in the adipose tissue have been shown to target genes involved in human adipogenesis (PENG et al., 2014). For example, miR-27b (KARBIENER et al., 2009) and miR-130 (LEE et al., 2011) target the peroxisome proliferator-activated receptor-gamma (PPARγ). On the other hand, the increased expression of miR-103 and miR-143 (ESAU et al., 2004) was associated with adipogenesis induction. Upregulation of miR-30c, miR-30d, and miR-30e has been found during adipocyte differentiation (WANG et al., 2013a). Other processes related to the cellular activity of adipocytes in which miRNAs are involved maturation, metabolism, and signaling (Figure 2).

Figure 2 - Adipocyte functions and examples of miRNA-mediated regulation



Adipocyte processes influenced by miRNAs: glucose uptake (miR-93 and miR-223), lipolysis and β-oxidation (miR-145), triglyceride synthesis (miR-125b), insulin signaling (miR-144), browning (miR-150 and miR-34a), adiponectin synthesis (miR-193b) and inflammation (miR-146b-5p in macrophages). Source: Brandão, Guerra e Mori (2017).

Although miRNAs are an essential part of transcriptomic regulation, epigenetic factors such as DNA methylation (WILSON et al., 2017; XU et al., 2018), histone modifications (NIE et al., 2017) have shown to contribute to obesity development. Other MetS components like as insulin resistance (ARNER et al., 2016) and hypertension (STOLL; WANG; QIU, 2018) have also shown to be influenced by epigenetics. It comes as no surprise that environmental factors can positively (ARMENISE et al., 2017) or negatively (MESSAOUDI et al., 2017)

influence metabolic diseases. Even the uterine environment can affect fetal epigenome in the early stages of human development (LING; RÖNN, 2019).

MetS is a multifactorial disease with a significant epidemiological, economic, and sociological impact. Even though several GWAS and candidate genes association studies have found obesity and MetS related genes, there are still divergences of the relevant genes among the studies. The identification of the MetS gene expression signature can help understand the role of miRNA in MetS and even impact the development of diagnostic/prognostic methods. Therefore, the use of a data-driven holistic method of analysis was proposed to investigate consensus target genes of miRNAs involved in this complex disease. For this, several microarray studies were integrated, gene modules associated with MetS were developed and the interactions of the differentially expressed genes with regulatory miRNAs were analyzed.

2. Aims

2.1. Main Aim

To use systems biology and meta-analysis tools to identify the molecular signaling pathways in MetS.

2.2. Specific Aims

To identify a consistent gene expression signature for obesity through a comprehensive meta-analysis of transcriptomic studies.

To identify consistent gene co-expression modules in MetS and obesity.

To reveal genes and signaling pathways not yet described as being related to MetS and obesity.

To analyze circulating miRNA profiles in patients with MetS using PCR miRNA array.

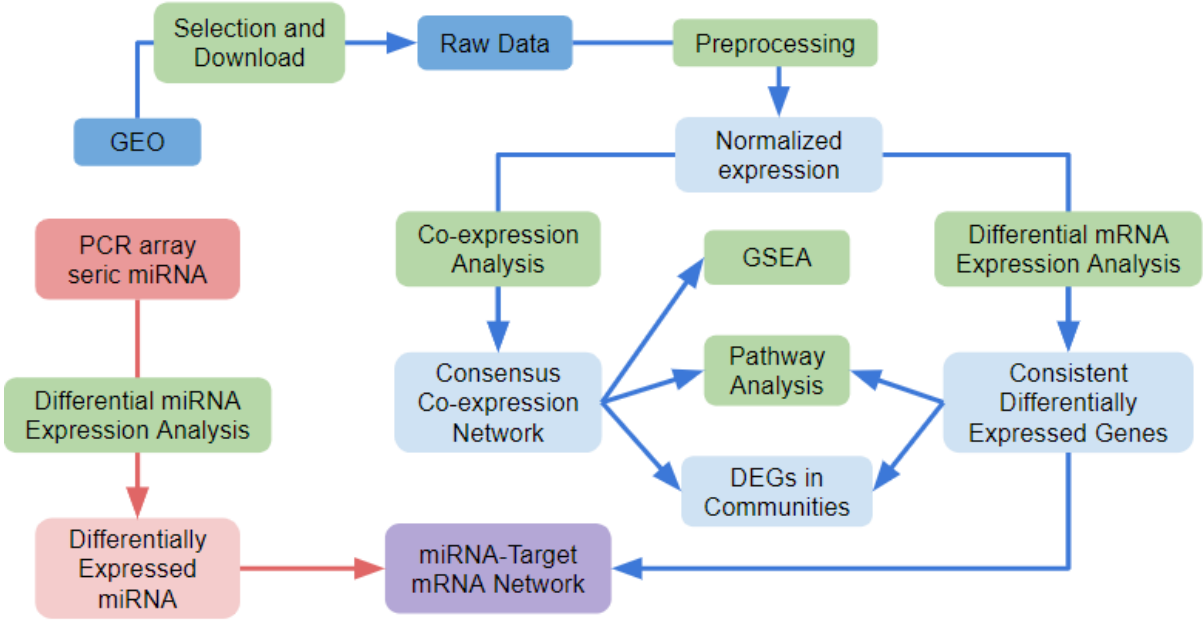
To identify potential regulatory miRNAs by integrating gene co-expression analysis of obesity and miRNAs associated with MetS.

3. Material and Methods

3.1. Study workflow

In order to reach the aims of this study, an analysis workflow was developed to integrate transcriptomic open-source data with the miRNA expression data obtained from a small Brazilian cohort. The main steps used to analyze the two datasets obtained are summarized in Figure 3. The first part of the bioinformatic analysis used publicly available datasets (Figure 3, blue arrows), where the microarray transcriptome data was selected, obtained, processed, and analyzed. In the second part, the miRNA dataset from MetS patients was normalized, analyzed, and integrated into the transcriptomic dataset.

Figure 3 - Flow chart of the summarized analysis workflow



GSEA: Gene Set Enrichment Analysis. GEO: Gene Expression, Omnibus. Green: Analysis method, blue: data obtained publicly, light blue: results from the public data, red: new unpublished data, light red: results from the unpublished data, purple: results combining the published and unpublished data. DEGs: differentially expressed genes. PCR: polymerase chain reaction. miRNA: microRNA. mRNA: messenger RNA.

3.2. Selection of mRNA expression microarray studies from a public database

Initially, we performed an online survey of gene expression studies in the GEO-NCBI on MetS, and related diseases: obesity, hypertension (SAH), dyslipidemia (DL), hypertriglyceridemia (HT) and insulin resistance. In this study, the terms used were: metabolic syndrome, obesity, hypertension, hypoalphalipoproteinemia, low high-density lipoprotein, low HDL, dyslipidemia, hypertriglyceridemia, and insulin resistance. The search was performed in Mar 2015 and updated in May 2018 with the following filters: “Series” (Entry Type), "Homo sapiens" (Organism); "Expression profiling by array" (Study Type). In this way, only studies performed in humans and with transcriptomic data analyzed by microarray technology were compiled.

In order to ensure correct classification of the microarray studies, the annotation of each study was performed manually. This annotation involved reading the GEO-NCBI Summary and Overall design fields and related scientific articles, identifying studies not consistent with the search criteria, classification according to disease, processed tissues, analysis platform used and the number of samples.

The following criteria were used to exclude studies from the analysis: no sample identification; non-human; less than 15 samples in total; derived from cell culture; derived from cell lineage; other disorders not characteristic of the metabolic syndrome (cancer, infections, polycystic ovary, autoimmune diseases, etc); and combination of more than 1 study (Superseries). Also, within the selected studies, samples collected after intervention or experimental procedures were excluded. A few examples of intervention were: physical activity, food supplementation, drug treatments, and surgical procedures.

3.3. The download of mRNA expression data

After study selection, the raw data were obtained from GEO using scripts developed by our research group at the Computational Laboratory of Systems Biology (CSBL). One of the scripts uses the Perl language to automatically

download the raw expression, sample annotation, and probe annotation files for each study. Also, when the raw expression file was not available, normalized expression files provided by the authors were downloaded.

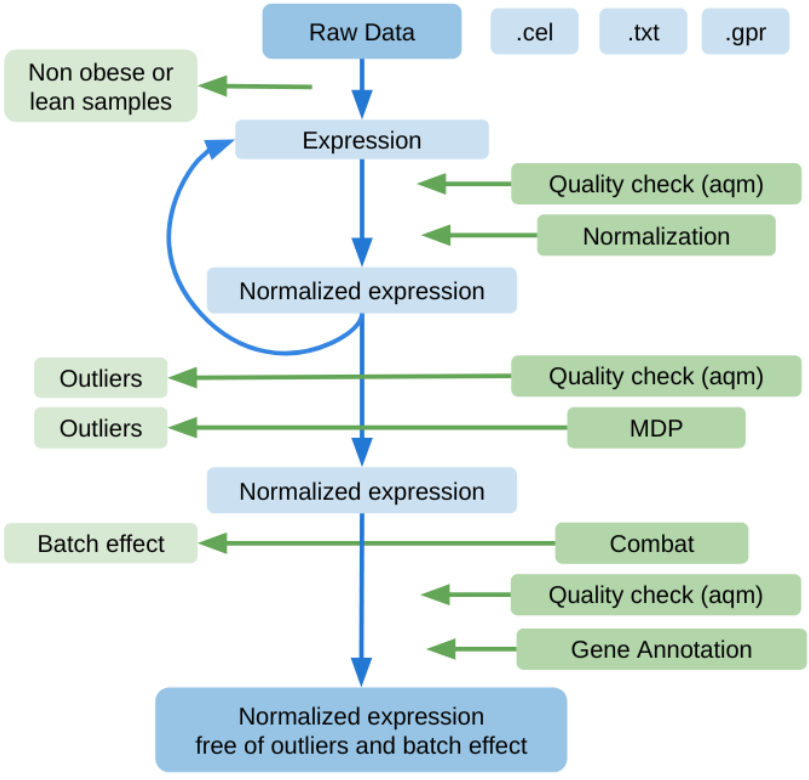
Next, duplicate samples between the studies were identified through a script that uses the "md5sum" program. The "md5sum" uses the MD5 algorithm to create a 128-bit code for any input file (RIVEST, 1992). This code works like a compact fingerprint that changes entirely by changing a single bit of the output file.

3.4. Pre-processing and Reanalysis from GEO Database

3.4.1. Normalization

After the study selection and download, the expression files were processed, as shown in Figure 4 that encompasses the preprocessing item in Figure 3.

Figure 4 - Flow chart of the summarized pre-processing steps



Aqm: ArrayQualityMetrics. MDP: the molecular degree of perturbation.

3.4.1.1 Normalization: Pre-processing: Affymetrix platforms

The samples processed by the Affymetrix gene expression platform were normalized by RMA (Robust Multi-array Average), using the affy version 1.6.0 data package present in Bioconductor (R language). This process consists of the following steps: raw reading files ".CEL", background correction of the gross values of the fluorescence intensity by whole array adjustment and normalization by quantile (BOLSTAD et al., 2003).

3.4.1.2. Normalization: Agilent platforms

The raw gene expression files (".gpr" or ".txt") from the Agilent platform normalized using the Linear Models for Microarray (LIMMA, version 3.38.3) package. This process consists of the following steps: loading the raw files (read.maimages), background correction (backgroundCorrect) and normalization by quantile (normalizeBetweenArrays) (BOLSTAD et al., 2003) (see Fig. 2).

3.4.1.3. Normalization: Illumina platforms

The studies obtained from Illumina gene expression platforms did not undergo normalization. The already normalized data by the authors was obtained because the upload of raw files in the GEO database is not as standard as the other microarray platforms.

3.4.2. Sample quality control

After normalization of the gene expression and annotation of the samples, quality control tests were necessary to evaluate the normalization of the data and identify batch effects. These tests were done by signal distribution analysis (using boxplot and histogram representation), Principal Component Analysis (PCA), use of the arrayQualityMetrics package (version 3.38.0) (KAUFFMAN et al., 2009) and correlation matrices between the samples. The samples that did not pass 3 or more out of 5 and had very different signals from the others were discarded. Also, the potential batch effects were corrected using the ComBat program (JOHNSON; LI;

RABINOVIC, 2007), which is part of the surrogate variable analysis (LEEK; STOREY, 2007).

3.4.3. Outlier Removal with Molecular Degree of Perturbation

Our research group has developed an R package to assess the Molecular Degree of Perturbation (MDP), which evaluates the heterogeneity of gene expression samples. In general terms, MDP calculates the degree of perturbation of each gene relative to the same gene identified in a healthy or control group of samples. Only with those highly disturbed genes, a representative disturbance score is set for each sample. Therefore, MDP can identify so-called sound samples that present some alteration in the transcriptome unidentified by phenotype.

The MDP tool identified outliers samples; in other words, samples in which the MDP score was outside the interval of the group scores. The outlier removal was performed by manually checking the ordered MDP values, and removing them with an R script since the package has not been automated to detect these outlier samples.

3.4.4. Annotate and collapse probes

The final step of the pre-processing was the annotation of the probe IDs into gene symbols and probe summarization. The probe annotation was performed using the most recent annotation file included in the package of each platform. The summarization of probes was performed using collapseRows from the WGCNA package (version 1.67). To represent each unique gene symbol, the probe with the highest average of expression was chosen (method = MaxMean).

3.4.5. Sample annotation

Each sample from all studies was manually classified into the studied phenotypic groups.

3.5. Differentially expressed genes related to MetS

3.5.1. LIMMA

Traditionally, the main focus of transcriptome data analysis is the differential expressed genes. Finding up or downregulated genes can shed light on driving the molecular processes and pathways of a specific condition. LIMMA (version 3.38.3) R package (RITCHIE et al., 2015) was used to detect differentially expressed genes (DEGs) between obese and non-obese patients for each study and tissue type. Array probes without gene symbol annotation were filtered out before LIMMA analysis. Genes were considered up or downregulated genes when fold-change (FC) was higher or lower than |1.5| and had an adjusted p-value lower than 0.05.

3.6. Gene co-expression analysis

3.6.1. Gene Co-expression Analysis with CEMiTool

The construction of highly correlated gene modules was generated by the WGCNA method adapted in the CEMiTool (version 1.7.9) R package (RUSSO et al., 2018). We developed this package to automate the WGCNA analysis by optimizing parameters and creating an easy to use Bioconductor package. The most important automatization is the selection of the beta parameter and consequently, the creation of the gene co-expression modules. For each study, the same standard parameters recommended in the package were used, including Pearson's Correlation Coefficient (PCC) for the correlation method and the use of automatic gene filtering. For each study, all the results from the CEMiTool analysis were stored in a cem object, including the module of co-expressed genes.

3.6.2. Identification of consensus communities between studies

The consensus module detection consisted of storing the cem objects into a list for each tissue, and applying the cem_overlap function. The resulting network was then processed to prioritize the edges (correlations between genes) of high confidence. This consisted of selecting the edges with present in: 1) at least 2

studies; 2) 2 studies and a PCC average > 0,8; 3) 3 studies and with a PCC average > 0,75; 4) 4 studies and with a PCC average > 0,7; 5) 5 studies and with a PCC average > 0,65; 6) 6 studies and with a PCC average > 0,6; 7) 7 studies and with a PCC average > 0,55; 8) 8 studies and with a PCC average > 0,5; 9) more than 9 studies with a PCC > 0,45.

The consensus co-expression network was partitioned in communities, dense interconnected parts of the network (REICHARDT; BORNHOLDT, 2006), using a method based on the spin-glass algorithm from the igraph package (version 1.2.4.1). We defined 10 genes as the minimum number of genes in the community. The communities of the consensus network were represented in a graph using Gephi software (version 0.9.2) (BATIAN et al., 2009). Next, functional and enrichment analyses were performed for each co-expression community of the consensus network. The following items describe both of the aforementioned analyses.

3.6.3. Protein-protein interaction between genes of modules

Protein-protein interaction (PPI) information was obtained from experiments validated by Western blot, co-immunoprecipitation, two-hybrid, among others available. For this purpose, the public database GeneMANIA: Multiple Association Network Integration Algorithm (http://genemania.org/data/) was being used. The genes pertaining to each module were connected to each other based on the protein-protein interactions of the above databases.

3.6.4. Identification of pathways related to MetS

In order to identify biological pathways related to each group of genes (modules or communities), we performed a functional enrichment analysis using the Over-Representation Analysis (ORA) method with the *clusterProfiler* package (version 3.10.1) (YU et al., 2012). Briefly, this analysis uses the hypergeometric statistical test to verify if a module overlaps (is enriched) with genes from a given biological pathway. The significance of the test is adjusted for multiple tests by the Benjamini-Hochberg method (BENJAMINI, HOCHBERG, 1995).

The genes belonging to metabolic pathways, cellular and molecular processes, were obtained in GMT format from the Reactome (http://www.reactome.org/) database. The adjusted p-values were ordered, and the pathways with -log10 greater than two were considered statistically significant. The result of this analysis was stored in a table of adjusted p-values, in which each row represents a module and each column a possible pathway or biological function associated with the module.

3.7.5. Gene Set Enrichment Analysis

The groups of co-expressed genes (modules or communities) underwent a Gene Set Enrichment Analysis (GSEA) to associate with a disease or a healthy control phenotype. The GSEA method determines if a gene set, or group of genes, shows statistically significant differences between two biological conditions (SUBRAMANIAN et al., 2005). The Lander and Mesirov group developed this algorithm to determine if the members of a set gene tend to occur in the upper (or lower) part of a list of genes ordered by degree of association to one of the two phenotype classes (TIAN et al., 2005). For this, the method applies the Kolmogorov-Smirnov test to find asymmetric distributions for defined gene blocks in the geneset distribution. It is interesting to note that the first article published with this method compared the gene expression profile of muscle biopsies between diabetic patients and healthy individuals (MOOTHA et al., 2003).

The GSEA software (http://www.broadinstitute.org/gsea) used in this study was implemented in the fgsea package (version 3.10.1) from Bioconductor (SERGUSHICHEV, 2016). The gene sets had the ".GMT" format and the expression data were passed to the program through 2 files: one containing the phenotype information of the samples (.CLS format) and the other containing the gene expression profile of the samples (.GCT format).

The GSEA results are enrichment scores (ES), which reflect how much the modules are represented at the top (positive value) or the bottom (negative value) of

a sorted list of genes. In this case, the lists were composed of genes ordered by increasing gene expression of each clinical condition.

The normalized enrichment score (NES) is preferred for comparing analyzes because it takes into account the module size and possible correlations between modules and gene expression data. Still, statistical significance was estimated by 1,000 permutations of the expression data of the members of the modules, and the false positive rate was controlled by the False Discovery Rate (FDR) of 0.005.

3.7. Analysis of miRNAs profile form Metabolic Syndrome patients

So far, no studies have performed miRNA profiling of Brazilian patients with MetS that compares to subjects without MetS. Also, scarce intervention studies are investigating the role of miRNAs in MetS (MARQUES-ROCHA et al., 2016). MiRNA profiling is important to identify possible biomarkers specific to this population.

3.7.1. MiRNAs expression profile in MetS patients

Sample collection, storage, preparation, and miRNA profiling were carried out by collaborators at the Laboratory of Molecular Investigation in Cardiology (LIMC) of the Dante Pazzanese Institute of Cardiology (IDPC). The results provided are from a collaborative project that was approved by the FCF-USP and IDCP Ethics Committee.

3.7.2. Subjects

Six volunteers who met the criteria of MetS according to IV Brazilian Guidelines on Dyslipidemias and Prevention of Atherosclerosis of the Department of Atherosclerosis of the Brazilian Society of Cardiology were selected for this study. Also, the control group was composed of six healthy volunteers (Table 2).

Table 2. Brazillian Cohort phenotype for miRNA profiling

Group	Gender	Age	Obese	SAH	IR	Low HDL-c	High TL
Control	Female	47	No	No	No	No	No
Control	Male	58	No	No	No	No	No
Control	Male	38	No	No	No	No	No
Control	Female	38	No	No	No	No	No
Control	Female	53	No	No	No	No	No
Control	Male	46	No	No	No	No	No
MetS	Male	47	Yes	No	Yes	Yes	Yes
MetS	Female	68	Yes	Yes	No	Yes	Yes
MetS	Male	37	Yes	Yes	Yes	Yes	Yes
MetS	Female	59	Yes	No	Yes	Yes	Yes
MetS	Male	61	Yes	Yes	Yes	Yes	Yes
MetS	Male	68	Yes	Yes	Yes	Yes	Yes

MetS: Metabolic Syndrome; SAH: Systemic Arterial Hypertension; IR: Insulin resistance; HDL-c: High-density lipoprotein cholesterol. TL: Triglyceride Levels.

3.7.3. Biological samples

After signing the consent forms, the research subjects underwent a clinical data interview and peripheral blood collection during the morning. The blood samples were in clinical analysis tests, while serum was pre-processed, stored at -80 freezer until miRNAs detection.

3.7.4. Analysis of miRNA expression profile in serum

“miRNeasy serum/plasma Kit™” (QIAGEN, GmbH, Hiden, Germany) was used as recommended by the manufacturer to extract serum miRNAs. MS2 Carrier (MS2 RNA, Roche) was used to increase miRNA extraction efficiency, and spike-in (*C. elegans* miR-39) was added for quality control and normalization purposes. MiRNA samples were quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, EUA), while purity was verified with the Nanodrop ND-1000 (NanoDrop

Tehnologies Inc., Wilmington, EUA). The miRNA was converted to cDNA using the miScript II Reverse Transcription (Qiagen) and then stored in -20ºC until the RT-qPCR.

The global miRNA expression analysis was performed by PCR *array* (Qiagen) according to the manufacturer's protocol. First, the miRNAs underwent reverse transcription to complementary DNA (cDNA) with the *miScript II RT Kit* (QIAGEN, GmbH, Hiden, Alemanha). The cDNAs were stored at -20ºC until PCR array.

The quality control of cDNA samples was performed with the miScript miRNA QC PCR array (código MIHS-989ZE-1, QIAGEN GmbH, Hilden, Alemanha) plate quat contains 4 control miRNAs (cel-miR-39-3p, cel-miR-16-5p, cel-miR-21-5p, cel-miR-191-5p), 3 non-coding RNA (SNORD 61, SNORD 95, SNORD 96A), miRTC (reverse transcription control) e PPC (PCR reaction positive control) for each sample. Only the samples that passed quality control checks were used in the PCR array.

The miRNA expression was analyzed using commercial miScript miRNA PCR Array Human miFinder 384HC (código MIHS-3001Z, QIAGEN GmbH, Hilden, Alemanha). This panel detects 372 miRNAs whose expression is abundant in most tissues and fluids, and are best characterized in the miRBase database registry (www.miRBase.org).

The QuantStudio ™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) system was used for the PCR reaction detection. The raw ".eds" files were initially processed in Expression Suite Software v1.0.3. This software allowed to remove the qPCR reactions with more than 2 peaks from the dissociation curves (TM) and automatically established one threshold for each miRNA applied to all samples and plaques. The sample Ct values were obtained and exported to the ".txt" format for the normalization with R written scripts.

The PCR efficiency was accessed if the Ct values of Positive PCR Control (PPC) comprised between 17 and 21. The reverse transcriptase (RT) inhibition also verified by subtracting the mean of the miRTC Ct values from the mean of the PPC Ct values [mean(Ct^{miRTC}) - mean(Ct^{PPC})]. The resulting values above 7 may indicate the presence of sample impurities that inhibit the RT reaction.

Variations of the qPCR reaction due to RNA extraction were corrected by the spike-in-control (cel-miR-39-3p) normalization. This normalization step of the Ct values was done by calculating the correction factor for each sample, and adding this factor to the Ct of all the miRNAs in that sample. The correction factor of a sample was equal to the mean of the Ct cel-miR-39-3p values of all samples subtracted by the mean of the cell-miR-39-3p of the same sample.

Next, we chose the normalization method based on housekeeping miRNAs. For this, all miRNAs that had missing Ct values were removed. Then the miRNA expression set was submitted to the function selectHKgenes implemented in the SLqPCR package (version 1.50.0). This function implements the geNorm method of reference transcript selection, in our case, miRNAs. All the miRNAs underwent the M-value (mean of expression stability) calculation. We chose the 5 most stable miRNAs, in other words, miRNAs with the lowest M-values, and normalized the all miRNAs Ct values with the average Ct of the normalizing miRNAs (VANDESOMPELE et al., 2002).

The mRNA expression was calculated by the relative quantification method with the formula $2^{-(\Delta\Delta Ct)}$, where ΔΔCt = ΔCt Disease - ΔCt Control), and ΔCt = Ct (each miRNA) – MeanCt (normalizing miRNAs). Differential expression analysis was performed with a T-test to find out which miRNAs had increased or decreased expression in the MetS concerning the group of patients not affected by the disease. The differentially expressed miRNAs were compared with the results of the bioinformatics analysis of microarray studies.

3.8. miRNA and target mRNA interactions

In order to identify miRNAs that regulate the differentially expressed genes, experimentally validated miRNA-mRNA interactions from the miRTarBase database (version 7.0, 15/09/2017 release) were used (CHOU et al., 2018). The visualization of the interaction networks of the differentially expressed miRNAs and their target genes were created either in Gephi (version 0.9.2) or Cytoscape (version 1.8.0) software.

4. Results

4.1. Selection of public microarrays mRNA expression studies

The GEO-NCBI search for gene expression studies of MetS and related diseases produced a total of 632 studies. However, only 43.35% (274/632) of these studies were derived from microarray technology. The study annotation and manual curation of microarray gene expression studies were followed by study removal according to the established inclusion and exclusion criteria. These criteria removed an astounding 80.3% (220/274) of the studies.

Most of the studies (45%, 99 studies) were excluded because patients had other diseases, such as cancer and infectious diseases. In addition, of the 220 excluded studies, 45 studies (20.45%) had less than 14 samples, 29 (13.18%) were of cell lineage or culture, 15 (6.82%) had no disease, 10 (4.55%) had no information on the samples, 9 (4.09%) were from non-commercial platforms, 7 (3.18%) were Superseries, and 6 (2.73%) studies were not from humans.

Of the 54 studies that passed the initial exclusion criteria, 28 studies did not have a control group, resulting in 26 studies that had patient information about obesity or MetS (Table 3). Still, some of these studies had patient samples that did not meet the inclusion criteria. For example, those patients who suffered some type of intervention (medical or nutritional), who had type 2 diabetes or other diseases were excluded. In the end, our study had a total of 815 samples, 339 were control patients, and 476 samples had diseases (Table 4). The studies were categorized according to the subjects' tissue samples: subcutaneous adipose tissue (SAT), liver, muscle, and blood (Figure 5).

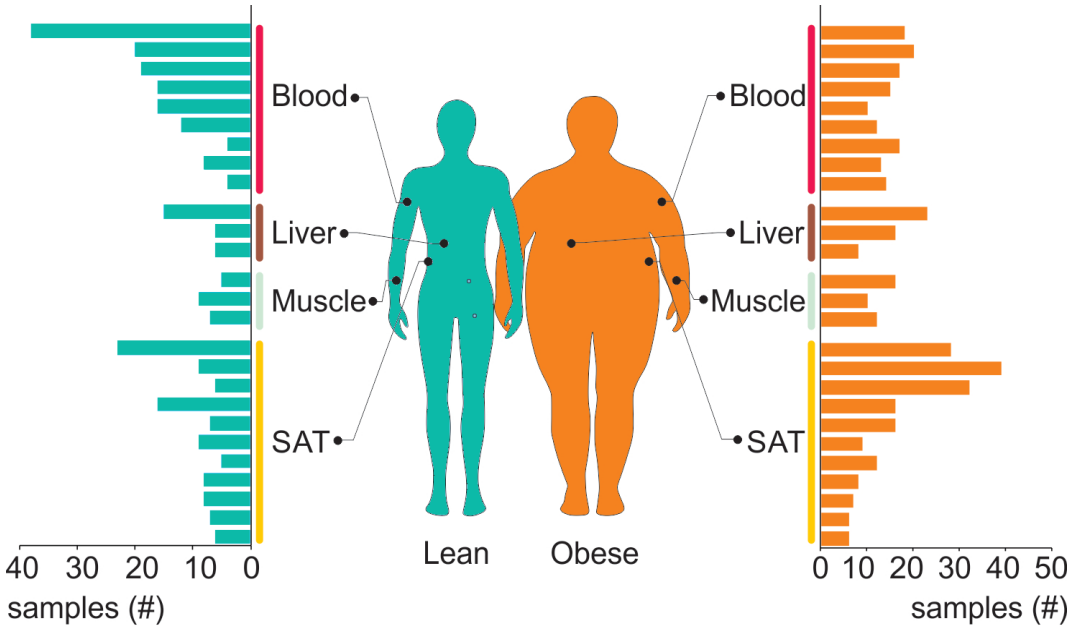
Due to all of these issues, were only able to find two studies that had patients with and without Metabolic Syndrome. Among all of the components of MetS, obesity had the highest number of studies in the database, the reason why those studies were chosen. Since the aim was to investigate miRNAs in MetS, we combined obesity transcriptomic studies with the miRNAs profile from MetS patients.

Table 3 - Number of human studies of Metabolic Syndrome and related diseases in Gene Expression Omnibus

Diseases	All types of Technologies	Microarray Technology	Selected for analysis	Analyzed
Metabolic Syndrome	64	25	3	2
Obesity	270	116	38	24
Hypertension	145	89	5	0
Insulin Resistance	102	37	8	0
Hypobetalipoproteinemia	2	2	0	0
Hypertriglyceridemia	6	5	0	0
Total	632	274	54	26

The search filters were: “Series” (*Entry Type*), “Homo sapiens” (Organism); “Expression profiling by array” (Study Type). The database research was done in May of 2015 and again in May 2018.

Figure 5 - Obesity studies: number of samples and studies for each tissue and condition



Twenty-six studies were included in our meta-analysis that included 4 tissue types: blood, liver, muscle, and SAT. The green bars represent the number of samples from lean individuals for each study and in orange the number of samples of obese patients. Source: Own authorship. SAT: subcutaneous adipose tissue.

4.2. Download and pre-processing of mRNA expression data

The most time-consuming part of the study was to learn, write, revise, test, and run all the scripts and code for the file manipulation, sample processing, and normalization. The majority of the code was written in R language using the Rstudio software (version 1.1.456) for Linux, and saved at the cloud service Github.

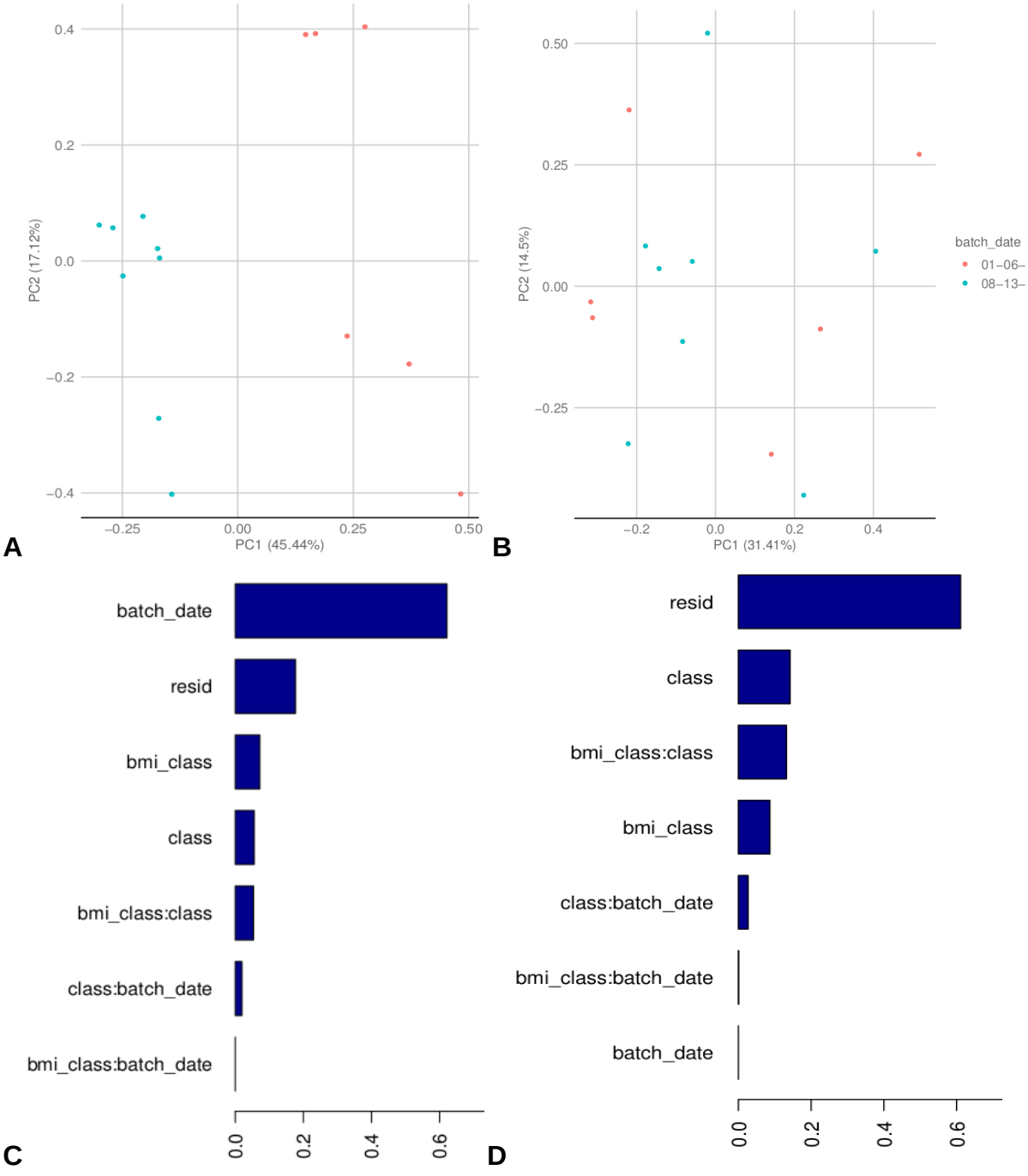
The PCA and PVCA were used to visualize and access the batch effect influence on the dispersion and variation of microarray expression data. The PCA plot shows data distribution of principal component (PC) 1 and PC2. In Figure 7 A, it is clear how the data from the same batch cluster together, whereas after the batch effect correction with the ComBat package the data did not show the same clustering (Figure 7 B). The PVCA analysis shows how the batch component “batch_date” significantly explains the sample variation (Figure 7 C). In contrast to after the batch effect removal in which the “batch_date” accounts for a small part of sample variation (Figure 7 D). All studies underwent quality control with arrayQualitymetrics (exemplified in Figure 6), and only 4 (GSE109597, GSE29718, GSE44000, GSE48452) studies had batch effects problems.

Table 4 - **Number of samples in each microarray transcriptome study**

Series	Platform	Tissue	Number of Samples			Reference
			Total	Healthy	Condition	
GSE109597	GPL570	Blood	84	43	41	(JOSEPH et al., 2018)
GSE12050	GPL7034	SAT	36	18	18	(MUTCH et al., 2009)
GSE18897	GPL570	Blood	40	20	20	(GHOSH et al., 2010)
GSE24883	GPL4133	SAT	48	16	32	(KLIMCÁKOVÁ et al., 2011)
GSE25401	GPL6244	SAT	56	26	30	(ARNER et al., 2012)
GSE25462	GPL570	Muscle	26	14	12	(JIN et al., 2011)
GSE27949	GPL570	SAT	21	5	16	(KELLER et al., 2011)
GSE29718	GPL6244	SAT	20	10	10	(TAM et al., 2011)
GSE32575	GPL6102	Blood	24	6	18	(HULSMANS et al., 2012)
GSE44000	GPL6480	SAT	14	7	7	(DENG et al., 2013)
GSE474	GPL96	Muscle	24	8	16	(PARK et al., 2006)
GSE48452	GPL11532	Liver	28	12	16	(AHRENS et al., 2013)
GSE53232	GPL11532	Blood	32	17	15	(ESSER et al., 2015)
GSE55200	GPL17692	SAT	23	7	16	(BADOUD et al., 2014)
GSE55205	GPL10558	Blood	13	6	7	(CHEN; LI; XU, 2015)
GSE59034	GPL11532	SAT	32	16	16	(PETRUS et al., 2018)
GSE61260	GPL11532	Liver	45	21	24	(HORVATH et al., 2014)
GSE64567	GPL10558	SAT	40	5	35	(WINNIER et al., 2015)
GSE64998	GPL11532	Liver	14	6	8	(KIRCHNER et al., 2016)
GSE69039	GPL10558	Blood	18	4	14	(JUNG et al., 2016)
GSE73034	GPL6480	Muscle	21	7	14	(CHAUDHURI et al., 2015)
GSE80654	GPL17586	SAT	14	7	7	(EHRLUND et al., 2017)
GSE83223	GPL10558	Blood	22	9	13	(PINHEL et al., 2018)
GSE87493	GPL6244	Blood	32	20	12	(STROJNY et al., 2017)
GSE94752	GPL11532	SAT	48	9	39	(KULYTÉ et al., 2017)
GSE98895	GPL6947	Blood	40	20	20	(D'AMORE et al., 2018)

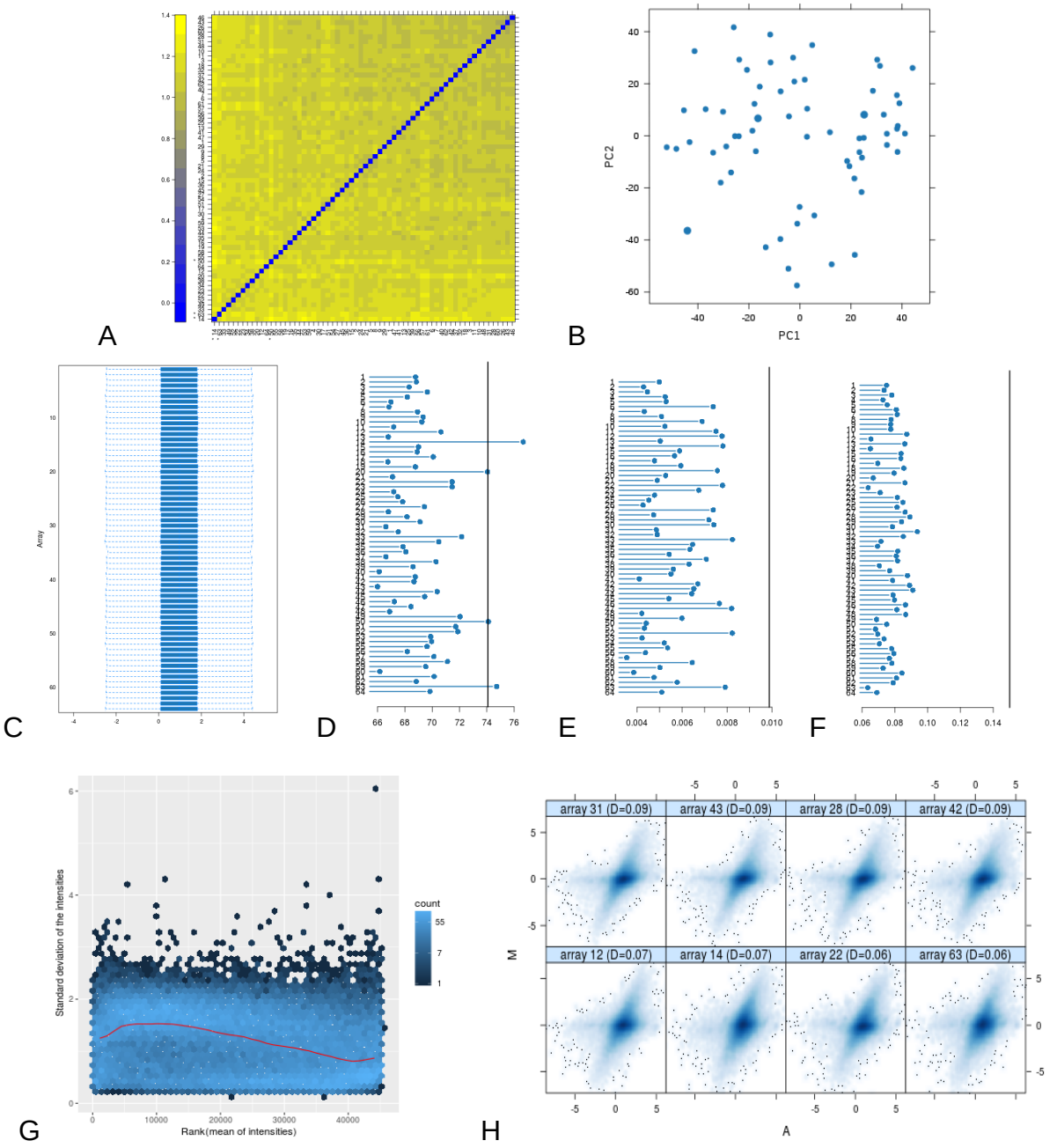
There was a total of 815 samples. SAT: Subcutaneous Adipose Tissue. GSE: GEO Study.

Figure 6 - PCA and PVCA before and after batch effect correction with ComBat



Principal Component Analysis (PCA) plots before (A) and after (B) ComBat batch effect correction. Each color represents a different batch. The Principal Variance Component Analysis (PVCA) plots before (C), and after (D) shows the percentage (x-axis) that the variables (y-axis) contribute to data variability.

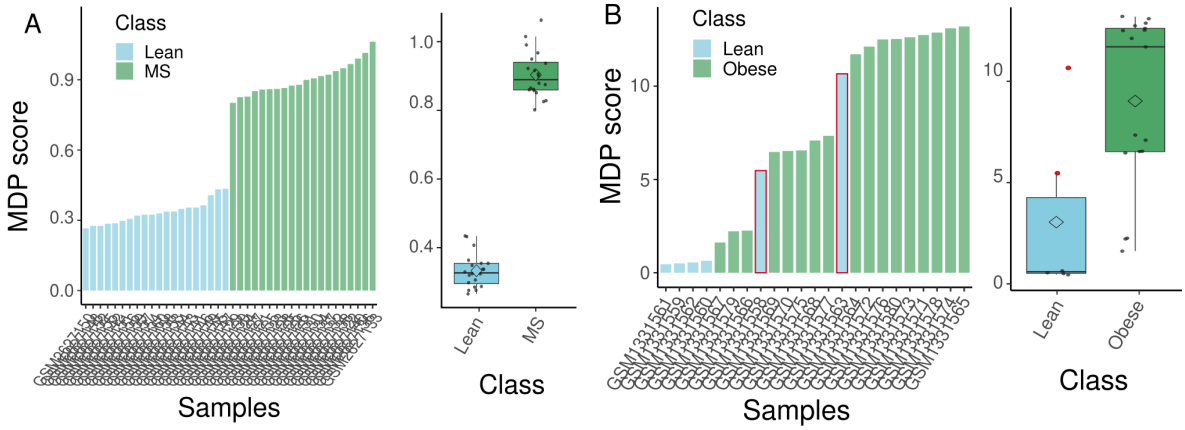
Figure 7 - Quality control by arrayQualityMetrics from study GSE27949 after quantile normalization



Legend: (A) Heatmap of the distance between arrays. (B) Principal Component Analysis (PCA) of the arrays. (C) Distribution of detected intensities of each array. Barplot of outlier detection criteria using the distance between arrays (D), boxplots (E) or by MA plots (F); the vertical bars represent the threshold of outlier sample. (G) Density graph of the standard deviation of intensities versus the median ranking of X intensities, the red dots are the standard deviation medians. (H) MA plots, array quality where M and I values were calculated by the formula M = log2(I1) - log2(I2), A = 1/2 (log2(I1) + log2(I2)).

After sample quality assessment, the MDP analysis was performed to remove samples with different from their phenotypic group (outliers). In most studies, there were no samples with MDP values different from their phenotypic group (Figure 8 A). The studies with discrepant samples had few samples to be removed (Figure 8 B). Due to the redundant microarray design, in which there is more than 1 probe for each transcript, the final number of transcripts is significantly reduced after gene annotation and summarization (Table 5).

Figure 8 - MDP values of each sample in histogram and boxplot



The Molecular Degree of Perturbation (MDP) analysis of study GSE98895 (A), and GSE55205 (B). Each lean (in blue) or obese (green) individual has an MDP value. By sorting the samples by the MDP values, it is possible to identify samples with MDP values different from their phenotype group. No samples were removed from the study GSE98895 (A), but two lean samples were removed from GSE55205 (B) and are highlighted in red.

We came about a few issues during sample annotation. First, not all samples were classified by the authors as obese or lean. In these cases, we used the BMI classification when it was available. Furthermore, some studies even classified obese patients as metabolically healthy (MHO) or metabolically unhealthy (MUO). However, in this study, we made no distinction between MHO and MUO because there is still contradictory evidence supporting the absence of cardiovascular disease risk in MHO patients (ECKEL et al., 2016; STEFAN et al., 2013).

Table 5 - Number of probes and transcripts per study platform

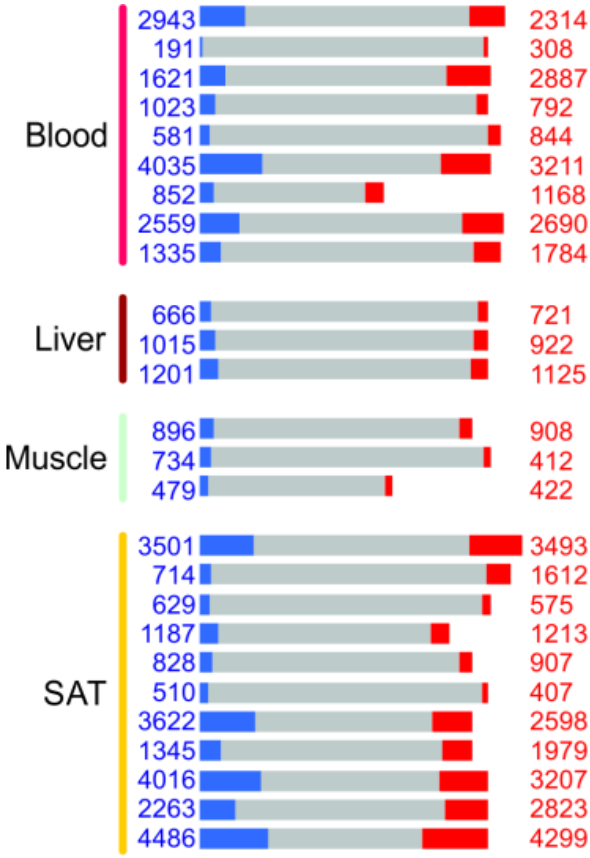
Company	Platform	Number of probes	Number of transcripts
Affymetrix	GPL11532	33,297	20,057
Affymetrix	GPL17586	70,753	23,987
Affymetrix	GPL17692	53,617	27,936
Affymetrix	GPL570	54,675	20,978
Affymetrix	GPL6244	33,297	20,057
Affymetrix	GPL96	22,283	12,993
Agilent	GPL4133	45,220	18,835
Agilent	GPL6480	41,108	18,835
Agilent	GPL7034	89,510	18,835
Illumina	GPL10558	48,107	25,993
Illumina	GPL6102	48,702	35,806
Illumina	GPL6947	49,576	32,674

GPL: GEO platform.

4.3. Differentially expressed genes related to MetS

In order to find a characteristic gene signature for obesity, the differential expression analysis was performed for each study with the LIMMA package (version 3.38.3). Independently of tissue type, most studies had over 1000 differentially expressed genes with a variable proportion of DEGs in each study (Figure 9). The SAT had the highest average number (4201.27) of DEGs, and the highest DEGs percentage (22.33%) per study, followed by blood (3459.78, 18.53%), liver (1883.33, 9.99 %), and muscle (1283.67, 7.78%).

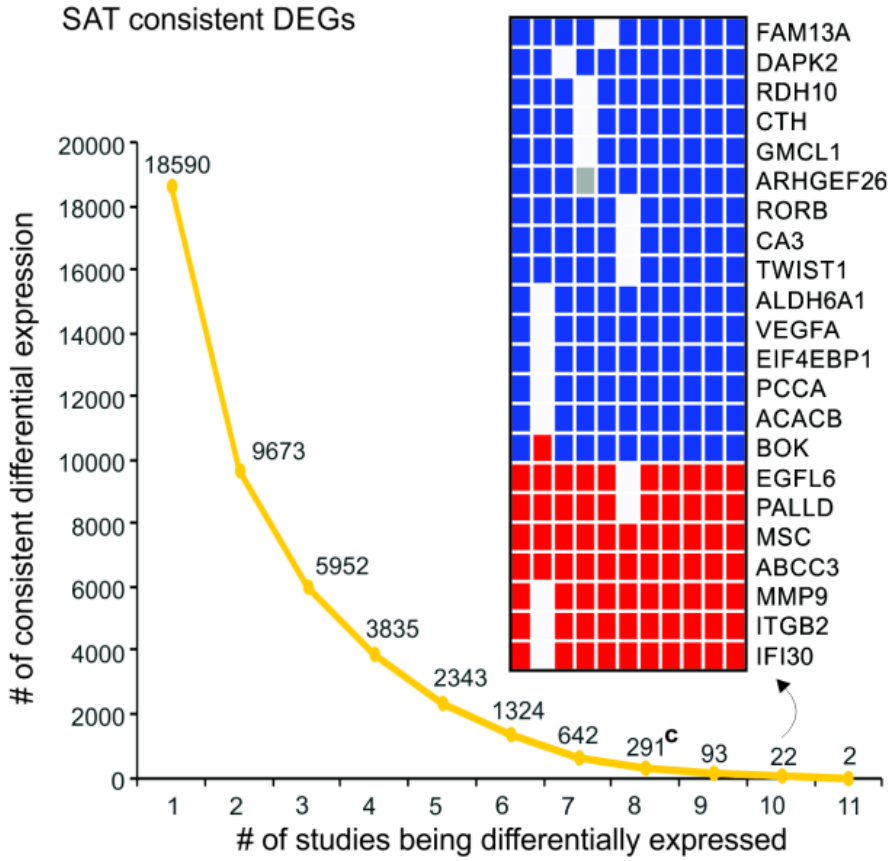
Figure 9 - Number of up and downregulated genes between Obese and Lean by tissue and dataset



The differential expression analysis was performed using limma and the same cut-offs for all studies (p-value <0.01 and FC < 1.5). The red bars represent the number of upregulated genes in the obese group compared to Lean, and blue bars represent downregulated genes. In grey are the genes that were not differentially expressed. The total size of the bars represents the number of total genes in the gene expression microarray platform. SAT: Subcutaneous adipose tissue.

The vote counting method was used to find overlapping DEGs. Besides having the largest number of studies (Figure 9), the SAT also appeared to have more common DEGs between studies (Figure 10) than the other tissues (Figure 11). In SAT studies, there were more downregulated than upregulated genes in the top 22 most consistent DEGs (Figure 10). Of those genes, two were upregulated in all SAT samples: musculin (*MSC*) and ATP Binding Cassette Subfamily C Member 3 (*ABCC3*).

Figure 10 - Number of consistent DEGs between Obese and Lean SAT studies



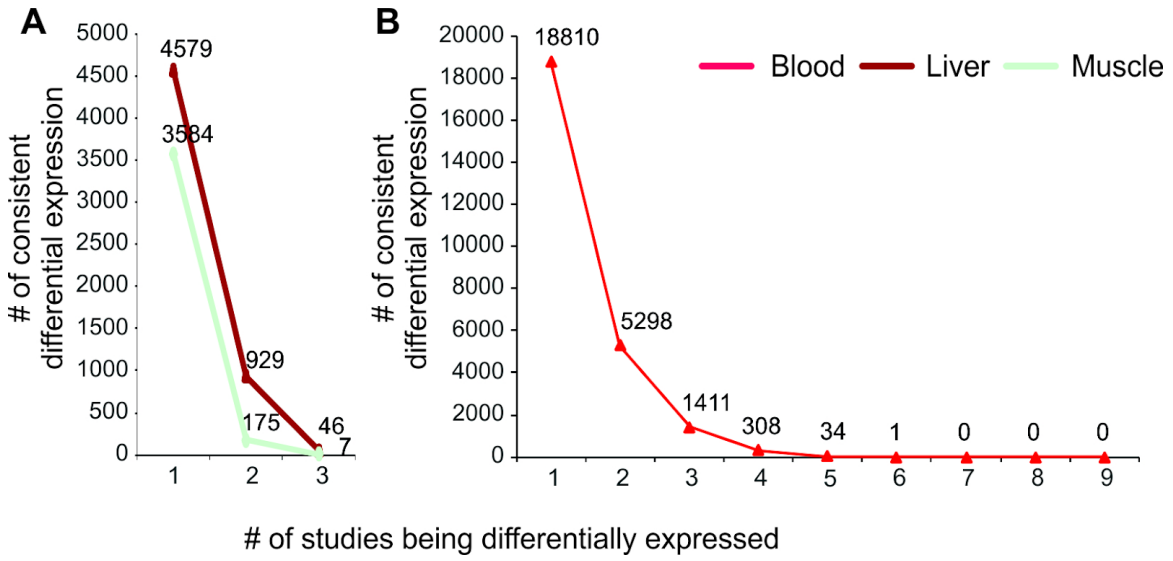
The differentially expressed genes (DEGs) between Obese and Lean was found for each subcutaneous adipose tissue (SAT) study. The vote counting method was used to find consistent DEGs. The consistently upregulated genes are represented in red for each study and downregulated genes in blue. C: the number of differentially expressed genes (DEGs) that comprised our gene expression signature for obesity.

Out of the 7 genes that were consensus DEGs in muscle tissue (Figure 11 A), *WDR7*, *SCPEP1*, *COPS5*, *GGPS1*, and *PSMD10* were upregulated while *PRODH* and *IGFBP3* were downregulated. The *ABCC3* gene was downregulated in 2 studies and upregulated on the third.

In the liver tissue, 58.7% (27/46) of consensus DEGs were upregulated (*ABHD1*, *ACOT1*, *ACOT2*, *CYP2U1*, *EXPH5*, *AM167B*, *FNDC5*, *FURIN*, *GRID1*, *HPGD*, *ILDR2*, *LRFN3*, *MEP1B*, *OLFM2*, *PRRG2*, *RDH16*, *RFTN1*, *SATB2*, *SCHIP1*, *SGCB*, *SLC2A13*, *SQLE*, *TMEM45B*, *TSPAN3*, *TSPAN33*, *TTC7B*, *UBE2H*). While 41.3% of DEGs (19/46) were downregulated (*ACKR2*, *BAMBI*,

EFCAB1, *EIF3E*, *GPR88*, *GPR89B*, *IGFBP2*, *P4HA1*, *PCF11*, *RP1*, *SCARNA9L*, SNORD14C, *SNORD47*, *TAF4B*, *TCTN2*, *ZNF347*, *ZNF507*, *ZNF600*, *ZNF880*).

Figure 11 - Number of consistent DEGs between Obese and Lean in Blood, Liver, and Muscle studies



The differentially expressed genes (DEGs) between Obese and Lean was found for each study. The vote counting method was used to find common DEGs among the studies of Blood, Liver and Muscle tissues.

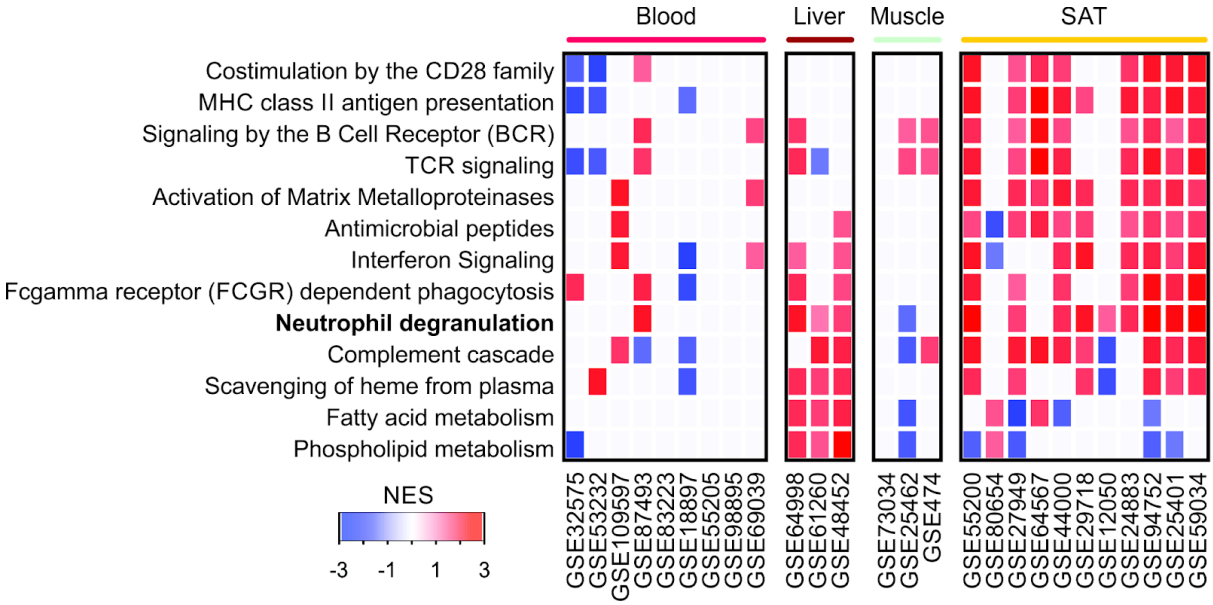
Even though studies performed in blood had the second-highest number of studies, there were not many genes in common higher than 5 out of 9 studies (Figure 11 B). The only gene to be differentially expressed in most blood studies was *TNIP1*; it was upregulated in six out of nine studies. There were 34 consensus DEGs in 5 out of 9 studies with blood, where 41.2% (14/34) were upregulated (*ADNP2*, *ATXN7L3*, *DLGAP4*, *GNG5*, *HBM*, *KLHL15*, *MUC1*, *RABAC1*, *RMND5A*, *SOCS3*, *TNIP1, TWF1*, *VWA5A*, *ZNF101*) and 58.8% (20/34) were downregulated (*AASDH*, *CPOX*, *E2F6*, *EARS2*, *ERI2*, *EXOC1*, *GLE1*, *HEATR1*, *INTS2*, *IPP*, *KLHL24*, *LRRC40*, *LTN1*, *POLR3A*, *RHBDL2*, *TDP1*, *TMEM38A*, *TRIM38*, *ZC3HC1*, *ZNF573*).

In order to see the consistency of the biological pathways and functions of the differentially expressed genes, a pathway enrichment analysis with GSEA was performed for each study (Figure 12). The blood tissue presented the least pathway enrichment consensus between the studies, with only “MHC class II antigen

presentation" pathway with a significant NES (Normalized Enrichment Score) in 3 out of 9 studies. The muscle presented similar problems, as only "TCR signaling" and "Signaling by the B Cell Receptor (BCR)" had positive NES in 2 out of 3 studies.

The liver and SAT had more compelling and consistent results. In the liver tissue, there was a positive NES for "fatty acid metabolism" and "phospholipid metabolism" pathways. There was also evidence of pro-inflammatory state in the liver and SAT: positive NES for "neutrophil degranulation", and "Interferon Signaling" pathways.

Figure 12 - Pathway enrichment analysis of all studies separated by tissue

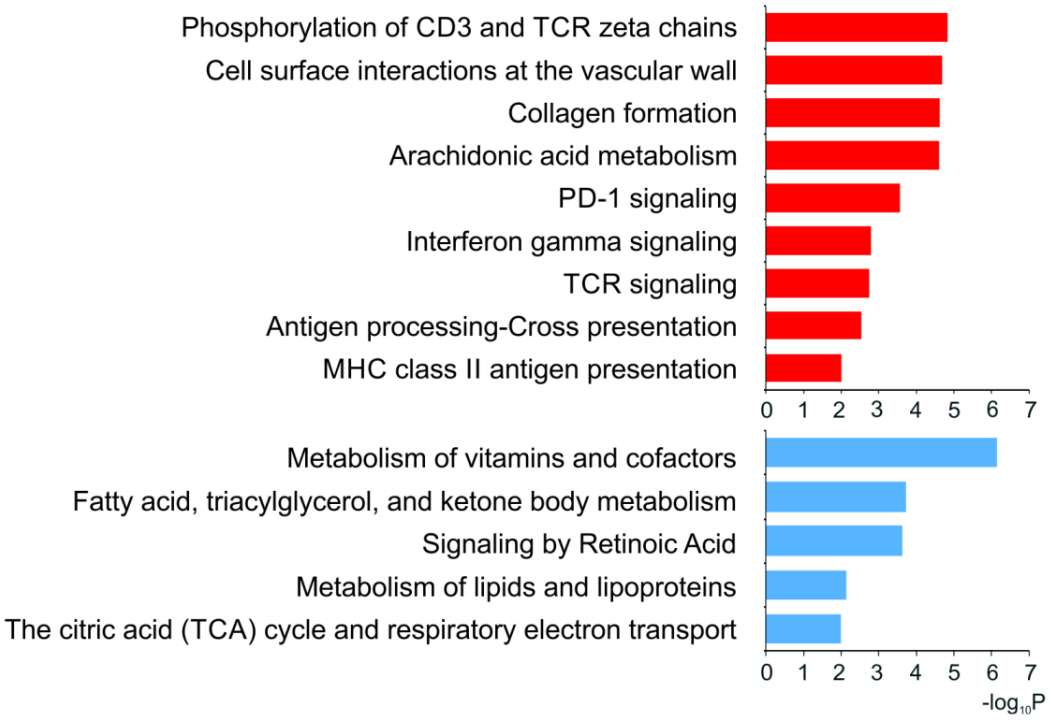


The differentially expressed genes (DEGs) from each tissue underwent a Pathway Enrichment Analysis using the Reactome database. Only the pathways with significant enrichment are shown (p-value < 0.01). The pathways in red had a positive NES and pathways in blue had negative NES. NES: normalized enrichment score.

The next step was to investigate the biological pathways and functions that the consistent differentially expressed genes had. The functional analysis showed that the upregulated genes in obese SAT had enrichment for the activation of adaptive immunological response pathways: PD-1 signaling, interferon-gamma signaling, TCR signaling, antigen processing-Cross presentation and MHC class II presentation (Figure 13). Whereas the downregulated genes in obese subjects were

related to metabolic processes and pathways: vitamins and cofactors, fatty acids, triacylglycerol and ketone body, lipids and lipoproteins.

Figure 13 - Significantly enriched Reactome pathways of the DEGs from SAT



The upregulated genes (in red) and downregulated genes (in blue) from subcutaneous adipose tissue (SAT) underwent a pathway enrichment analysis using the Reactome database. For each Reactome pathway, the bars show the significance of enrichment of differentially expressed genes (DEGs). Only the pathways with enrichment of $-\log_{10}P > 2$ are shown.

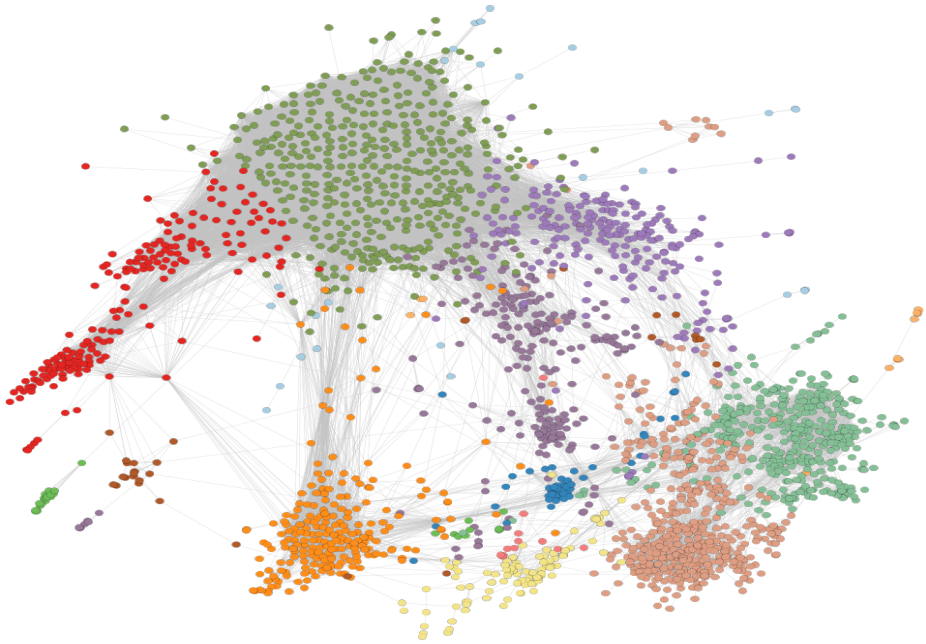
4.4. Gene co-expression analysis

4.4.1. Consensus communities of the co-expression network

After the studies were grouped by tissue and phenotype groups, the cem object for each co-expression analysis was integrated with the “cem_overlap” function, which creates a consensus network and also performs a community analysis. In SAT studies, three large communities containing more than 300 genes were found, five communities between 100 and 33 genes, and five small communities with under 100 genes each (Figures 14 and 15). The gene

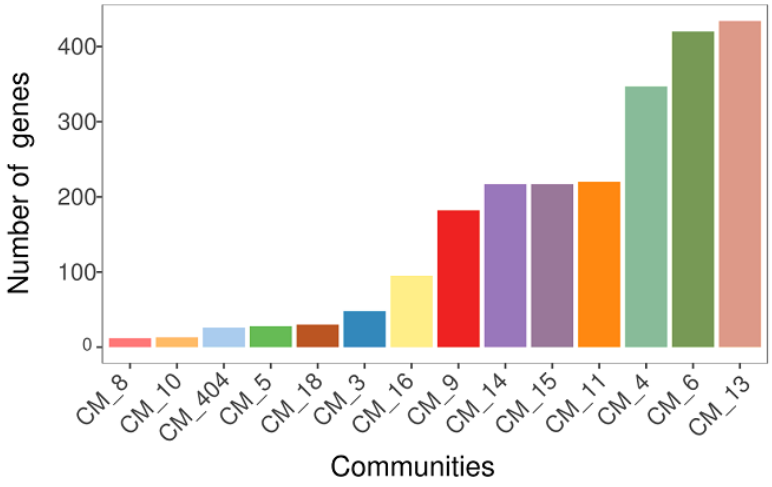
co-expression consensus network uncovered 16 communities for the liver, 14 communities for blood, and four small communities for muscle.

Figure 14 - SAT Consensus Co-expression Network



The consensus co-expression network for Subcutaneous Adipose Tissue (SAT) has 14 communities. Each color represents a different community, a densely interconnected part of the network (REICHARDT; BORNHOLDT, 2006). We defined the community with a minimum of 10 genes. The graph was created with Gephi software (version 0.9.2).

Figure 15 - Number of genes in each CM of the SAT Consensus Co-expression Network



Each color represents a different community (CM) from the Consensus co-expression network for the SAT. The same colors were used for figures 14 and 16. SAT: subcutaneous Adipose Tissue. CM_404: a group of genes that were not part of any community.

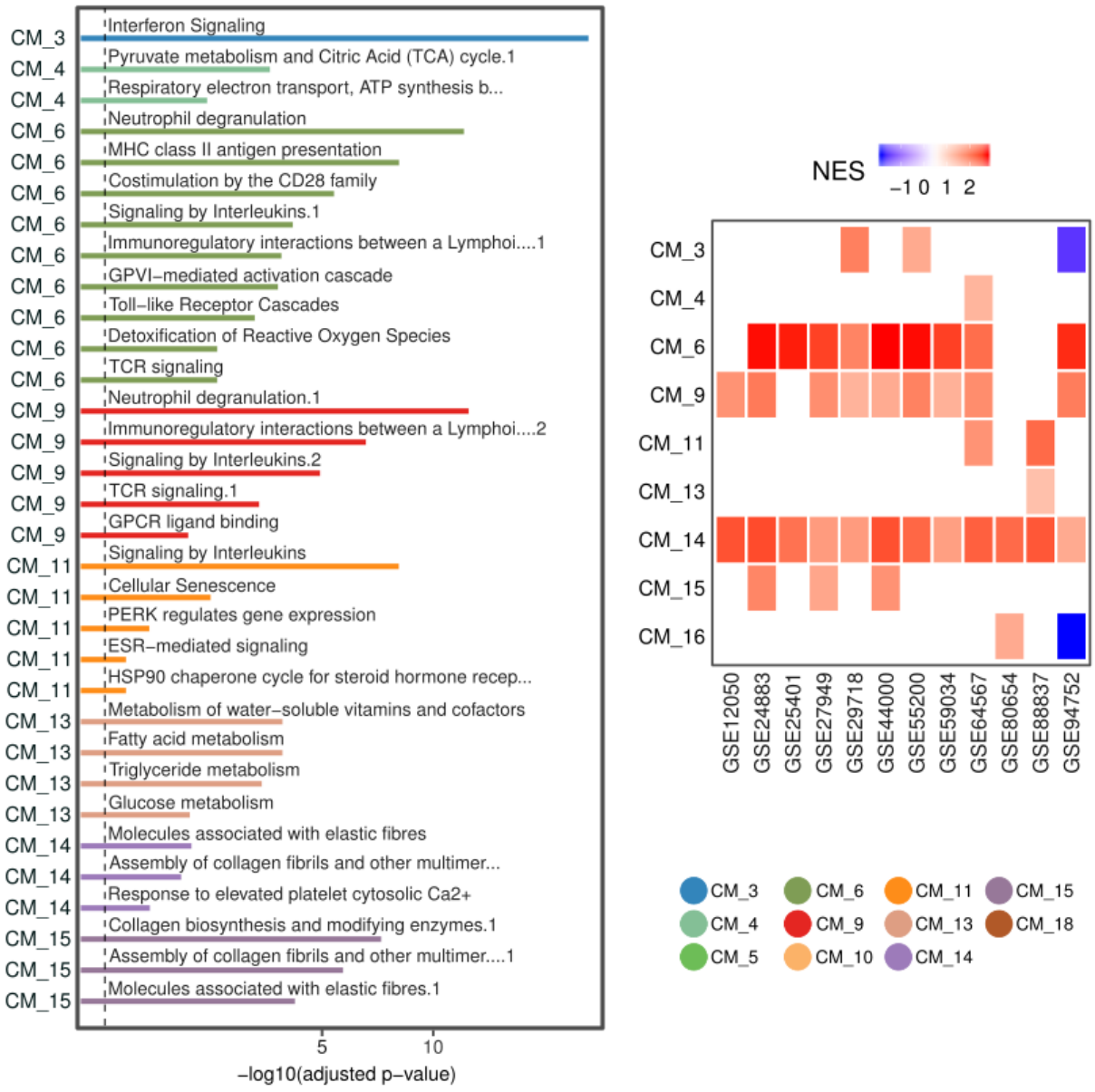
4.4.2. Functional Analysis of the Consensus Co-expression Communities

The co-expression communities for each tissue underwent functional annotation analysis and GSEA. The consensus co-expression communities for blood, muscle, and liver did not show any consistent results for pathway enrichment analysis or GSEA (data not shown). On the other hand, it was able to find consistent pathways and genotype enrichment for SAT communities from the consensus co-expression network (Figure 16).

The SAT had 11 communities with more than ten genes. Eight communities had enriched pathways, 2 of which had enrichment for neutrophil degranulation. Many of the pathways shown are related to inflammatory processes that occur in obesity. For example, interferon-gamma is one of the cytokines of the inflammatory T cell response associated with obesity-induced by diet (ROCHA et al., 2008).

The GSEA showed three communities had a positive enrichment in the majority of the studies with SAT (Figure 16, right). Two of those communities had the neutrophil degranulation enriched pathways (CM 6 and CM 9).

Figure 16 - Functional annotation analysis by ORA and GSEA of SAT Consensus Co-expression Modules

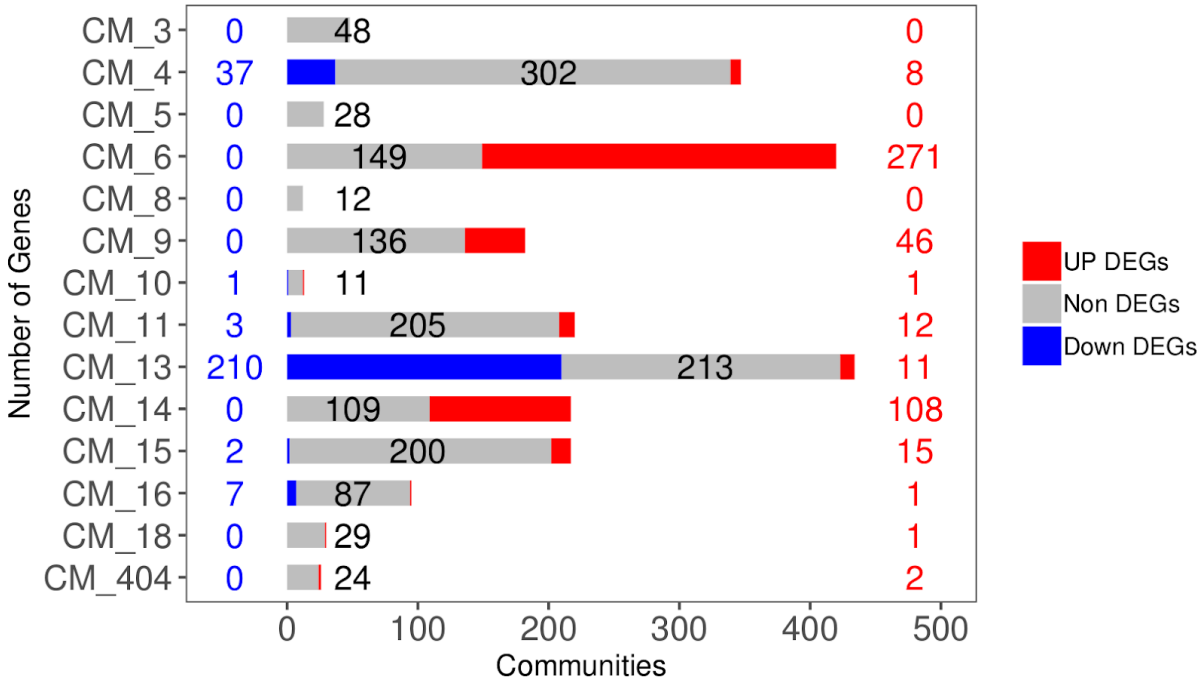


Pathway enrichment analysis (left) and Gene Set Enrichment Analysis (right) of the communities (CM) within the consensus network. The adjusted p-value was converted into -log10 and shown a color scale from blue to red. ORA: Over-Representation Analysis. CM: community. SAT: subcutaneous adipose tissue.

Combining the information from the co-expression analysis and differential expression analysis, we found which communities had more upregulated and downregulated genes (Figure 17). Communities 13 and 4 had the most number of

commonly downregulated genes, whereas communities 6, 14, and 9 had the most genes with upregulated genes.

Figure 17 - The number of DEGs in each CM of the SAT Consensus Co-expression Network



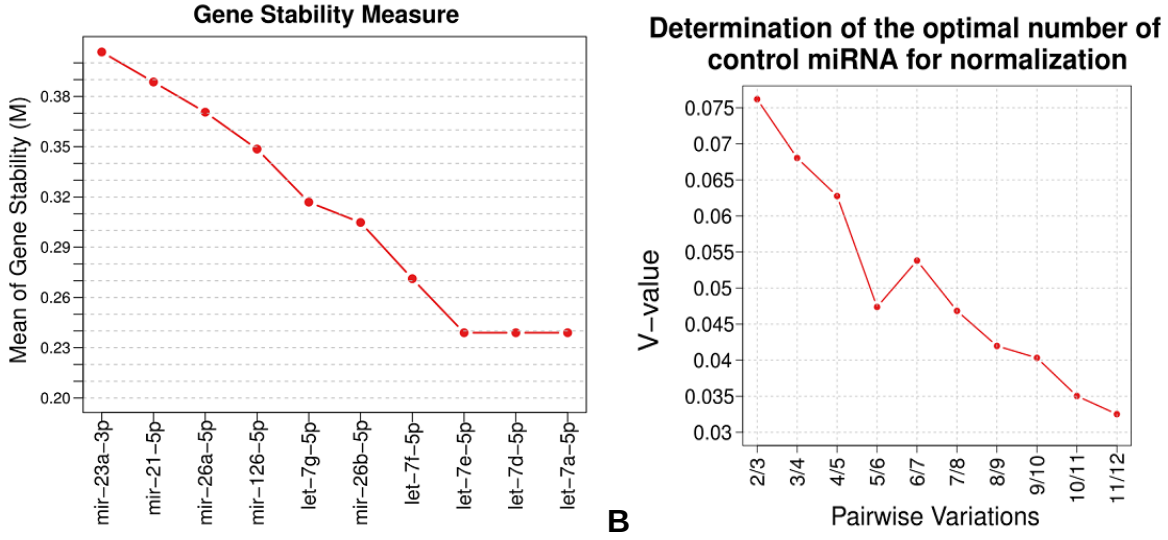
Blue: downregulated genes, red: upregulated genes, grey: genes not differentially expressed. CM_404: a group of genes that were not part of any community. CM: community. SAT: subcutaneous adipose tissue.

4.5. Analysis of circulating miRNAs in MetS patients

The quality control steps of the miRNA qPCR experiments did not exclude any samples. In the PCR efficiency check, the PPC values of all samples varied between 18.02 and 18.66 with a median of 18.36, in other words, all samples had values within the recommended interval from the manufacturer (17 to 21). The values from the transcriptase inhibition check [Average(Ct^{miRTC}) - Média(Ct^{PPC})] were between 4.53 and 6.03 (median of 5.08). According to the manufacturer, values below 7 indicates a profound influence of impurities in the reaction. Next, we performed the normalization of the Ct (cycle threshold) values with the correction factors calculated from the Ct values of cell-miR-39-3p. The median of the normalization factors was -0.0595 (minimum = -1.4683, maximum = 1.2212).

The normalization method performed in the qPCR array was by housekeeping miRNAs. After removal of the quality control miRNAs (miRTC, PPC, cel-mir-39-3p), and miRNAs with incomplete Ct values, there were 124 unique miRNA left to choose the most stable normalizing miRNAs. The lower the gene stability mean (M) value, the more stable is the miRNA. The M values of 10 most stable miRNAs from the experiments are shown in Figure 18 A. The choice of the number of housekeeping miRNAs was made based on the graph of Pairwise variations (Figure 18 B).

Figure 18 - Determination of normalizing circulating miRNAs

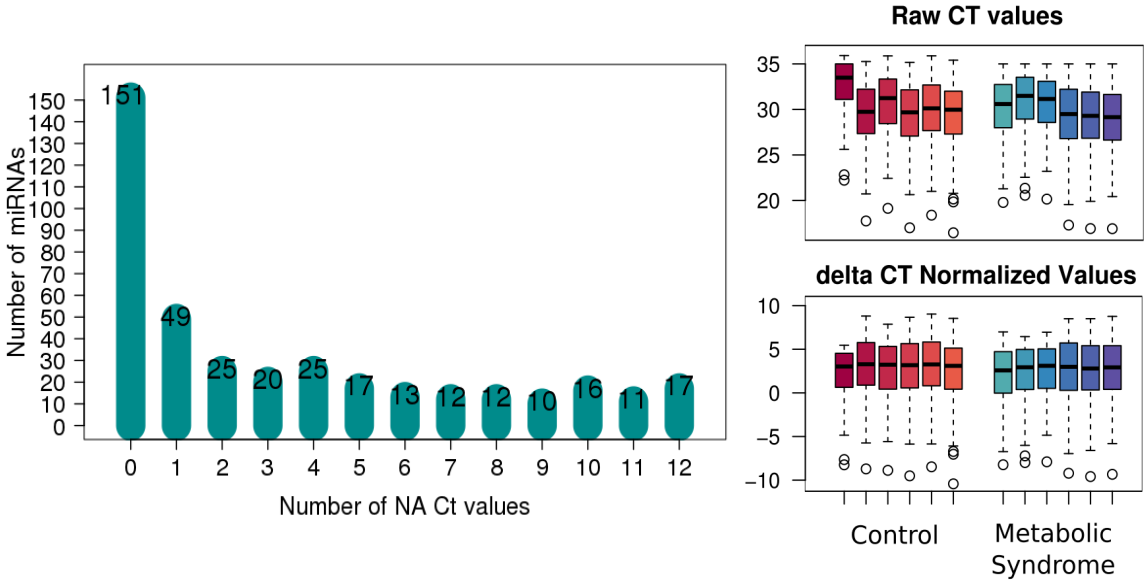


A: The ten miRNAs with the lowest Gene Stability Mean (M). B: determination of the optimal number of control miRNAs for normalization. Pairwise variations are based on the $V_{n/n+1}$ calculation between 2 sequential normalizing factors. For a cut-off of v-value = 0.05, five miRNAs were necessary for the normalization of these samples. The five miRNAs are: hsa-let-7a-5p, hsa-let-7d-5p, hsa-let-7e-5p, hsa-let-7f-5p and hsa-miR-26b-5p.

Out of the 378 miRNAs from the miRNA PCR array, 33 miRNAs had an average CT value over 35, and 17 miRNAs were undetectable in all 12 samples. Figure 19 shows the number of reactions with undetected miRNAs from MetS serum. In total, there were 151 miRNAs with detected reactions for all samples, most miRNAs with undetected reactions (49) had only one undetected reaction. After normalization, the miRNA differential expression analysis with 222 miRNAs

uncovered six significantly upregulated miRNAs (p-value <0.01) in MetS patients compared with healthy controls (Table 6).

Figure 19 - MiRNAs PCR array quality control and Ct values



The number of undetected miRNAs (left panel), a boxplot of CT values of all miRNAs for each sample before and after normalization (right panel). PCR: polymerase chain reaction. Ct: cycle threshold.

Table 6 - Circulating miRNAs differentially expressed in MetS patients

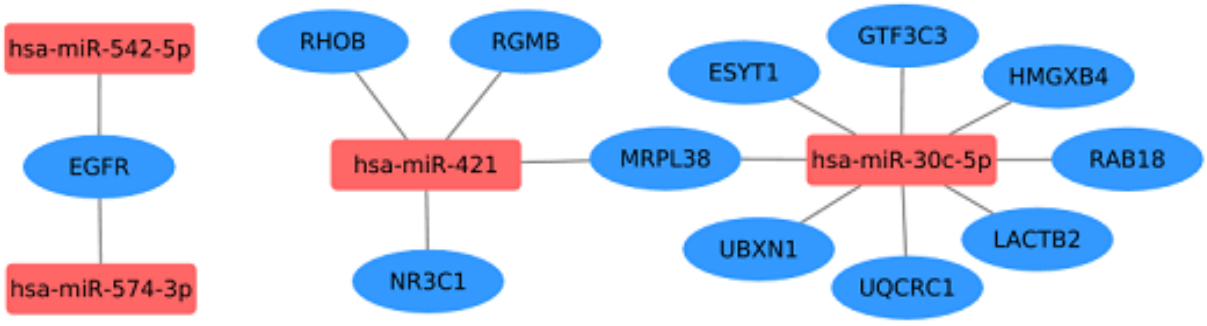
miRNA	*fold change*	p-value	Lower CI	Upper CI	BH adj. p-value
miR-574-3p	2.37	0.00040	0.7381	1.75	0.08884
miR-542-5p	3.40	0.00103	0.9091	2.627	0.11421
miR-421	2.22	0.00271	0.5081	1.793	0.20054
miR-30c-5p	1.62	0.00918	0.2155	1.180	0.28786

The miRNAs were normalized by the most stable housekeeping miRNAs using the geNorm method. Only the miRNAs with a p-value of 0.01 were considered differentially expressed. CI: confidence interval. BH adj. P-value: p-value adjusted by Benjamini-Hochberg. MetS: Metabolic Syndrome.

4.6. MiRNA-mRNA Integrated Analysis

An integrative analysis was carried out to analyze the interactions between the consistently downregulated genes from the SAT studies and the upregulated circulating miRNAs from MetS patients. There were 562 genes downregulated in six or more studies out of 11, only 12 genes of those were targets of the 6 differently expressed miRNAs (Figures 20 and 21).

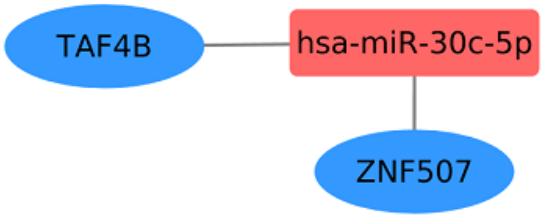
Figure 20 - MiRNA-target regulation network from consensus DEGs from SAT



Twelve downregulated genes from SAT (blue) were also targets of the upregulated circulating miRNAs of MetS patients (red). The interaction between the miRNAs and target mRNA were experimentally validated from the mirTarBase (release 7.0). The network was generated with Cytoscape (version 1.8.0). DEGs: differentially expressed genes. SAT: subcutaneous adipose tissue.

Next, the integrative analysis between the consistently downregulated genes from the liver studies and the upregulated circulating miRNAs from MetS patients was performed. From the 19 downregulated genes in all three studies, only three genes were targets of the six differently expressed circulating miRNAs (Figure 21).

Figure 21 - MiRNA-target regulation network from consensus DEGs from liver



Three downregulated genes from the liver (blue) were also targets of the upregulated circulating miRNAs of MetS (red). The interaction between the miRNAs and target mRNA were experimentally validated from the miRTarBase (release 7.0). The network was generated with Cytoscape (version 1.8.0). DEGs: differentially expressed genes.

No interactions were identified between the two consistently downregulated genes from the muscle (*PRODH* and *IGFBP3*) and the upregulated circulating miRNAs from MetS patients (from Table 6). For blood, the number of consensus DEGs was also to low to create a miRNA-mRNA interaction network.

5. Discussion

Here we utilized system biology tools to integrate data from a comprehensive transcriptomic meta-analysis with circulating miRNA of MetS patients. MetS gene signatures from 4 different tissues were using differential expression analysis, gene co-expression analysis, gene set enrichment analysis, and network analysis. We also performed a differential expression analysis of circulating miRNAs from MetS patients compared to healthy controls. Finally, we integrated both results by comparing the MetS circulating miRNAs with their tissue-specific targets.

The GEO-NCBI database has over 90,000 transcriptomic studies performed with a multitude of high-throughput technologies, experimental designs, types of samples, and for a broad range of biological conditions. The GEO-NCBI search for microarray studies of MetS and related diseases produced a total of 632 studies. After careful manual curating, only 4% of studies passed the study selection exclusion criteria. This high exclusion rate of 93.8% (257/274) is typical in many kinds of meta-analysis studies (EDINGER; COHEN, 2013) (MANSFIELD et al., 2016) (ORTEGA-BERNAL et al., 2018).

We had to manually curate each one of these studies even after using the available filters of the search tool. The GEO query system only allows the use of broad terms for sample characterization, study summary and description. The lack of specific metadata fields makes the search for a specific disease very inefficient, with a high percentage of the output including unwanted results. The need for a specific field just for disease classification has recently pushed a researcher team to create a revamped searchable tool for the GEO database named ReGEO (CHEN et al., 2019).

Although most authors follow the MIAME guidelines for the submission of transcriptome datasets, the authors provide limited clinical information (NOOKAEW et al., 2013). In some cases, authors did not even provide basic sample

characteristics, making it difficult or even impossible to use the studies (NOOKAEW et al., 2013).

Our differential expression analyses between obese and lean subjects of 26 studies in four tissue types (blood, liver, muscle, SAT), retrieved a varying number of DEGs. SAT analysis showed the highest number and percentage of DEGs per study, and this may explain the higher number of consistent DEGs. However, in a multi-tissue study (HAO et al., 2018), a higher number of DEGs was found in muscle (584) and liver (367) than adipose tissue (283). This difference was due to a high percentage of downregulated genes since the number of upregulated genes in the muscle (79.79%, 466/584, 118 upregulated) and in the liver (65.4%, 240/367, 127 upregulated) and was lower than in the adipose tissue (167 upregulated) (HAO et al., 2018). Although we identified many consistent DEGs for SAT and liver, a limited number of consistent DEGs or no consistent DEGs at all were found in muscle and blood, respectively.

Two genes *MSC* and *ABCC3* were consistently upregulated in all SAT studies analyzed. The *MSC* encodes the musculin protein, which is also known as activated B-cell factor-1 (ABF-1). This transcriptional repressor is highly expressed in activated B lymphocytes and capable of binding to an E-box element (ROMAGNANI et al., 1981). According to a few transcriptomic experiments from the Expression Atlas, *MSC* is highly expressed in adipose tissue (PAPATHEODOROU et al., 2018).

Hishikawa et al. (2005) have shown that the expression of *MSC* is inversely correlated with the expression of Leukemia Inhibitory Factor (LIF), a member of the interleukin 6 (IL-6) family whose primary function is the inhibition of cell proliferation (GOUGH et al., 1988). In a more recent mice study, it was shown that the hypothalamus inhibition of LIF leads to decreased protection against diet-induced obesity (FIORAVANTE et al., 2017). Thus, the upregulation of *MSC* observed in this study could lead to inhibition of the LIF and, consequently, to increased susceptibility to diet-induced obesity.

The *ABCC3* encodes the protein canalicular multispecific organic anion transporter two, also formerly known as multidrug resistance-associated protein 3

(MRP3) (ALLIKMETS et al., 1996). It is a part of a group of efflux proteins that transports various molecules across cellular membranes. In the liver, ABC proteins have a role in the absorption, disposition, and distribution of organic anions, such as xenobiotics and endogenous substances to bile (BELINSKY et al., 1998). Although ABCC3 function is not entirely known, its upregulation in blood-derived NK cells may be associated with a protective cell response to cytotoxic drug treatments (PESSINA et al., 2016).

According to the Expression Atlas, *ABCC3* is highly expressed in the adrenal gland, stomach, liver, and small intestine. In contrast, experiments showed a low expression of *ABCC3* in adipose tissue and immune cells (PAPATHEODOROU et al., 2018). This may be indicative of inter-tissue regulation, or infiltration of immune cells with altered *ABCC3* expression. Nonetheless, little attention in the literature is given to its involvement in immune processes and their active secretion of inflammatory mediators.

We did not find a consensus gene signature in blood for obesity (i.e. no DEGs were found in at least 70% of the studies containing blood samples). This variability between studies might be due to the inherent inter-individual differences of blood cells. According to some authors, the variation of gene expression from blood cells can come from different sources and confounding signals (EADY et al., 2005). These include technical and experimental design, inter-individual (RADICH et al., 2004) and exposure factors (DUMEAUX et al., 2010; WHITNEY et al., 2003).

A looser definition of consensus DEGs for blood, in which a gene is identified as DEG in at least 5 out of 9 studies, results in a group of 34 genes. Among those genes, *TNIP1*, also known as Naf1 or ABIN-1, was the top consistently upregulated. TNIP1 has the capacity of dampening TNF-α induced NF-κB activity (HEYNINCK et al., 1999) and acts as a corepressor of ligand-bound PPARs (FLORES et al., 2011). The increased expression of *TNIP1* rheumatoid arthritis induced by TNF-α (GALLAGHER et al., 2003) is suggestive of countermeasure against a pro-inflammatory condition. Furthermore, SNPs reported in *TNIP1* has shown to increase the risk of coronary artery disease in a Chinese cohort (SONG et al., 2017).

The muscle-derived studies showed the lowest percentage of DEGs among all tissues (7.78%). In an attempt to generate a consensus gene signature for muscle tissue, only 7 DEGs were detected in all three studies. In this small gene signature, geranylgeranyl diphosphate synthase 1 (*GGPS1*) was found consistently upregulated. The overexpression of *GGPS1* has been observed in fat and skeletal muscle tissues, and further investigated in a knockout model which showed GGPS1's mediation of lipid-induced systemic insulin resistance in obese mice (TAO et al., 2015).

The liver gene signature had 46 consensus DEGs of which 41.3% was downregulated. This included genes encoding the bone morphogenetic protein (*BMP*), and the pseudo receptor activin membrane-bound inhibitor (*BAMBI*). In LPS exposed hepatic stellate cells, transforming growth factor (TGF)-β signaling was enhanced by TLR4 activation and *BAMBI* downregulation (SEKI et al., 2007). Altered gene expression levels of TGF-β and their antagonists in adipose tissues is described in obesity (LEE, 2018).

The TATA-Box binding protein-associated factor 4b gene (*TAF4B*) was also found consistently downregulated in the liver studies. This gene is involved in the control of cell proliferation acting as an NF-kB co-activator in response to TNF-α (YAMIT-HEZI; DIKSTEIN, 1998), and TGFβ signaling mediator (MENGUS et al., 2005) of anti-apoptotic genes and pathways. In mice models, diet-induced obesity has shown to upregulate liver expression of TNF-alpha (BORST; CONOVER, 2005) and NF-kB (CARLSEN et al., 2009). NF-κB upregulation can increase serine/threonine phosphorylation of the insulin receptor substrate 1 (IRS1), which results in insulin resistance (SAAD et al., 1992), as well as elevate hepatic production of proinflammatory cytokines, including TNF-α, IL-6, and interleukin 1 beta (IL-1β).

We performed a co-expression analysis to generate a consensus network of genes related to MetS and obesity from transcriptome datasets of human tissues. We used an innovative approach that combines several studies to generate a

consensus co-expression network with our recently published package CEMiTool (RUSSO et al., 2018).

Some co-expression studies performed on adipose tissue from obese patients have used a similar meta-analysis approach. However, the authors used a module preservation method and candidate gene selection based on the connectivity of the co-expression network (HAAS et al., 2012; HE et al., 2017).

The integration of co-expression analysis with GWAS findings has found that pituitary, pancreas, esophagus, nerve, skin, and adipose tissue were a worthy investigation for obesity pathogenesis. However, with the exception of skin and adipose tissue, most of these tissues are hard to collect due to significant health risks (HAO et al., 2018). Few studies have performed co-expression analysis in adipose tissue (PRAVENEC et al., 2018), and in obese patients (WANG et al., 2017) (HAO et al., 2018), and one has even integrated co-expression analysis with miRNA-gene interaction (MIAO et al., 2019). However, none of these studies combined multiple studies into a consensus signature and compared it with differentially expressed miRNAs in circulation.

The co-expression analysis of SAT studies generated 13 densely interconnected communities. The largest communities containing over 300 genes were CM 13, CM 6, and CM 4. CM 6 had the highest number of genes found in different immune system cells. The presence of resident immune cells in the adipose tissue is well known (CILDIR; AKINCILAR; TERGAONKAR, 2013; SCHIPPER et al., 2012). These cells are responsible for apoptotic cell clearance and extracellular matrix remodeling. Their activation can lead to inflammatory processes, insulin resistance, and other metabolic complications found in obesity and MetS (FAIN, 2006; HOTAMISLIGIL et al., 1995).

The pathway most enriched in the immunometabolism co-expression community (CM 6) of the SAT was neutrophil degranulation. Neutrophils have a critical effector role in innate and humoral immunity and are part of the first line of defense against microorganisms and foreign particles (BURG; PILLINGER, 2001). Previous studies have shown that patients with elevated adiposity had higher

circulating neutrophil counts, and neutrophils degranulation (REYES et al., 2015). Even proteins secreted mainly by neutrophil granules, such as plasmatic myeloperoxidase and calprotectin, have even been found increased in a human obese study (NIJHUIS et al., 2009).

The enrichment of the detoxification of reactive oxygen species pathway is consistent with the oxidative stress and elevated superoxide production associated with obesity and MetS (FURUKAWA et al., 2004). This process can be due to the increase of neutrophils in the adipose tissue. In obese patients, peripheral blood neutrophils are prone to elevated superoxide production and chemotactic activity (BROTFAIN et al., 2015).

Cells from the adaptive immune system were also uncovered in the CM 6. The pathways of immunoregulatory interactions between a Lymphoid and a non-lymphoid cell, MHC class II antigen presentation, co-stimulation by the CD28 family, and TCR signaling are evidence of T-lymphocytes presence in SAT. The CD28 co-stimulation pathway has been shown to aid T-lymphocytes nutrient uptake by increasing the glycolytic flux and levels of glucose transporters aiding the activation of T-lymphocytes (FRAUWIRTH et al., 2002). Also, TCR signaling is responsible for T-lymphocyte proliferation, differentiation, and effector function (VAN LEEUWEN; SAMELSON, 1999).

One of the shortcomings of our analysis is that it was not possible to distinguish or quantify specific types of lymphocytes present in the SAT. However, studies have already shown the increased presence of CD8+, CD4+, Th1, Th1:Treg, Th17 lymphocytes in SAT from obese patients (YANG et al., 2010; ZÚÑIGA et al., 2010) and also mice (IP; HOGAN; NIKOLAJCZYK, 2015).

Obesity induces low-grade chronic inflammation, also known as a meta-inflammatory state (LUMENG; SALTIEL, 2011). The positive enrichment of the pathway “Toll-like Receptor Cascades” in the co-expression community CM 6 brings forth pattern recognition receptors’ (PRRs) role in the inflammatory process. PPRs can sense different molecules, such as pathogen-associated molecular patterns (PAMPs) and initiate an innate immune system response. It was shown that some

PRRs can sense endogenous ligands, as damage-associated molecular patterns (DAMPs) and generate proinflammatory signals (FESSLER; RUDEL; BROWN, 2009). For example, saturated fatty acids (FFAs) may potentially bind to toll-like receptor 4 (TLR-4), and activated its signaling pathways (HUANG et al., 2012). In adipocytes, the activation of TLR-4 promotes NF-κB translocation and leads to the production of proinflammatory cytokines (SUGANAMI et al., 2007). Furthermore, the elevated FFAs in obese patients also induces adipose tissue macrophage infiltration (NGUYEN et al., 2007).

The analysis of this study also showed positive enrichment of the signaling by proinflammatory interleukins in CM 6. Interleukins and other inflammatory mediators were shown to partake in the macrophage-adipocyte crosstalk (ENGIN, 2017), leading to a pro-inflammatory state and adipose insulin resistance in obese patients (BING, 2015). This crosstalk secrets IL-6 and directly interferes with insulin signaling (WEISBERG et al., 2003), and it has also been correlated with obesity-induced insulin resistance (PRADHAN et al., 2001).

Another cytokine produced by the adipose tissue macrophages is IL-1β (SIMS; SMITH, 2010), which has been shown to reduce the expression of proteins from the insulin signaling pathway, such as IRS-1 and glucose uptake (GLUT4), and impair downstream insulin signaling (GAO et al., 2014). Interestingly, both IL-6 and IL-1β have also been identified as highly differentially co-expressed genes between MHO and MUO individuals in the liver, muscle, and adipose tissues (KOGELMAN et al., 2016).

When combining the SAT gene expression signature with the consensus co-expression network, it was no surprise to have found CM 6 and CM 14 with high numbers of DEGs since they both had high NES throughout most studies (Figure 16). In contrast, CM 13 had the greatest number of DEGs among all co-expression communities, and yet no consistent and significant pathway enrichment was found.

The pathway enrichment analysis and GSEA failed to find a consensus for liver, muscle, and blood studies. Previous reports have performed co-expression analysis for blood samples (FATIMA et al., 2018; NAKAYA et al., 2015; OBEIDAT et

al., 2017; VOIGT et al., 2018) and even for blood from obese patients (GHOSH et al., 2010) (CROTEAU-CHONKA et al., 2018). These studies find multiple pathways implicated in the gene co-expression network profiles. Although it is possible to combine multiple co-expression analyses (LANGFELDER; HORVATH, 2007), this is still a novel approach in the field (REINHOLD et al., 2017) (SUN et al., 2017).

The lack of significant and consistent pathways in the blood co-expression communities might have been due to individual-specific factors involved in gene expression variability (RADICH et al., 2004). Even in healthy volunteers, gene expression variation from blood can be explained by varying proportions of leukocyte subsets (EADY et al., 2005), and the time of day of sample collection (WHITNEY et al., 2003). For liver and muscle studies, it is possible that having only three studies for each tissue may have impaired the creation of the consensus co-expression network and consequently the enrichment analysis. Therefore, it can be beneficial to have a more significant number of studies of liver and muscle co-expression analysis.

We detected 151 out of 378 miRNAs in the serum of MetS patients and controls. The number of undetected miRNAs may result from the loss of exosomes, which also carry miRNAs, during the extraction of miRNAs from serum. Some miRNAs have been shown to only be undetectable in the whole serum compared to exosome serum pellets (GALLO et al., 2012). Also, processing blood into plasma or serum may result in different quantities of miRNA in these liquid biopsies (THORSEN; BLONDAL; MOURITZEN, 2017; WANG et al., 2012).

In our MetS cohort, four members of the let-7 family were selected to normalize miRNA expression due to its low variability among all samples. Though some members of the let-7 family have known to regulate glucose metabolism (FROST; OLSON, 2011), the less variable miRNAs in our study (let-7a-5p and let-7d-5p) have been used as housekeeping miRNAs (RICE et al., 2015)

The differential miRNA analysis of MetS serum resulted in the upregulation of miR-542-5p, miR-574-3p, miR-421, and miR-30c-5p. None of these miRNAs have been shown to be dysregulated in MetS. Previous studies reported dysregulation of

other circulating miRNAs in MetS, such as: miR-23a, miR-27a, miR-130, miR-195, miR-197, miR-320a, miR-509-5p (KAROLINA et al., 2012), miR-140-5p, miR-142-3p, miR-143, miR-222, miR-15a, miR-146a, miR-423-5p, miR-520c-3p, miR-532-5p (AL-RAWAF, 2018), miR-16, miR-33, miR-107, and miR-150 (MA; FU; GARVEY, 2018).

The integrative analysis showed that 12 consistently downregulated DEGs from SAT were also potential targets of 4 upregulated miRNAs form MetS patients (Figure 20). Transcriptomic studies have shown that miRNA interactions with target genes can potentially be involved in the pathogenesis of obesity (JORDAN et al., 2011; LI et al., 2015).

The consistent downregulated *EGFR* encodes the epidermal growth factor (EGF) receptor, also known as *ERBB1* or *HER1*, a receptor tyrosine kinase that is activated by binding of EGF, transforming growth factor α (TGF-α) and amphiregulin (*AREG*) (RIESE; STERN, 1998). The downregulation of this gene may be associated with insulin resistance since protein *EGFR* expression in adipose tissue has shown to be positively correlated with insulin level and insulin sensitivity (ROGERS et al., 2012).

The miR-542-5p interaction with *EGFR* was suggested to have a role in oncogenesis. It was observed that miR-542-5p inhibited the proliferation of human lung cancer cells, and had an inverse correlation with EGFR protein levels (YAMAGUCHI et al., 2012). The directionality of miR-542-5p expression in other types of cancers is still controversial (CHENG et al., 2015).

The miR-574-3p also targets *EGFR,* and it was discovered to be a tumor suppressor miRNA because of its downregulation in many types of cancer (CUI et al., 2014) (TATARANO et al., 2012). However, the role of miR-574-3p in MetS is still controversial. In subjects from the Framingham Heart Study, the miR-574-3p expression in plasma was associated with diabetic nephropathy (BIJKERK et al., 2015), and insulin resistance (SHAH et al., 2017). In a small cohort, circulating levels of miR-574-3p temporarily increased in the first month after Roux-en-Y gastric bypass surgery (ALKANDARI et al., 2018) which was not observed in a similar study

(ATKIN et al., 2018). In contrast, serum expression of miR-574-3p was significantly downregulated in type 2 diabetes patients (BALDEÓN ROJAS et al., 2016). Furthermore, the expression of circulating miR-574-3p is also unlikely to be correlated with its levels in the adipose tissue since it has been found downregulated in visceral adipose tissue from obese patients with non-alcoholic steatohepatitis (NASH) (ESTEP et al., 2010).

The upregulated miR-421 from MetS had the following four target genes that were consistently downregulated in obese SAT: *NRC31*, *RHOB*, *RGMB*, and *MRLP38*.

The miR-421 has been extensively described as a regulator of cellular proliferation in many types of cancer (LIU et al., 2017; MENG et al., 2016; ZHOU et al., 2016). Nevertheless, its involvement in metabolic diseases is yet to be discussed appropriately. The targeted genes of this miRNA appear to have essential functions in MetS development. For example, SNPs on the coding gene for the nuclear receptor subfamily 3 group C member 1 (*NR3C1*) were associated with the susceptibility for MetS, though its molecular mechanism is not fully elucidated (YAN et al., 2014).

Another gene targeted by miR-421 is the *ras* homolog gene family member B gene (*RHOB*). This gene encodes RhoB, a small GTPase that regulates the cytoskeletal organization and membrane trafficking (JAFFE; HALL, 2005). Also, RhoB acts through protein-related kinase 1 (*PRK1*) to regulate the kinetics of *EGFR* trafficking. (GAMPEL; PARKER; MELLOR, 1999). In mice models, knocked out of *RHOB* significantly decreased the levels of proinflammatory cytokines such as IL-6, IL-1β, and TNF-α (HUANG et al., 2017).

The repulsive guidance molecule bone morphogenetic protein (BMP) co-receptor b (*RGMB*, *DRAGON*) gene acts as a co-receptor that potentiates BMP signaling (SAMAD et al., 2005). *RGMB* has been mostly studied in cancer and has shown to regulate negatively (LI et al., 2012) or promote cancer growth (SHI et al., 2015). Although the role in *RGMB* in adipose tissue is still undefined, it is highly expressed in macrophages. In mice models, the *RGMB* knockout resulted in the

upregulation of IL-6 in macrophages and lung and dendritic cells (XIA et al., 2011). The *RGMB* consistent downregulation in adipose tissue studies is indicative of its proinflammatory role due to the negative regulation of IL-6.

MRPL38 encodes the large subunit 38 of the mitochondrial ribosomal protein (MRP) family. The MRPs are encoded in the nuclear genome, imported into the mitochondria where they are translated 13 mitochondrial protein components of the oxidative phosphorylation (KENMOCHI et al., 2001). A study investigating mitochondrial biogenesis in human SAT in acquired obesity found downregulation of *MRPL* transcripts, mtDNA amount and oxidative phosphorylation proteins, as well as downregulation and methylation of *MRPL38* (HEINONEN et al., 2015). These findings are suggestive that *MRPL38* downregulation in SAT may result from the targeting of miR-421 found upregulated in our MetS study.

Upregulation of miR-30c-5p, besides other members of the miR-30 family, was reported to promote adipogenesis and inhibit osteogenesis (WANG et al., 2013a). During adipogenesis, it repressed the expression of *SERPINE1* and *ACVR1*, which encode plasminogen activation inhibitor 1 (PAI-1) and activin receptor-like kinase 2 (ALK2), respectively. Also, miR-30c-5p plasma levels were strongly associated with age (AMELING et al., 2015) and inversely correlated with total and LDL cholesterol (CEOLOTTO et al., 2017). The miR-30c family (-1, -2, -1-3p, -2-3p or -5p) were found to be downregulated in the SAT of HIV infected patients (SQUILLACE et al., 2014) and obese patients (ARNER et al., 2012).

Some of the miR-30c-5p target genes have been found dysregulated in obesity and mediate lipogenic responses. The gene encoding the general transcription factor IIIC subunit 3 (*GTF3C3*) was found downregulated in adipose tissue from obese subjects (SKINKYTE-JUSKIENE; KOGELMAN; KADARMIDEEN, 2018). The gene *RAB18*, member ras oncogene family, can mediate lipogenesis and lipolysis and when silence it has been found to impair lipogenic response to insulin in adipocytes (PULIDO et al., 2011). Ubiquinol-cytochrome c reductase core protein 1 (*UQCRC1*) is responsible for mitochondrial energy metabolism and has been found associated with the development of obesity (KUNEJ et al., 2007).

The high mobility group (HMG)-box containing 4 (HMGXB4) is a nonhistone chromosomal protein and also targeted by miR-30c-5p. In a GWAS meta-analysis study, significant variants were identified in HMGXB4 associated with waist-to-hip ratio adjusted for body mass index in SAT (SHUNGIN et al., 2015). The UBX Domain Protein 1 (UBXN1) is a known modulator of the innate immune response by blocking the canonical NF-kappa-B pathway (HU et al., 2017). It has been studied viral replication and appears to also negatively regulate IFN-β expression after viral infection (YUAN et al., 2019). By targeting these anti-inflammatory modulators miR-30c-5p may contribute to predisposing SAT to a pro-inflammatory state.

Little is know about the other miR-30c-5p targets: extended synaptotagmin 1 (*ESYT1*) and lactamase beta 2 (*LACTB2*). *ESYT1* is responsible for lipid binding and transport through the endoplasmic reticulum membrane (FERNÁNDEZ-BUSNADIEGO; SAHEKI; DE CAMILLI, 2015; MAEDA et al., 2013). The protein encoded from *ESYT1* has been shown to negatively impact Herpes Simplex Virus 1 membrane fusion in host cells (EL KASMI et al., 2018). *LACTB2* is required for normal mitochondrial function and cell viability. Recently, its fusion with gene NCOA2 was identified in colorectal cancer (YU et al., 2016). The downregulation in the SAT of these 2 genes has still to be revealed in the scope of obesity and MetS.

The miRNA-mRNA regulation network for the liver showed two interactions: miR-30c-5p targeting *ZNF507* and *TAF4B*. The consistently downregulated genes TATA-Box binding protein associated factor 4b (*TAF4B*) and Zinc finger protein 507 (*ZNF507*) in the liver were found to be potential targets of miR-30c-5p. However both of these genes are highly expressed in testis and ovaries (LIZIO et al., 2019). Since obesity has been shown to be detrimental to the reproductive system (DAĞ; DILBAZ, 2015; KLENOV; JUNGHEIM, 2014), it is possible that the upregulation of miR-30c-5p may target other tissues and contribute to infertility. Targeted inactivation of TAF4B leads to female and male mice sterility (FALENDER et al., 2005). Therefore, the miR-30c-5p by targeting *TAF4B* and *ZNF507* may contribute to obesity-induced infertility.

In the literature, miR-30c was found to be potentially beneficial in treating hyperlipidemias as it was discovered to reduce lipid biosynthesis and lipoprotein secretion (SOH et al., 2013). A study with the non-alcoholic fatty liver disease found downregulation of circulating miR-30c (MEHTA et al., 2016), though no mention was made of the miRNA type. Furthermore, the liver upregulation of miR-30c-5p in leptin-deficient mice reduced triglyceride accumulation and hepatic steatosis by counterbalancing fatty acid biosynthesis (FAN et al., 2017). Since not all obese patients have signs of developing hepatic steatosis (BACON et al., 1994) (STEFAN; HÄRING; CUSI, 2019), upregulation of miR-30c-5p can be a potential mediator of this process.

Even though the same differentially expressed miRNAs target distinct tissues, for example, miR-30c-5p can regulate genes in SAT and Liver, the targeted genes are different in each tissue. Thus showing the complexity of miRNA's gene expression regulation.

In summary, this study provides preliminary research findings regarding the unique miRNA-gene regulatory network expressed in MetS. However, research is still needed to characterize the relationship between the miRNA and their target genes uncovered in our analysis as well as their mechanism of action in the immune and metabolic pathways.

6. Conclusions and Future direction

In this study, bioinformatics analysis was used to determine a gene expression signature, gene co-expression modules, an integrative miRNA-mRNA analysis. These methods showed that combining multiple transcriptomic studies can uncover possible new molecular interactions even in a complex disease as MetS.

A consistent gene expression signature for obesity was found for subcutaneous adipose tissue and liver. The SAT gene signature showed evidence of local infiltrating immune cells, including neutrophils, and meta-inflammation pathways. The liver gene signature uncovered fatty acid and phospholipid metabolism pathways and neutrophil presence in hepatic tissue.

A consensus co-expression analysis successfully detected consistent co-expression communities in SAT that were passive of finding meaningful enriched pathways. These pathways confirm increased immune cells and a pro-inflammatory environment in SAT. It was not possible to uncover meaningful co-expression communities from the consensus co-expression networks of blood, liver, and muscle due to analytical limitations including the number of studies.

The circulating levels of miR-574-3p, miR-542-5p, miR-421, and miR-30c-5p were found upregulated in serum from MetS patients. Since the first 3 aforementioned miRNAs have not been described in MetS until now, additional validation in a larger cohort is needed for their potential use as metabolic alteration biomarkers in obese or MetS patients.

The same miRNA can potentially regulate different tissues through distinct targets for each tissue. Obese SAT has a distinct proinflammatory signature compared to the lean SAT as shown through integrative analysis. Thus, proving possible to integrate transcriptomic data with miRNA profiling for MetS.

The Systems Biology strategy was able to identify dysregulated mRNAs in four types of tissues that could be under the influence of upregulated circulating miRNA from MetS patients.

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8. Attachments

A. Ficha do Aluno

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Programa:	Farmácia (Fisiopatologia e Toxicologia)
Área:	Fisiopatologia
Data de Matrícula:	04/02/2015
Início da Contagem de Prazo:	04/02/2015
Data Limite para o Depósito:	04/06/2019
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Proficiência em Línguas:	Inglês, Aprovado em 04/02/2015
Prorrogação(ões):	120 dias Período de 04/02/2019 até 04/06/2019
Data de Aprovação no Exame de Qualificação:	Aprovado em 30/03/2017
Estágio no Exterior:	QIMR Berghofer Medical Research Institute, Austrália - Período de 01/10/2017 até 30/09/2018
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor de 20/04/2013 até 28/03/2018).
Última ocorrência: Matrícula de Acompanhamento em 04/02/2019
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Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBC5792-3/2	Tópicos em Análises Clínicas III	03/03/2015	16/06/2015	15	1	100	A	N	Concluída
FBC5766-4/2	Tópicos em Análises Clínicas IV	04/08/2015	16/11/2015	15	1	100	A	N	Concluída
IBI5035-1/4	Biologia Molecular Computacional (Curso Interunidades: Bioinformática - Universidade de São Paulo)	06/08/2015	04/12/2015	120	8	100	A	N	Concluída
IBI5037-1/2	Algoritmos em Bioinformática (Curso Interunidades: Bioinformática - Universidade de São Paulo)	10/08/2015	30/11/2015	120	8	90	B	N	Concluída
FBC5708-5/4	Farmacogenômica Cardiovascular	19/10/2015	02/12/2015	90	6	100	A	N	Concluída
BMI5862-9/2	Seminário Didático-Cientifico em Imunologia I (Instituto de Ciências Biomédicas - Universidade de São Paulo)	10/03/2016	30/06/2016	60	4	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
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Disciplinas:	0	25	28
Estágios:			
Total:	0	25	28

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B. Curículo Lattes

Currículo **Lattes**

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2015 Doutorado em Farmácia (Fisiopatologia e Toxicologia).
Universidade de São Paulo, USP, Sao Paulo, Brasil
Título: Análise dos Mecanismos Regulatórios Transcricionais Mediados por microRNAs na Síndrome Metabólica
Orientador: Helder Takashi Imoto Nakaya
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2004 - 2011 Graduação em Farmácia e Bioquímica.
Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, FCFUSP, Sao Paulo, Brasil
Título: Perfil Epidemiológico de pacientes pediátricos com suspeita de H1N1
Orientador: Eliane Ribeiro

Atuação profissional

1. Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP

Vínculo institucional

2016 - Atual Vínculo: Bolsista , Enquadramento funcional: Doutorado Direto na FCF-USP, Regime: Dedicação exclusiva

2. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES

Vínculo institucional

2015 - 2016 Vínculo: Bolsista , Enquadramento funcional: Doutorado Direto

na FCF-USP, Regime: Dedicação exclusiva

3. Fundação Adib Jatene - ADIB JATENE

Vínculo institucional

2012 - 2015 Vínculo: Celetista , Enquadramento funcional: Biologista Molecular , Carga horária: 40, Regime: Dedicação exclusiva
Outras informações:
Área de atuação: farmacogenética e expressão gênica de doenças cardiovasculares. Atividades: Supervisão e acompanhamento do andamento de projetos FAPESP, auxílio na orientação de alunos de iniciação científica com bolsa FAPESP e PIBIC. Procedimentos de extração, controle quantitativo e qualitativo de ácidos nucléicos. Auxílio na implementação de POPs, boas práticas e biossegurança no laboratório. Organização de treinamentos para funcionários e alunos. Padronização e experimentos de pirosequenciamento em PyroMark Q24 (Qiagen), padronização e realização de expressão gênica nos equipamentos Fast 7500(ABI) e Rotor-GeneQ (Qiagen), preparação de bibliotecas Nextera e sequenciamento de próxima geração na plataforma MiSeq (illumina). Análise de dados de expressão gênica e de sequenciamento de exômas utilizando softwares de bioinformática como CLCbio e Galaxy.

4. Drogasil S.A. - DROGASIL

Vínculo institucional

2011 - 2012 Enquadramento funcional: Farmacêutico , Carga horária: 48, Regime: Integral
Outras informações:
Area de atuação: atenção farmacêutica, dispensação, farmaceutico responsável, controle de processos, treinamentos, relatórios a vigilância sanitária.

5. Fundação Oswaldo Ramos - FOR

Vínculo institucional

2010 - 2011 Vínculo: Bolsista , Enquadramento funcional: Estagiário , Carga horária: 40, Regime: Dedicação exclusiva
Outras informações:
Área de atuação: banco de dados, transplante

6. Hospital Universitário da Universidade de São Paulo - HU-USP

Vínculo institucional

2009 - 2009 Vínculo: Estágio , Enquadramento funcional: Estagiário , Carga horária: 40, Regime: Dedicação exclusiva
Outras informações:
Área de atuação: Farmácia Clínica e Farmacovigilância sob orientação da Profa. Dr. Eliane Ribeiro

7. Novartis Biociencias Sa - NOVARTIS

Vínculo institucional

2007 - 2009 Vínculo: Estágio , Enquadramento funcional: Estagiário , Carga horária: 40, Regime: Dedicação exclusiva
Outras informações:
Área de atuação: Farmacovigilância.

8. Instituto de Ciências Biomédicas da Universidade de São Paulo - ICB-USP

Vínculo institucional

2005 - 2007 Vínculo: Bolsista , Enquadramento funcional: Estagiário/ Iniciação científica , Carga horária: 40, Regime: Dedicação exclusiva
Outras informações:
Local: Laboratório de Fisiologia e Biofísica, sob orientação do Prof. Dr. Rui Curi foi desenvolvido o seguinte projeto: "O estudo do PPAR-gamma na morte de células Jurkat utilizando RNAi " com auxílio de bolsa PIBIC - CNPq. Atividades desenvolvidas: cultura celular, experimentos de inibição de expresssão gência por RNA de interferência, utilização de citometria de fluxo para avaliação de viabilidade celular.

Producão

Produção bibliográfica

Artigos completos publicados em periódicos

1. UINT, LUCIANA; BASTOS, GISELE MEDEIROS; THUROW, HELENA STRELOW; BORGES, JESSICA BASSANI; **HIRATA, THIAGO DOMINGUEZ CRESPO**; FRANÇA, JOÃO ITALO DIAS; HIRATA, MARIO HIROYUKI; SOUSA, AMANDA GUERRA DE MORAES REGO
Increased levels of plasma IL-1b and BDNF can predict resistant depression patients. REVISTA DA ASSOCIAÇÃO MÉDICA BRASILEIRA. JCR, v.65, p.361 - 369, 2019.

2. RUSSO, PEDRO S. T.; FERREIRA, GUSTAVO R.; CARDOZO, LUCAS E.; BÜRGER, MATHEUS C.; ARIAS-CARRASCO, RAUL; MARUYAMA, SANDRA R.; **HIRATA, THIAGO D. C.**; LIMA, DIÓGENES S.; PASSOS, FERNANDO M.; FUKUTANI, KIYOSHI F.; LEVER, MELISSA; SILVA, JOÃO S.; MARACAJA-COUTINHO, VINICIUS; NAKAYA, HELDER I.
CEMiTool: a Bioconductor package for performing comprehensive modular co-expression analyses. BMC BIOINFORMATICS. JCR, v.19, p.56 - , 2018.

3. **DC HIRATA, THIAGO**; NAKAYA, HELDER; TT LE, THUY; S POO, YEE; SUHRBIER, ANDREAS
Early pregnancy factor, chaperonin 10 and rheumatoid arthritis; the story unravels. Journal of Translational Science. , v.4, p.1 - , 2018.

4. PROW, NATALIE A.; TANG, BING; GARDNER, JOY; LE, THUY T.; TAYLOR, ADAM; POO, YEE S.; NAKAYAMA, ERI; **HIRATA, THIAGO D. C.**; NAKAYA, HELDER I.; SLONCHAK, ANDRII; MUKHOPADHYAY, PAMELA; MAHALINGAM, SURESH; SCHRODER, WAYNE A.; KLIMSTRA, WILLIAM; SUHRBIER, ANDREAS
Lower temperatures reduce type I interferon activity and promote alphaviral arthritis. PLoS Pathogens. JCR, v.13, p.e1006788 - , 2017.

5. GABRIEL, FABÍOLA SANTOS; ALMEIDA-SANTOS, MARCOS ANTONIO; **HIRATA, THIAGO DOMINGUEZ CRESPO**; HIRATA, MARIO HIROYUKI; PINTO, IBRAIM MASCIARELLI FRANCISCO; SOUSA, ANTÔNIO CARLOS SOBRAL; MOTA, FLAVIA BIANCA SUICA; OLIVEIRA, DANIEL PIO DE; OLIVEIRA, JOSELINA LUZIA MENEZES
Coronary Computed Tomography Angiography and C-Reactive Protein in the Evaluation of Coronary Artery Disease. International Journal of Cardiovascular Sciences. , v.29, p.338 - 347, 2016.

6. Schroder, W.; ITRAU, A.; LE, T.; **HIRATA, T.D.C.**; NAKAYA, H. T. I.; MAJOR, L.; ELLIS, J.; SUHRBIER, A.
SerpinB2 Deficiency Results in a Stratum Corneum Defect and

Cardiologia: Volume Biologia Molecular.1 ed.São Paulo : Atheneu, 2013, v.1, p. 215-240.

Livros organizados

1. SOUSA, A. G. M. R.; HIRATA, M. H.; HIRATA, R. D. C.; SAMPAIO, M. F.; **HIRATA, T.D.C.**
Cardiologia: Volume Biologia Molecular. São Paulo : Atheneu, 2013, v.1. p.268.

Trabalhos publicados em anais de eventos (resumo)

1. CERDA A; Genvigir, FDV; LEITE, G. G.; FAJARDO CM; **HIRATA, T.D.C.**; Dorea, EL; Bernik, MMS; HIRATA, M. H.; HIRATA, R. D. C.
microRNAs como marcadores moleculares de los efectos pleiotrópicos de las estatinas en individuos hipercolesterolómicos tratados con atorvastatina In: 43o Congresso Brasileiro de Análises Clínicas, 2016, São Paulo. **Anais de Resumo do 43o Congresso Brasileiro de Análises Clínicas**. Sao Paulo: Sociedade Brasileira de Análises Clinicas, 2016. v.48. p.46 - 47