Molecular studies on microbes and Toll like receptors signaling pathway in obesity associated inflammation

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Dedication

Dedicated To my Loving Parents, my Supervisor for providing will to aspire, to my best friend (Maleeha), my loving Sisters (Kokab and Farah) and my caring Husband for giving me courage to fight against odds.

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List of Acronym/abbreviations (alphabetically)

Abbreviation	Full form
AP-1	Activator Protein-1
ATMs	Adipose Tissue Macrophages
BMI	Body Mass Index
CDA	*Candida* Differential Agar
CDC	Centre For Disease Control
CFU	Colony Forming Unit
CLSI	Clinical and laboratory Standard Institute
CRA	Congo Red Assay
DALYs	Disability Adjusted Life Year
DAMPs	Damage Associated Molecular Patterns
DUBA	Deubiquitination Enzyme
ECD	Extracellular Domain
EMB	Eosin Methylene Blue
FADD	FAS-associated death domain-containing protein
FFAs	Free Fatty Acids
GIT	Gastrointestinal Tract
Hsp 60	heat shock protein 60
Hsp 70	heat shock protein 70
IBD	Inflammatory Bowel Disease
IKK	IkB kinase
IL-6	Interleukin 6
iNOS	nitric oxide synthase
IQR	Inter Quartile Range
IRAK	IL-1 receptor associated kinase
JNKs	c-Jun N terminal kinases
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAL	Limulus Amebocyte Lysate
LBP	LPS-binding protein
LDA	Linear Discriminant Analysis
LPS	lipo-polysaccharides
LRR	Leucine Rich Repeat
MAPKs	mitogen activated protein kinase
MD	Microbial Dysbiosis
MH	Muller Hinton
MSA	Mannitol salt agar
MTP	Microliter Plate Assay
MyD88	Myeloid differentiation primary response 88
NCD-RisC	NCD Risk Factor Collaboration
NMDS	Non-metric Multidimensional Scaling
OR	Odd Ratio
PAGE	Polyacrylamide Gel Electrophoresis
PAMPs	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer saline
PCI	Phenol-Chloroform Isoamyl Alcohol
PCO's	Polycystic Ovarian Syndrome
PCoA	Principal Coordinates analysis
PCR	Polymerase Chain Reaction
PLM	Phospholipomannan

PRRs	Pattern Recognition Receptors
RHIM	Homotypic Interaction Motif
RIP	Receptor-Interacting Protein
RLE	Relative Log Expression
RMR	Resting Metabolic Rate
SD	Standard Deviation
SDA	Sabouraud`s Dextrose Agar
SDB	Sabouraud`s Dextrose Broth (
SDS	Sodium Dodecyl Sulphate
SFA	Saturated Fatty Acids
SNPs	Single Nucleotide Polymorphisms
SS agar	Salmonella Shigella agar
SSCP	Single Stranded Conformational Polymerase
TAG	TRAM adaptor with GOLD domain
TBE	Tris Boric EDTA buffer
TBK1	TANK-binding kinase 1
TEF	Thermic Effect of Food
TFTC	Too Few to Count
TIR	Toll-Interleukin-1 Receptor
TLR-2	Toll Like Receptor-2
TLR-4	Toll Like Receptor-4
TLRs	Toll Like Receptors
TNF	Tumour Necrosis Factor
TNTC	Too Numerous To Count
TRADD	TNFR-associated death domain protein
TRAF6	TNF receptor associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon-β
UK	United Kingdom
USA	United State of America
VAT	Visceral Adipose Tissue
VEP	Variant Effect Predictor
WHO	World Health Organization
ZO-1	Zonula Occludens-1
χ^2	Chi Square

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Mehreen

Abstract

Obesity is a complicated disease that affects equally populations in industrial and developing countries. Europe and USA currently have the highest prevalence of obesity (60%) which accounts for 2.8 million of all global fatalities. It is predicted that adult obesity to increase up to 78% by 2030. In recent years, an upward trend in obesity prevalence is even observed in Pakistan placing it at 145th position as per Global observatory report after an overall obesity increase from 5.1 to 8.60% in 2022, where 11.3% are females and 6% males. Obesity being a multifactorial metabolic disorder, it involves interplay of multiple risk factors including dietary components, environmental, immunological and genetic attributes along with most recent addition of gut microbial diversity. Present study was designed to decipher the role of *TLR-2* and *TLR-4* associated polymorphisms to understand the genetic susceptibility. Also, possible dysbiosis of gut microbiota, dietary choices, socio-demographic and lifestyle habits as risk for obesity in local Pakistani population.

The present study was conducted on total 687 obese and healthy controls, in which various predisposing risk factors for obesity were assessed after recording data in designed questionnaire. Among various demographic factors, marital status [p <0.01, OR= 2.529(1.683-3.802)], area of residence [p= 0.001, OR=2.090(1.370-3.189)], lower and middle socioeconomic strata [p=0.002, OR= 2.900(1.461-5.757)] and [p=0.011, OR=2.250(1.207-4.193)] respectively were posing risk for obesity. Upon regression analysis, increasing portion of meals [p=0.006, OR= 46.45 (3.05-705.45)], snacks [p <0.01, OR= 0.002 (0.01-0.05)] and fruits/vegetable [p<0.01, OR=26.54(5.72-123.06)] were positively associated as well as showing increased risk for obesity. The regular beef consumption at least 1-2 times/week [p=0.016, OR=3.60 (1.26-10.25)] had three fold odds for obesity.

Detection of genetic variations was done by using polymerase chain reaction and Single Stranded Conformational Polymerase followed by Sanger sequencing. By using Bio edit software four novel variants in *TLR-2* gene (153688371 T >C, 153702295 T >C, 153703504 T >C and 153705074 C >A) and 7 variants in *TLR-4* gene (117707870 G>A, 117708080 A>G, 117708777 C>G, 117708780 G>A, 117713024 A>G, 117715449 T>A and 117715853 G>C) were detected. The genotypic and allelic frequencies have also shown positive association with obesity susceptibility.

Different *in silico* analysis were performed to predict effects of detected genetic variations on the mRNA structure and gene regulation by miRNAs. Mutation taster showed that the 4 detected *TLR-2* variants changed polyA signal and the splice sites. miRBase predicted creation of novel binding site for hsa-miR-4523 in variant 153688371 T >C coding region. Moreover, Visual gene graphic predicted that the variants 153688371 T >C and 153702295 T >C were increasing mRNA stability while variant 153703504 T >C and 153705074 C >A decreased mRNA stability. In the 7 detected *TLR-4* gene variants, in four variants there was loss of splice site and other were predicted a change in TLR-4 protein. Most of genetic variation either led to loss or dislocation of miRNA binding sites; variant 117707870 G>A (hsa-miR-576-5p and hsa-miR-122-3p), 117708780 G>A (hsa-miR-215-3p, hsa-miR-130b-3p) and 117715449 T>A (hsa-miR-1246). Also, for detected *TLR-4* polymorphisms in variants 117707870 G>A, 117713024 A>G and 117715449 T>A, decreased stability of mRNA was predicted. However, variants 117708080 A>G, 117708777 C>G and 117715853 G>C enhanced stability of predicted mRNA. The variant 153703504 T >C of *TLR-2* and 117708780 G>A *TLR-4* both upon *in-silico* were affecting the LRR region, which are ligand binding site of the TLRs. Also, *TLR-4*'s variant 117713024 A>G disrupted the disulphide bond formation and hence might affect the ligand binding. The variant in *TLR-2* encoding the cytoplasmic domain was also detected, which can affect the downstream signaling and pro-inflammatory cytokines formation. All the *TLR-2* and *TLR-4* gene variants` after mutational and *in-silico* analysis found change in mRNAs and some were annotated to have alteration in their protein. Most of these genetic variations had strong association for their involvement in obesity associated inflammation.

The culturable fungi was isolated using standard microbiological techniques from fecal samples of the obese and control study individuals. From all samples, different *Candida* species were identified which were *Candida kefyr, Teunomyces krusei* and *Candida albican. C.kefyr* was most prevalent with increased abundance in obese gut. Moreover, increased diversity was present in obese gut with additional species including *Candida glabrata*, *Candida dubliensis* and *Candida parapsilosis*. The percentage positivity of many virulence factors was high among isolates from obese group where majorly expressed virulence factors were; biofilm formation 95.2% and phospholipase 84.6% with high resistance to Fluconazole 31.7%. The pathogenic potential in *Candida* (*C.kefyr, C. albicans* and *T. krusei*) isolated from obese individuals was very potent, which can be trigger for low grade inflammation by these opportunistic pathogens in the obesity.

The gut microbial diversity was checked by both culture dependent approaches using microbiological standard techniques and the culture independent approach using 16SrRNA based sequencing. Among the culturable bacterial gut colonizers, change in bacterial abundance and diversity was observed with increase in *Klebsiella pneumonia* (16.1%), *Pseudomonas aeruginosa* 64 (19.5), *Shigella species* (6.4%) and *Acinetobacter* (1.5%). The change in diversity was seen with *Proteus* vulgaris (0.9%) and *Streptococcus specie* (19.0%) that were present in obese group only. Based on 16SrRNA sequencing relative abundance of *Firmicutes* (obese=42.31% and controls=6.59%) was increased and Phylum *Bacteroidota* (obese= 40.53% and control= 90.95%) was decreased among obese subjects. Moreover, the Phylum *Proteobacteria* (11.2%), *Verrucomicrobiota* (1.31%), *Cyanobacteria* (0.4%), *Desulfobacterota* (0.17%) and *Patescibacteria* (0.01%) were detected only in obese samples showing increased diversity in obese faecal samples with dysbiosis in case of obesity.

According to *alpha* and *beta* diversity analysis, the change in bacterial gut diversity was significantly associated with the obesity. Phylum *Proteobacteria* population was significantly increased in the obese group. Moreover, the member of *Proteobacteria* namely *E.coli* and *Enterobacter*'s presence was associated with detection of lower levels of LPS (23.8EU/mL) in obese serum samples only, which were indicative of endotoxemia. The detection of LPS and *Proteobacteria* point toward possible role of secreted LPS by gut microbiota for TLR-mediated inflammation in obesity. Also the gut bacterial diversity had intricate and complex network of interactions, which need to be further evaluate to understand their role in digestion and energy harvesting in case of obesity. There was dysbiosis in gut bacterial diversity with more Gram negative in core microbiome and change in culturable gut microflora with Gram negatives too with detection of LPS in serum of obese individuals' points towards triggering of TLRs for generation of low grade inflammation in obesity.

All the risk factors were further studied for their association with the each another as multifactorial risk for the obesity. The detected single nucleotide polymorphisms of *TLR-2* and *TLR-4* were common in females. Interestingly age 30 years for men and above 40 for women was risk in individuals carry polymorphisms in study population. There was association of detected polymorphisms with demographic risk factors like lower to middle-socioeconomic strata, secondary level and urbanization with obesity. Moreover, three variants 117708080 A>G, 117707870 G>A and 117715853 G>C were significantly

associated with both culturable dysbiosis as well as dietary choices (beef, poultry and fruits/vegetable) and lifestyle (no physical exercise) changes. Moreover, dysbiosis of uncultured bacteria was associated with gender, beef intake and marital status. These analysis showed there was strong association of gut microbial dysbiosis (culturable and unculturable) with the demographics, dietary habits and lifestyle choices linking TLRs with obesity.

Overall, it can be concluded that all the risk factors were associated to each other including Toll like receptor SNPs, dietary habits, demographics, lifestyle, fungal and bacterial diversity change and increased LPS with the obesity. All these were posing risk for sustaining obesity and may have been triggered by low grade inflammation in the obesity. At the end it can be inferred that there is need of comprehensive study including genetics, demographics and dietary habits to control the increasing trends of obesity around the globe and in various regions of Pakistan. Based on this work, it is recommended that there is need for nationwide awareness campaign to reduce prevalence of obesity in Pakistan for better weight management and healthy life style to combat this pandemic.

CHAPTER 1: GENERAL INTRODUCTION

Obesity is characterized on the basis of excessive accumulation and storage of fat in the adipose tissues posing risk for health. According to the World Health Organization (WHO), obesity is defined and categorized based on Body mass index (BMI) (WHO, 2020). BMI is a universally accepted method for obesity and pre-obesity classification based on weight, height and gender of an individual person. Based on BMI, the following groups of health status are defined as; underweight BMI less than 18.5 kg/m^2, healthy weight between 18.5-24.9 kg/m^2, pre-obesity between 25 and 29.9 kg/m^2, and obesity ≥ 30 kg/m^2. Obesity is further classified into three groups: obesity class I when BMI is between 30.0 and 34.9 kg/m^2, obesity class II between 35.0 and 39.9, and obesity class III over 40.0 kg/m^2. Class III obesity is further subdivided into morbid obese BMI > 40 kg/m^2, super obese > 50 kg/m^2, and super - super obese > 60 kg/m^2 (Purnell, 2000; Weir & Jan, 2019). It is a chronic, multifactorial illness that results from excession of energy intake compared to energy expenditure. This excess energy is stored in adipose tissue as triglycerides, that ultimately causes increase in the body fat with weight gain (Chooi *et al.,* 2019).

An increasing trend of obesity has been observed continuously in different countries (Finucanes *et al.,* 2011) including United State of America (USA), Egypt, Australia, New Zealand, South Africa, Iran, China, India, and Pakistan. Obesity increased threefold globally between 1975 and 2016, affecting different regions equally (Hossain *et al.,* 2007). In adults, every 2 out of 5 individuals is obese and 1 out of 11 have severe obesity. The prevalence of obesity in Pakistan, according to WHO report (2016) is 4.8% obese with 20.8% overweight (Hasan & Hasan, 2017). An increased trend of obesity in Pakistan is observed where it is more in females than males (Amin Baig, 2018). Obesity has been on the rising, causing overall 60–70% of deaths annually due to its association with other metabolic disorders. Recently, the World Obesity Federation's Global Obesity Observatory (2022) has shown that among adult Pakistani individuals 6.5/10 person are at risk of obesity.

Obesity is a heterogeneous disorder that happens due to the interaction of various behavioural, environmental, and biological factors. All of these factors also influence one's energy expenditures and metabolism. The resting metabolic rate (RMR) and thermic effect of food (TEF) along with physical exercise are involved in energy expenditure and storage that maintains energy balance of

the body. If energy expenditure equals energy consumption, body weight remains stable; if energy intake exceeds energy outflow, adiposity increases. All these factors disturb and contribute to obesity by altering energy balance.

In the past few decades, obesity prevalence has risen which is directly associated with the changing social setups, shifting to more crowded places with built-in environments with less activity and more access to fancy foods due to the availability of restaurants and food courts. Due to hectic schedules, people have turned to sweet beverages, fizzy drinks, and inactive lifestyles. The combination of factors like regular intake of junk food, snacks, and sugary beverages along with sedentary habits contributes to the dramatic increase in obesity (Kadouh and Acosta 2017). Furthermore, "obesogens" are certain environmental chemicals that are known to disrupt the endocrine system, therefore causing hormonal imbalance and potentially increasing the risk of obesity. One example of an obesogen is phthalates, which interact with receptors involved in fat development (Romieu *et al.,* 2017)

Obesity prevalence also varies among individuals and populations depending on their demographic features. For instance, the gender-based prevalence of obesity shows that the obesity is higher in women than men. Among different ethnic groups, black and Hispanic populations are at a greater risk than the white population. Socioeconomic status in relation to ethnicity and race also acts as a risk factor for obesity, as evident from high obesity susceptibility in low income non-Hispanic group than in the white women (Cynthia *et al.,* 2010). Differences in geographical regions have a role in increasing obesity prevalence around the world. A study of the Canadian population found higher rates of obesity in urban areas and pre-obesity due to easy access to a variety of food resources with sedentary lifestyle (Atek *et al.,* 2013; Rundle and Heymsfield, 2016). Although in general, rural area residency is thought to be more active lifestyle with pure sources of food. But in 80% of low- and middle-income nations, rural areas contributed towards 55% of the global obesity, as per NCD Risk Factor Collaboration report (Bentham *et al.,* 2017).

The behavioural factors contributing to fat accumulation are eating habits, lifestyle, daily routine, and habits. According to worldwide data, mortality rate among obese was 15% in 2000, which was found to be associated with poor dietary habits and an inactive lifestyle. Eating habits such as meal type, portion size, and time of meal consumption all have a significant impact on body weight

imbalance. Significant associations have been found with large portion sizes, frequent consumption of high-fat and sugar diets, frequent consumption of fast foods, skipping breakfast, and frequent restaurant visits (Affenito *et al.,* 2012).

Furthermore, red meat and processed meat consumption was found to be linked to obesity progression and increases odds for obesity by 37 fold (Rouhani *et al.,* 2014). Some studies have recorded fast food as a risk factor for obesity in comparison with non fast food intake (Alkerwi *et al.,* 2015; Kant *et al.,* 2015). Based on these findings, several studies linked specific dietary habits, such as eating meals with caloric restriction and caloric counting with clinical weight management strategies. Over the last decade, even some obesity-reducing foods have been investigated, including wholegrain foods, preferably low-fat cooked vegetables, legumes, fruit, and nuts, which are also significantly associated with a lower risk of obesity (Fogelholm *et al.,* 2012). Remarkable reductions in weight have been reported in clinical trials with caloric restriction. Inactive lifestyle with poor eating choices in weight management program shows diet as one of the vital player in increasing the obesity (Hruby and Hu, 2016; Amarasinghe and D'Souza, 2012).

The gastrointestinal tract (GIT) and adipose tissue are both involved in the brain-gut axis that regulates homeostatic and non-homeostatic signals to regulate food consumption (Buhmann *et al.,* 2014). Prenatal factors, such as gestational weight and a high pregnancy BMI typically rises with each gestation which is an indicator of future childhood obesity. Gestational diabetes predisposes the newborn to future obesity. Smoking is another associated risk for weight gain in children, especially if it is done during pregnancy. Additionally, menopause in women is risk for weight gain (Liao *et al.,* 2019). Neuroendocrine condition like hypothalamic dysfunction, Cushing's syndrome, hypothyroidism, and polycystic ovarian syndrome (PCO's) can have role in increasing obesity (Weaver, 2007; Wilding, 2020). Many widely used drugs act as obesogenic medicines which are anti-psychotic drugs, β-receptor antagonists, corticosteroids, neurotropic drugs, and HIV treatment drugs (Verhaegen & Van Gaal, 2000).

Biological variables are also investigated for their role in the initiation and progression of obesity. These biological components interact with behavioural and environmental factors, all of which contribute to disease outcomes. Obesity is a polygenic disorder, up to now several mutations/single nucleotide polymorphisms (SNPs) have been reported (Thaker, 2017). There are several potential

genes and their mutations found to be linked to obesity like leptin/melanocortin pathway genes which results in severe early-onset of obesity an uncommon type of obesity. It is characterized by dysregulated adipokine secretion profiles, increased recruitment of inflammatory mediators, cells, markers and altered metabolic homeostasis, which is further connected to gut microbiota dysbiosis.

Beyond lipid build up and energy imbalance, the pathophysiology of obesity is incredibly complex. Various studies have revealed involvement of gut microbes as a vital player in chronic inflammation and a causative factor for obesity via roles in nutrient absorption, polysaccharide breakdown, inflammatory responses, and gut permeability (Mathur & Barlow, 2015; Tseng & Wu, 2019)

The changes in gut microbiota composition elicit qualitative and quantitative immune cells by resident adipocytes, making it a centre of immunopathological activation that also plays a role in low grade inflammation, referred to as meta inflammation (Daryabor *et al.,* 2019). Obesity was thought to be due to increased accumulation of fatty acids in adipose tissues and chronic activation of inflammatory pathways. The content of free fatty acids and glucose in plasma as well as the metabolism of nutrients in different tissues are all impacted by triglycerides, which are stored in adipose tissue in the human body. Increased fatty acid accumulation in adipose tissues has been seen in cases of obesity (Cildir *et al.,* 2013).

The amount of hormones and adipokines released by adipocytes affect the amount and distribution of adipose tissue in a body (Cinti, 2018). The adipose tissue due to various stimuli (nutritional and microbial) become infiltrated with peripheral monocytes and these later develop into adipose tissue macrophages (ATMs). It is seen that 50% of the cells in adipose tissue are ATMs in obese people but only 5% in a healthy person (Ahmad *et al.,* 2016). Also certain nutrients induce raise in pro-inflammatory markers such as C-reactive protein, Interleukin 6 (IL-6), and tumour necrosis factor (TNF) in humans (Galland, 2010). In case of obese individuals, increased activation of kinases, transcriptional factors [Nuclear Factor *κ*appa-B (NF*κ*B), transcriptional factors activator protein-1 (AP-1)], and interferon regulatory factors up regulate the pro-inflammatory cytokines and chemokines (Sheng *et al.,* 2012; Douglass *et al.,* 2017).

Adipose tissue bulk and levels of pro-inflammatory cytokines vary among obese individuals. Its development is influenced by numerous endogenous and exogenous variables. These endogenous

factors that affect immune responses include damage-associated molecular patterns (DAMPs) even dietary components like fatty acids and exogenous components from gut bacteria, such as pathogen-associated molecular patterns (PAMPs). PAMPs and DAMPs are recognized by pattern recognition receptors (PRRs), including scavenger receptors, RIG-like receptors, NOD-like receptors and Toll Like Receptors (TLRs) (Luthans *et al.,* 2007; El-Zayat *et al.,* 2019). The expression of TLRs has been reported to be high in the peripheral blood monocytes of obese individuals which are primarily PRR to identify such PAMPs and DAMPs (Ahmad *et al*. 2012).

TLRs are present on a range of immune cells including leukocytic and non-leukocytic cells, where these are expressed as type 1 transmembrane proteins (Hardy *et al.,* 2013). These PRR, can recognize as variety of exogenous and endogenous antigens. TLRs (TLR-2 and TLR-4) use adoption protein fetuin A to indirectly recognize the microbial antigen LPS or nutritional free fatty acids (FFAs), which triggers the pro-inflammatory response by activation of transcription factors like NF-kB (Hwang *et al.,* 2016) via Myeloid differentiation primary response 88 (MyD88) dependent pathway. The local microbiota also regulates intestinal inflammation and activates regulatory cells to control pro inflammatory cytokines for promotion and maintenance of intestinal homeostasis (Mishima *et al.,* 2019). Recently, such inflammation has been seen in various illnesses and was postulated on the basis of work Parkinson's disease, that the reduction in integrity of intestinal barrier was associated with microbial dysbiosis which in turn linked to varied interaction with TLR-2 and TLR-4 (Gorecki *et al.,* 2021).

Numerous studies have shown that single nucleotide variations in the TLRs (TLR-2 and TLR-4) gene can be associated with an increased risk of a variety of inflammatory disorders, including diabetes, tuberculosis, heart issues, sepsis, malignancies, and leprosy (Gao *et al.,* 2015; Malhotra *et al.,* 2005; Sharma *et al.,* 2016; Thuong *et al.,* 2007; Zhu *et al.,* 2013). In the etiology of inflammatory bowel disease (IBD), TLRs are also thought to act as an immune modulator in response to gut bacteria. The genetic polymorphisms in TLRs can cause change inflammation and immune responses after change in gut microbiota composition (Lu *et al.,* 2018).

In the metabolic syndrome, diet-induced obesity and associated inflammation have been linked to higher M1:M2 macrophage ratios and pro-inflammatory mediators. Furthermore, disruptions in

intestine-microbiota interactions were observed when M1:M2 ratio varied and resulted in innate immunity activation (Tran *et al.,* 2020).

Only few studies in the Pakistani population regarding the prevalence of obesity (Shah et al., 2004; Bahadur et al., 2013; Tanzil & Jamali, 2016), and only one (Asif *et al.,* 2020) has linked the demographic features of obesity so far in the literature. Previous research documented more obesity in children in Karachi, Pakistan's largest city (Afzal & Naveed., 2004; Jafar *et al*., 2008). Other studies recorded adult obesity from the Khyber Pakhtunkhwa, Punjab, and Sindh areas of Pakistan (Imran *et al.,* 2019; Randhawa et al., 2021). Also provided rough estimates of the gender-based increasing trend of obesity. Based on findings in other metabolic diseases, it is hypothesized that functional polymorphisms in the TLR gene, along with changes in gut diversity, might have a role in obesity which is influenced by individuals' dietary habits, lifestyle changes, and demographics and promotes the development of inflammation in obesity.

Up to now, no study has been carried on genetic variations (mutation/ SNPs) in TLR-2 and TLR-4 to determine role of these PRRs in obesity for triggering the innate immunity to initiate and sustain the low-grade inflammation. There is utmost need to decipher the role of these genes in the local Pakistani population as obesity has an increased trend here. As obesity is multi factorial disease, the other potential risk factors such as dietary patterns, lifestyle, and human gut microflora (an endogenous source of inflammatory ligands for TLRs) are integral to investigate for understanding meanful part in imparting obesity. The data on various potential risk factors would be helpful in designing strategies for obesity control and devising future surveillance systems. The microbial culturable and unculturable profiling would further add to the knowledge of obesity-associated gut flora of the local Pakistani population with genetic variation in the TLRs along with link to dietary and life style. Thus the hypothesis of the study could be as follows:

“Type of microbial diversity along with mutations/SNPs in genes of TLRs can lead to aberrant signaling which might perpetuate and sustain inflammation and have role in obesity”

AIM AND OBJECTIVES

There are many inflammatory pathways that contribute to obesity, but the increased expression of TLRs in obese individuals indicates that these PRRs which recognize a variety of endogenous and exogenous triggers including dietary components and gut flora might be activating proinflammatory responses. To date, there is no study on association of *TLR-2* and *-4* genes or any mutations/SNPs along with microbiota in obesity.

Therefore, the aim of the present study was to detect gut microbes (culturable and un-culturable) and mutations/single nucleotide polymorphisms in *TLR-2* and *TLR-4* genes along with the contributing risk factors, in order to achieve this aim following were objectives:

1. Collection of data by questionnaire designed to gather information about sociodemographic, food patterns and lifestyle choices as obesity risk.
2. Detection of mutations or polymorphisms in *TLR* (*2* and *4*) genes for their potential role in inflammation related to obesity by using PCR based Sanger sequencing technique.
3. Investigate the culturable fungal diversity using standard microbiological procedures and their pathogenic potential.
4. Investigate the dysbiosis of culturable diversity using microbiological methods and noncu turable diversity using 16SrRNA-based sequencing.

CHAPTER 2: REVIEW OF LITERATURE

Obesity is defined by excessive accumulation of body fats that can have deleterious effects on health especially in adults (Panuganti *et al.,* 2022). BMI has been long considered as useful measure for assessing obesity according to WHO and it is a good indicator for all groups and gender (Nuttall, 2015). After long debate, now there is consensus on obesity being a disease. World Health Organization has declared obesity, a disease which require intervention. Now, it is declared as a global epidemic which is associated with various underlying health conditions (Müller & Geisler, 2017). Research advancements in understanding the obesity have revealed that there are multiple factor involved in its pathogenesis, among these there is association of obesity with immunity especially inflammation (Saltiel & Olefsky, 2017). Obesity is also considered as chronic disease as it affects millions around the globe belonging to all races, gender, ethnicity, socioeconomic strata, and all ages equally.

The obesity has nearly tripled in time period of 1975 to 2016 all around the world(Hossain *et al.,* 2007). Increasing trend of obesity has been observed continuously worldwide. It has been observed that the obesity in the modern world is not only an issue for the developed countries but also for the underdeveloped countries (Finucane *et al.,* 2011). About two third of the World`s population has been estimated to live in the most endemic regions of obesity and causing mortality more than malnutrition (Frühbeck *et al.,* 2013). Moreover high mortality rate of individuals having obesity during comorbidities like infections and diseases also increases the rate (Saab & Salvatore, 2015). In population based studies, the country with higher BMI population 1/10 adults with infection die in comparison with healthy weight population (O'hearn *et al.,* 2021).

In 2008, a study recorded obesity rate of 10% in Western Africa (Abubakari *et al.,* 2008). In 2020, the obesity in the Libya was observed to be 32.5% in adults and Libya is ranked at 16th position among most obese country worldwide. The other countries with high obesity in the Africa after Libya are Egypt and South Africa (Kengne *et al.,* 2017; Merwe & Pepper, 2006). In case of Iran, the obesity prevalence was 26.3% in 2008 which has been increased up to 36.7% in the year 2016 (Fakhrzadeh *et al.,* 2016; Pour *et al.,* 2009)

In China, the overall obesity prevalence was recorded as 5% across the country while it was high as 20% in some large cities. Within span of 10 years, obesity across China has increased 3 times up to 16.4% in adults (Pan *et al.,* 2021; Zhang *et al.,* 2020). In New Zealand, every 1 in 3 adults is obese and has risen since 1988 from 12% to 32% according to national survey in 2016-17 (Fini *et al.,* 2019; Lal *et al.,* 2012). In United States of America (USA), about 42.5% adults over the age of 20 years are obese and with overall 9.0% severely obese and 31.1% pre-obese (Fryar *et al.,* 2020). The USA Center for Disease and Control (CDC) found that the 48% patients causalities during hospitalization were in obese individuals (Finer *et al.,* 2020; Garg *et al.,* 2020; Petrilli *et al.,* 2020). A population based cohort study from United Kingdom (UK) recorded 36% hospitalizations during recent COVID pandemic of patients obesity (Hamer *et al.,* 2020). Another study of CDC reported that in US population from 2000 to 2017, a drastic increase in obesity was seen from 30.5% to 41.9%. In the same time duration, the severe obesity also increased from 4.7% to 9.2%. Moreover, highest prevalence (44.3%.) of obesity was in age 40-59 years (Ward *et al.,* 2021). In 2016, a high global burden of 650 million obese individuals lead to initiation of obesity control program under WHO supervision but this programme is unable to achieve the required targets and rather an estimated 1 billion individuals are expected to be obese by 2030. About 1/3rd of the world population is obese while pre-obese population reached to 39% overall recently. The gender based estimates showed that by 2030 1/5 female and 1/7 males would be obese by 2030 (Bentham *et al*., 2017; Lim *et al*., 2020)

The rise of obesity has not been restricted to one region but affecting the whole world equally, even Pakistanis constituting in global obesity epidemic. In Pakistan, prevalence of obesity with the passing time has been recorded to be on rise. In a study from Northern areas of Pakistan, age-adjusted obesity prevalence was 13.5% in men and 14.1% in women. Also, annual obesity increase on gender basis has same trend (Shah *et al.,* 2004). According to the WHO report of 2011, 26% Pakistani women and 16% men were obese. Another 2013 report showed rise in obesity among males and females as 28% men and 38% women were obese. This difference in prevalence was observe with area of residence in the country, as it was higher in urban localities (56% in males and 67% in females) as compared to rural localities (Bahadur *et al.,* 2013). According to a 2014 global disease estimates, Pakistan ranked at 8th position among top 10 obese countries with half million obese population worldwide (Ng *et al.,* 2014). A prevalence up to 46% was given based

on Asian cut off values for obesity (Tanzil & Jamali, 2016). In 2016, WHO reported 4.8% nation wide obesity (Hasan & Hasan, 2017). The general trend in Pakistan is more obesity in females than males (Amin Baig, 2018). The Malakand region of Khyber Pakhtunkhwa has 40% obesity and 38% for pre-obesity in age 15-61 years, showing a high burden. These studies show that there is high obesity in different part of our country, it may be due lack of awareness and knowledge (Imran *et al.,* 2019). A study based on household survey to analyse obesity level in Pakistan was conducted on 10,063 participants comprising of 3,916 males and 6,147 females found 22.8% pre-obesity and 5.1% obese with females (6.3%) than males (3.2%) (Asif *et al.,* 2020). All of these studies have shown increasing trend of obesity with the passage of time in Pakistan in urban and rural areas of the country. A Forbes reports from 2007, ranked Pakistan at 165 position among 194 countries with 22.2% obesity in age ≥15years, however, recently World Obesity Federation Global Obesity Observatory (2022) positioned Pakistan at 145 number as prevalence increased with time to overall 8.60% in age ≥20years, with females having 11.30% and males 6.00% prevalence. Also, the risk of obesity among adult Pakistani individuals was observed to be 6.5/10.

The obesity kills about 8% people annually due to underlying comorbid conditions along with the increased weight. Obesity is one of the leading risks of premature deaths, which kills 4.72 million people globally. Obesity has increased health care burden worldwide and has been affecting economies, hence is now considered as pandemic (Bentham *et al.,* 2017). Obesity and associated complications affect the economy by affecting 36 billion disability-adjusted life year (DALYs), which contributes total 2-6% of health care budget in various countries. In Pakistan, this cost has been estimated to be 1.5% of the health care cost (Seidell & Halberstadt, 2015; Tanzil & Jamali, 2016).

Clinically obesity due to the accumulation of extra fats depositions in various parts of the body has been categorized as systemic disorder that involves and affects multiple organs due to low grade inflammation. The most common co-morbidities that are associated with the obesity are Type 2 diabetes mellitus, dyslipidemia, polycystic ovary syndrome, hypertension, obstructive sleep apnea, gastro oesophageal reflux, asthma, backache, stroke, infertility (Apovian, 2014; Khaodhiar *et al.,* 1999), non-alcoholic fatty liver disease (Fabbrini *et al.,* 2010) and cancers including breast, ovarian, endometrial oesophageal, colorectal, kidney, prostate, pancreatic (Hall *et al.,* 2021;

Ramdass *et al.,* 2021; Sun *et al.,* 2019) These associated complications, further lower down the quality of life in addition to more mortality. Highest mortality rate due to obesity is observed due to associated cancers and cardiovascular diseases (Kivimäki *et al.,* 2017). There are several risk factors that can influence the mortality and morbidity rate in obese individuals are low socioeconomic status, ethnicity, eating behaviours, smoking alongside age and gender (Porras *et al.,* 2022; Mehta & Preston, 2016). The increase in BMI has been associated with increased mortality rate showing 45% risk of death increases with BMI >30kg/m^2 (Mehta & Chang, 2012). Similarly, a report have shown that the more cost of hospitalization in co-morbid obese individuals up to 17.2% (Balsera *et al.,* 2013; Zhang *et al.,* 2014).

Obesity being a metabolic disease, it is has been currently observed to be a closely related to changes in immune and metabolic pathways together as these are mutually dependent on one another for their proper functioning. There need to understand immune mechanisms got better risk factors the disease and its associated consequences. The adipose tissue inflammation and metabolic dysfunction are widely studied in the obesity progression (Ghosh *et al.,* 2017). Immune system is orchestrated by various signalling pathways, which are activated after nutrient intake and in presence of pathogens. These systems are greatly integrated and evolutionary conserved (Kirwan *et al.,* 2017). Various types of exogenous and endogenous stimuli are recognized by the cells of innate immunity with the help of PRRs, which could be also triggered by DAMPS derived from excessive nutrients too. It is seen in such case, these DAMPs initiate and sustain a chronic low intensity inflammation which termed as metabolic or meta inflammation (Rogero and Calder, 2018). The most common PRRs which recognizes these DAMPs of the body but also recognizes the PAMPs are TLRs. Studies have shown that these receptors get activated in response to various metabolic signals for example saturated fatty acids (SFAs) and their signalling pathways activates downstream kinases to regulate inflammation by releasing pro-inflammatory mediators. The activation process of TLRs has been associated with the innate immune response that usually produce cytokines and chemokine to initiate inflammation in the body (Hong *et al.,* 2015). These TLRs have been studied in detail for their involvement in metabolic tissue associated inflammation also their role in obesity also (Tao *et al.,* 2017).

Toll like receptors are present in different types of immune cells and tissues and specialized to recognize variety of pathogens including viruses, bacteria, protozoa and fungi. About 12 functional TLRs have been identified in the human in which TLR3, TLR7, TLR8 and TLR9 are intracellular receptors which recognizes the processed nucleic acid pathogens or free nucleic acids. The TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are transmembrane receptors which recognizes the pathogenic membrane components like LPS, flagellin and peptidoglycan. The presence of TLR-2 and TLR-4 in the plasma cell membrane of monocytic cells has been confirmed by the help of flow cytometry. They recognise peptidoglycan and LPS to generate immunity against them as a first line defence (Cole *et al.,* 2010). These all TLRs are recognised to important in immunity but among these TLR-2 and TLR-4 are getting attention for their role obesity. As there was altered higher expression of TLR-2 and TLR 4 detected in case of obese individuals in different studies.

TLR-4 gene (20,561 bases) is localized on chromosome 9, q33.1 arm and consist of four exons on the basis of ENSEMBLE gene data (Figure 2.1). *TLR-4* gene encodes a 100 kDa type I transmembrane glycoprotein, that contains three domain involved in recognition, binding and activation of protein. *TLR-2* gene (26,568 bases) is localized on chromosome 4, q31.3 arm and consist of four exons on the basis of ENSEMBLE gene data (Figure 2.2). The TLR-2 protein consists of 580 amino acids and predicts a molecular mass of 65.8kDa.

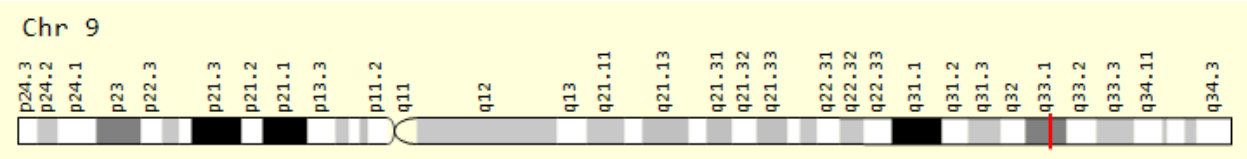


Figure 2.1: Genomic location of *TLR-4* gene (Adapted from https://www.genecards.org/)

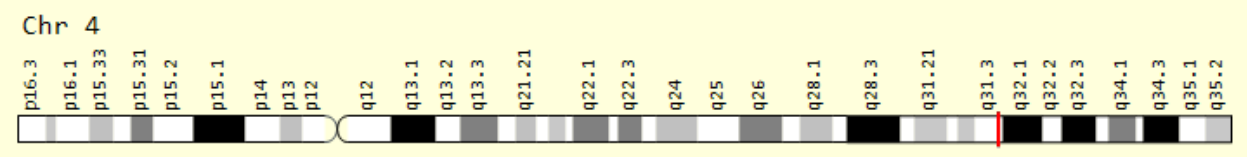


Figure 2.2: Genomic location of *TLR-2* gene (Adapted from https://www.genecards.org/)

These TLRs are present on variety of cells including macrophages, monocytes, and dendritic cells, as well as in muscle cells, enterocytes, and adipocytes. These TLR-2 and TLR-4 are also functional in peripheral monocytes immune cells and the subcutaneous adipocytes where their activation led to formation of IL-6, TNF-α and chemokine (Katanani and Lazar, 2007). These TLRs are composed of three domains: an extracellular domain (ECD), transmembrane helical domain, and intracellular TIR domain. The ECD lie in N-terminal region, its three dimensional crystal structure is reported to have a horseshoe like arrangements with concave and convex surfaces. The concave surfaces are made up of a number of parallel β-sheets whereas the loops and helices are involved in the making of convex surfaces. The N-terminal domain is highly hydrophobic containing leucine-rich repeats (LRRs), a hypervariable region is involved in the recognition of different environmental stimuli. There C-terminal region has a transmembrane double helical domain, which anchors these receptors in the membrane. The intracellular TIR/IL-1 domain is involved in signal transduction by activating the mediators. A general structure for TLRs has adapted from Sameer & Nissar, (2021) and is shown in Figure 2.3.

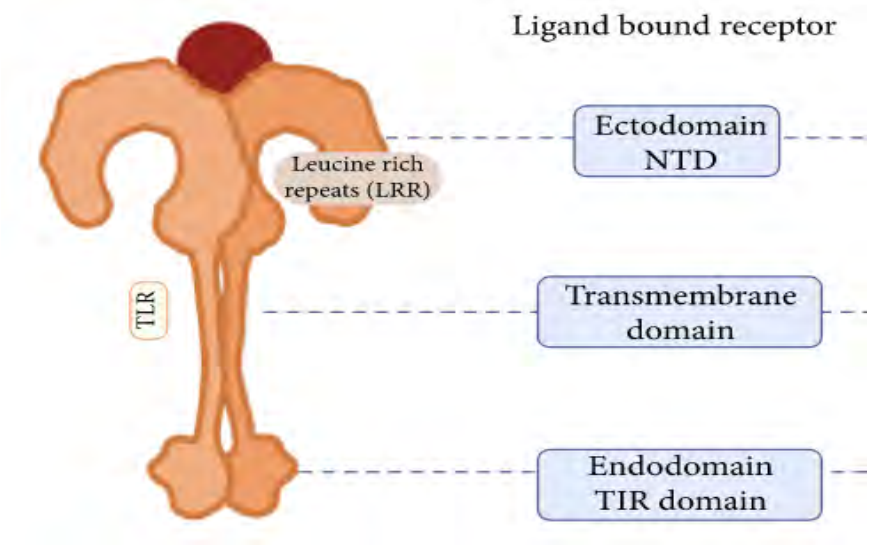


Figure 2.3: Structure of Toll Like Receptor

The signalling of TLR-2 starts after recognizing fungal cell walls, lipoproteins, peptidoglycan, mycobacterial lipoarabinomannan and GPI anchors from parasitic trypanosomes (Underhill *et al.,* 1999;Lien *et al.,* 1999). The TLR-2 can recognize a wide range of PAMPs and DAMPs due to its ability to form homo or heterodimer with TLR1 and TLR6 (Ozinsky *et al.,* 2000). The complexes TLR2/1 and TLR2/6 recognize different bacterial products like triacylated lipopeptides and diacylated lipopeptides, respectively (Takeuchi *et al.,* 2001;Takeuchi *et al.,* 2002).

The TLR-4 was the first reported TLR of human, the primary agonist is lipopolysaccharides but also recognize different saturated fatty acids too (Tramullas *et al.,* 2016). Some other endogenous ligands of TLR-4 are oligosaccharides of hyaluronic acid, heat shock protein (Hsp) 70, heat shock protein (Hsp) 60, polysaccharide fragments of heparan sulfate, type III repeat extra domain A of fibronectin and fibrinogen (Tsan & Gao, 2004; Erridge, 2010). TLR-4 activation starts after binding with the ligands, and it was observed as circulating LPS in the body (Karczewski *et al.,* 2018). LPS in Gram-negative is found in their outer membrane; and the intestinal LPS is produced by commensals of gut microflora too. This integral component of the bacteria is also a primary ligand for the TLR-4 receptor.

The LPS binds with the help of LPS-binding protein (LBP), which enhances its binding with the CD14 molecule, a protein present in the lipid bilayer of cells. After binding with CD14, the protein LBP dissociates itself and a complex of LPS-CD14 is formed with TLR-4. Then the extracellular complex of TLR-4 associated with MD2 accessory as it recognizes LPS. Thus, TLR-4 complex with CD14 and MD2 which causes the dimerization of TLR with conformational changes in it. These changes activate the cytosolic TIR domain and interaction with MyD88. A series of phosphorylation events taking place to phosphorylate the proteins IRAK4 and IRAK1 along with association with TNF receptor associated factor 6 (TRAF6) for MAPK activation and with IkB degradation for activation of transcription factor NFkB. The NFkB regulated genes of TNF-α, IL-1β, IL-6, COX-2, and an enzyme known as inducible nitric oxide synthase (iNOS). The TLR-4 also use another TRIF dependent pathway for more expression of interferon type-1 gene (Shalaby, 2014; Rogero & Calder, 2018). The activation of downstream adopter like MyD88 after interaction with TIR domain also induces TIR-domain-containing adapter-inducing interferon-β (TRIF) protein, which in turns activates the IkB kinase (IKK), again for the activation of NFkB, including

p38, c-Jun N terminal kinases (JNKs) and extracellular signal regulated kinase. Hence transcription and translation occur for the generation of inflammatory mediators; cytokines, chemokines, histamines, leukotriene and interferon (Fessler *et al.,* 2009).

There are now various studies on TLRs indicating a possible role of TLR-2 and TLR-4 with the inflammatory responses to nutritional stimuli. Obesity is also characterised as to be insulin resistance. In case of obesity, the insulin resistance has regulatory effects on the *TLR-4* gene expression and also on the PU.1 transcription factor, which regulates the activation and expression of the MyD88 through which pro-inflammatory cytokines expression is altered (Haehnel *et al.,* 2002; Ghanim *et al.,* 2008)

TLRs' various pathways have been studied by the help of cell cultures *invitro* models, in which TLR-4 derived cytokines were found to have negative impact on the glucose uptake and metabolism of fatty acids (Jager & Kim *et al.,* 2007). These SCFA activates TLR and enhances the NFkB mediated signalling in human macrophages (Suganami *et al.,* 2007) in the adipose tissue with increased level of TNF- α and IL-6 in the plasma. In addition to FFA, Pam3CSK4 and LPS acts agonist of TLR-2 and TLR-4 respectively for activation of inflammation in the various cells like macrophages, adipocytes and adipose tissue (Creely *et al.,* 2007; Fresno *et al.,* 2011; Shi *et al.,* 2006; Vitseva *et al.,* 2008). A mouse model study for assessing the role of high fat diet was carried out and an increased TLR-2 and TNF-α expression in visceral adipose tissue (VAT) was detected in comparison with control (Murakami *et al.,* 2007). In obese mouse model, when TLR-2 signalling was suppressed with antisense oligonucleotide, an improvement of insulin sensitivity with decreases in the IKK-β and MAPK8 within white adipose tissue was seen (Caricilli *et al.,* 2008). In case of these mice with obesity, circulating free fatty acids and proinflammatory mediators was observed and this situation was promoting insulin resistance (Boden, 2008). An elevated expression of TLR-2 and TLR-4 in monocyte cells with high abundance of MyD88 and IRAK was detected in patients in comparison with the control individuals (Devaraj *et al.,* 2008). Another murine model study on high fat diet fed mice, has shown protective effects against inflammation after deletion of TLR-4 gene from its adipose tissue. Such work points that the TLR-4 might be involvement in causing metabolic impairment in obesity with excessive intake of food (Saberi *et al.,* 2009).

A *TLR-2* knockout mice when kept at high fat diet, their glucose tolerance and secretion of insulin was improved, that showed increased insulin sensitivity with reduced inflammation inside the tissue (Ehses *et al.,* 2010). Dasu *et al.,* (2010) confirmed, a high expression of TLR-2, TLR-4 and MyD88 in mononuclear cells of white adipose tissue in abdominal subcutaneous region of the diabetic group. Moreover, the expression was increased with increasing BMI in pre-obese and obese individuals in comparison with the normal weight control group.

Macrophages are also considered to be involved in the obesity development as they release whole sets of interleukins and chemokines in response to bacterial LPS induced activation of TLR-4. In these macrophages form enzymes, prostaglandins, platelet activation factor, reactive oxygen and nitrogen species (nitric oxide NO and superoxide anion). These mediators are involved in inhibition of pathogen dissemination to other site of body. In order to control the growth and to eliminate them from the host body directly or indirectly through inducing adaptive immunity (Könner & Brüning, 2011). The expression of TLR-2 and TLR-4 was found to be significantly higher in peripheral blood mononuclear as well as subcutaneous adipose tissue cells with increased abundance of TNF-α, NFκB and IL-6 in case of individuals with higher BMI as compared to lean subjects pointing towards there direct relationship with the obesity(Ahmad *et al.,* 2012; Rada *et al.,* 2018).

Ingestion of meals with higher calorie lipid and carbs contents by moderate weight individuals causes significantly high TLR-2 and -4 expression on mononuclear blood cells in the post prandial period. These events points towards, there is post-prandial inflammation in case of obesity. Also high fat meal has been observed to increase NFkB activation in postprandial period along with the activation of leukocytes and abundance; it was assessed through blood plasma levels of the CD11a, CD11b, CD62L and metabolic endotoxemia or increased bacterial LPS in plasma (Rogero & Calder, 2018).

In another study, positive correlation of TLR-2 with IL-18 was seen in comorbid case of obesity plus diabetes. In these individuals, free fatty acids and modified LDLs were detected to bind with TLR-2, which was leading to activation of MAPK-pathway through MyD88 dependent as well as insulin signalling. In insulin signalling, serine phosphorylation of insulin receptor substrate (IRS)

was involved in the activation of transcription of pro-inflammatory cytokines (Mohamed & Elraheem, 2016).

In case of *TLR-4* -/- mice models, Shi *et al.,* (2006) there was reduction in cytokines in the adipose tissue despite high fat diet. Another study found association of *TLR-4* and high IL-6 levels with obesity in individual with high FFA intake (Zhang *et al.,* 2018). A novel and potential regulator of age-related obesity development was seen to hypothalamic neurons along with *TLR-2* gene expression (Shechter *et al.,* 2013). *TLR-2* based inflammation also positively correlates to disease progression through manipulation of glycolytic pathway (McGarry *et al.,* 2017). These engagement of TLRs if continue for longer time, can results in a state of low grade inflammation in the body (Li *et al.,* 2020).

The inflammatory cascades take part in progression of various other metabolic and infectious diseases, however, it is now becoming evident that the outcome of these cascades in such diseases can be affected by the presence of genetic variations in *TLRs* genes. SNPs have been observed in exonic, intronic or intergenic regions of *TLRs* in other diseases (Andersson *et al.,* 2014; Teama, 2018). SNPs mostly observed in the intronic regions of the candidate genes can direct or indirectly interfere with the binding of various regulator factors for gene expression (Ahmad *et al.,* 2018). In *TLR-2* gene, SNP at the position 2029 C > T substitution and variant rs121917864 detected in patients with leprosy, were significantly associated with only patients with leprosy and not with the controls, suggesting that the SNPs might have role in disease susceptibility and pathology (Kang & Chae, 2001; Ben-Ali *et al.,* 2004). Multiple studies have reported, a SNP rs5743708 (R753Q) to be involved in different disease progression. In case of Lyme disease, SNP rs5743708 (R753Q) lead to impairment of *TLR-2* signalling with no TNF-α induction and dampened inflammatory responses (Schröder *et al.,* 2005). Another *TLR-2`* SNP (T597C) was significantly associated with developing of Tubercles meningitis, with dissemination of *M. tuberculosis* (Thuong *et al.,* 2007). The *TLR-2* SNP (R753Q) in infective endocarditis was found to increase risk of susceptibility to endocarditis by co-dominant (odds ratio=13.33), recessive (odds ratio=9.12) and dominant (odds ratio=3.65) genotypes (Bustamante *et al.,* 2011). All these studies showed that SNPs or mutations, can affect the infectious disease outcomes.

Majority of SNPs reported in *TLR-4* gene, make aberrant signalling pathways due to altered ligand binding, such changes either increasing susceptibility or resistance to disease. According to Feng *et al.,* (2005), 5 novel SNPs across the length of *TLR-4* gene as observed in Chinese population. These were present in both 5`UTR and 3`UTR region, an important part of gene for gene regulation. The *TLR-4* mutational analysis, detected four of the SNPS (rs10759931, rs11536889, rs1927911 and rs6478317) in case of lymphoblastic leukaemia with positive association with increased risk of developing neutropenia as a result of chemotherapy (Miedema *et al.,* 2011). In case of chronic obstructive pulmonary disease, SNPs in both *TLR-2* and *TLR-4* were observed to linked with disease (Budulac *et al.,* 2012).

A meta-analysis of SNPs in *TLR-4* gene has been highlighted their role as risk factor for developing cancer. Two TLR-4 SNPs rs4986790 and rs4986791 were found to be involved in peptic cancer progression (Zhang *et al.,*, 2013). The rs4986790 SNP in exon 4 of *TLR-4* due to substitution of A>G at 896bp results in amino acid change from aspartic acid to glycine at site of 299. This variant has a protective role against *P. vivax* infection by interfering in the inflammatory responses (Rani *et al.,*, 2018). In case of pulmonary disorder, polymorphism in *TLR-2* and *TLR-4* gene showed inverse relation with the chronic obstructive pulmonary disease and pulmonary tuberculosis (Li *et al.,*, 2019). In a study on tuberculosis, positively associated with the SNPs (rs4986790, rs4986791) present in *TLR-4* gene that cause the activation of the infection. These SNPs were shown to affect the susceptibility for suffering active tuberculosis disease (Ortega *et al.,* 2020). The work on type 2 diabetes mellitus and rheumatoid arthritis association of chronic inflammations with allele C of SNP rs7873784 in *TLR-4* gene. Due to SNP rs7873784 a novel binding site for PU.1 transcription factor was created, it increased gene *TLR-4* expression in the monocytic cell lines. This data has suggested high expression of *TLR-4* due to SNPs lead to more immune responses for in initiation and progression of inflammation (Korneev *et al.,* 2020). In another study on Qatari females with *TLR-4* variants D299G (rs4986790) and T399I (rs4986791), more insulin resistance especially in females with increased BMI was recorded (Sharif *et al.,* 2020).

Mutation and SNPs are responsible for making diverse outcomes of disease associated phenotypes, and traits. In case of non-synonymous SNPs, effects are seen in the protein structure, function, conformation, expression or stability. Nowadays, the sequenced data can be used to detected

variations in the genes, inter or intra gene region *in silico* to predict the changes in RNA or proteins. Such *in silico* tools are mathematically valid and computationally efficient, which are powerful way to study the impact of SNPs induced RNA structural alterations on gene expression and function. Some of the SNPs are found to effect the secondary or super secondary structure of mRNA, such change can have ability to interfere with transcript's splicing and translational regulation including mRNA's stability (Shastry, 2009; Meyer, 2017).The various region of mRNA like 3` or 5`UTR region, if have SNPs affect mRNA structure with disease causing potential. Halvorsen *et al.,* (2010) found in various disease like beta-thalassemia, Hyperferritinemia cataract syndrome, retinoblastoma, cartilage hair hypoplasia, chronic obstructive pulmonary disease, and hypertensions, SNPs in UTRs affecting the mRNA structure of the genes. A common amino-acid deletion in cystic fibrosis coupled with synonymous mutation was seen to change mRNA structure which was responsible for altered inflammation (Bartoszewski *et al.,* 2010).

The changes in the genes can be synonymous or non-synonymous mutations, such changes in nucleotides can affect protein structure, interfere with post transcriptional events like folding of mRNA, creation or loss of binding sites for regulatory machinery. Among the known non-coding RNAs, miRNAs are small nucleotide sequence which regulate gene expression by binding to transcript. There are specific binding sites for the miRNAs in the mRNA of various genes, due to SNPs formation, either new binding sites of miRNAs is formed or sometime lost. The SNPs can be also in miRNA genes, or it target site. If miRNA target site on the respective gene is altered, it can modify gene regulation with significant effects on the diseases phenotypes and susceptibility (Ryan *et al.,* 2010). Due to regulatory role of miRNA, these RNAs are widely studied in different diseases.

In case of metabolic processes, miRNAs play an important role as key mediators for regulating energy balance and homeostasis. The regulatory role of miRNA in obesity has been studied using model organisms. *In silico* analysis of miRNA expression in adipocytes and in other biological processes associated with obesity revealed that there is alteration in miRNAs expression levels. The identified miRNA in obesity were involved in the regulation of insulin signalling pathway, lipid/carbohydrate metabolism, adipocytes differentiation and fat metabolism (McGregor & S. Choi, 2011;Peng *et al.,* 2014;Amri & Scheideler, 2017; Dosal *et al.,* 2019). The regulation by

miRNA also governs normal functioning of organs and tissues like adipose tissue, liver, pancreas and muscles. The altered miRNA expression in some studies on obese animals and human found link to adipose tissue functioning, showing that they are involved in dysregulation of the metabolic processes and homeostasis.

miRNAs are detected in circulating plasma and other body fluids, therefore, are used for diagnosis of diseases as biomarker (Iacomino & Siani, 2017). In case of obesity, miR-143 was involved in controlling the adipocyte differentiation. But the miR-143 expression within the adipocytes and its function in the obesity development is not yet fully elucidated. However, the miR-143 was found to be associated with mesenteric fat and gain of body weight. Furthermore, miR-143 expression was directly correlated to differentiation markers of adipocytes and plasma leptin levels. In case of high fat diet fed mice, up regulated expression of miR-143 in mesenteric fat, is an indirect evidence that miRNAs have association with obesity. This miRNA might be regulating the adipocytes genes in obesity pathophysiology (Takanabe *et al.,* 2008)

Other studies on miRNA regulation of genes were focused on multiple pathways that were related to obesity. Kyoto Encyclopaedia of Genes and Genomes (KEGG) studies has revealed statistically significant association of metabolic pathways leading to obesity with miRNAs. KEGG study found 6 miRNAs (hsamiR-4523, hsa-miR-3615, hsa-miR-671-3p, hsa-miR-922, hsa-miR-1307-3p and hsa-miR-198) in regulation of NF-kappa B signalling pathways. miR hsa-miR-1307-3p and hsa-miR-922 had strong association with metabolism and fatty acids biosynthesis. A mirPath gene ontology (GO) analysis of miRNAs predicted that the hsa-miR-922, hsa-miR-3615, hsa-miR-671-3p, hsa-miR-637, hsa-miR-1307-3p and hsa-miR-198 were link to metabolic process, cellular nitrogen compound, organelle/RNA binding and poly (A) RNA binding. Additionally, five miRNAs hsa-miR-637, hsamiR-922, hsa-miR-3615, hsa-miR-198 and hsa-miR-1307-3p were also associated with biosynthetic process, cytosol, and mitotic cell cycle. Lastly four miRNAs (hsa-miR-922, hsa-miR-3615, hsa-miR-1307-3p and hsa-miR-198) were found to be linked with molecular function like gene expression, protein complex, nucleoplasm, and cellular components in case of obesity (Donato *et al.,* 2018).

Mutations in TLR-2 and TLR-4 along with nutritional factors impact the metabolic and inflammatory responses. In addition, directly or indirectly obesity development is also affected by

gut microflora. The human gut microbiota has low diversity of microbial communities in comparison with other bodily sites showing functional redundancy of the resident flora (Costello *et al.,* 2009; Schluter & Foster, 2012; Pérez-Cobas *et al.,* 2013). After a country-specific microbial communities identification, revealed that there a signatures microbiome which is shaped by diet, and host genetics (Li *et al.,* 2014). This resident gut microbiota has some function in harvesting the energy and orchestrating the host immune responses. The interaction of specific bacterial species with the obesity development need investigation for better understanding of this disease.

The development of gut microbiota starts with infancy. After intake the solids, it starts to mature which after 3 years of age show more complex and stable community with predominant being *Firmicutes* in case of adults (Koenig *et al.,* 2011; Flores *et al.,* 2012; Yatsunenko *et al.,* 2012). In case of formula-fed infants, the microbiota is more complex and comprise of enterobacterial genera including *Streptococcus*, *Bacteroides*, *Clostridium* as well as *Bifidobacterium* (Bezirtzoglou & Stavropoulou, 2011). The normal gut microbiome consists of 5 phyla that includes *Actinobacteriota*, *Proteobacteria*, *Verrucomicrobia*, *Firmicutes*, and *Bacteroidota*. These Gram positive *Firmicutes* and Gram negative *Bacteroidota* makeup 90% of the bacterial species (Endo *et al.,* 2015). These species are categorized into the most prominent enterotypes. The enterotypes are dominated by three different genera: *Bacteroides*, *Prevotella*, and *Ruminococcus*, independent of age, gender, ethnicity, or body mass index (Arumugam *et al.,* 2011;Benson, 2016).

The microbiota in adults is relatively stable and reduction in stability occurs with the age. This microbial community's composition in gut relies entirely upon the host and can be modified by a number of both exogenous and endogenous factors. These resident microbial gut communities maintains a symbiotic relationship with host where both mutually evolve along the time and is influenced by different factors like living environments, genetics, sex, age, immune system, and gut motility (Andoh, 2016). Due to its diverse nature, gut microbiota from the last few years has attracted so much importance in context of its involvement in health status. The dysbiosis of the gut microbiota have been shown to participate in the etiology of obesity (Guarner & Malagelada, 2003)(Debédat *et al.,* 2019). The composition of gut microbiota in obesity in a study had predominantly *Firmicutes* and *Bacteroidota* phyla followed by *Proteobacteria* and *Actinobacteriota*. Ley *et al.,* (2005) reported in genetically modified mouse, an increased

abundance of *Firmicutes*/*Bacteroidota* (F/B) ratio in obesity. The gut microbiota profile of obese people in a study was seen to be characterised by reduced abundance of phylum *Bacteroidota* and an increased prevalence of the phylum *Firmicutes* in comparison with the controls. Also transfer of the gut microbiota from obese mice significantly increased the adipose tissue development in recipient mice in comparison with the gut material transfer from healthy controls. Furthermore, in study on high-fat diet fed mice there was an increase in specific type of bacteria which were *Deltaproteobacteria*, *Gammaproteobacteria*, and pathobionts (*Staphylococcus, Odoribacter, Neisseria*, and *Propionibacterium* spp.)(Singh *et al.,* 2017). An increased ratio of F/B in both obese mice and obese individuals as result of high fat diet intake is recorded (Ley *et al.,* 2006; Hildebrandt *et al.,* 2009).

Turnbaugh *et al.,* (2009), found increase in body weight after transplantation of cecal material from obese mice to germ free mice (Turnbaugh *et al.,* 2009). Up to now, the gut microbiota composition and obesity seem to have some association. Moreover, gut microbiota shift with the change in the dietary components is found in mice on methionine-choline-deficient diet in which F/B ratio in the gut was altered (Ishioka *et al.,* 2017). Potentially, gut flora with a distinct and specific gut community has ability to harness energy and different flora can give different obesity phenotypes. The gut flora participates in harvesting the energy by metabolism of complex indigestible carbohydrates and as a result produces the SCFA. These SCFAs play an important role in immune responses as they reduce the inflammatory responses (Canfora *et al.,* 2017; Venegas *et al.,* 2019) SCFAs consists of acetate, propionate, and butyrate, among these acetate plays a role in energy metabolism of the host by releasing glucagon-like peptide-1, peptide YY, and other intestinal hormones. These hormones have anti-inflammatory by reducing pro-inflammatory cytokines and lipolysis. In case of energy harness process they increase the energy production and lipid oxidation (Hernández *et al.,* 2019).

The propionate intestinal lipolysis and energy homeostasis in mice model is regulated through the AMPK/LSD1 pathway (Wang *et al.,* 2019). Butyrate act as main energy source for the intestinal epithelial cells by its oxidation, the butyrate-producing bacteria in the gastrointestinal tract increases the level of butyrate which in turn improves the lipid metabolism through the butyrate

pathway SESN2/CRTC2. However, the increase of butyrate has been observed to interfere the probiotics and impose reverse metabolic effects on host (Yu *et al.,* 2019).

More advancement in the field of gut microbiota has drawn attention due to their beneficial effects on intestinal homeostasis, energy metabolism and gut permeability as well as negative effects in various disease like obesity for their use as metabolic disorder markers (Zuluaga *et al.,* 2019). Teixeira *et al.,* (2013) identified a positive correlation of faecal SCFA with female obesity, waist circumference and other metabolic indicators. Overall, SCFA perform dual function by either protecting the host from diet-induced obesity or on the other hand excessive SCFA provide extra energy to host resulting in obesity.

Beside these SCFA, the other bacterial components noteworthy gut microflora products are LPS, enzymes and endotoxins. All of these bacterial products have different functions inside the host body and function to maintain the integrity of gut epithelium, homeostasis, interactions with immune cells and act as signalling molecules (Wells *et al.,* 2017). Various studies have shown that LPS an outer membrane part of Gram-negative bacteria can be released into host body and become part of the circulation. An increased levels of circulating LPS in case of obese rodents and humans is detected (Creely *et al.,* 2007; Cani *et al.,* 2008; Oliveira *et al.,* 2011). Although, the increase in LPS due to gut microflora in obesity is controversial as the *Firmicutes* are the most abundant phyla found in microbiota which are Gram positives. Another proposed explanation is that this increase of LPS in case of obesity is associated with the increased intestinal permeability. The altered permeability is attributed to reduced zonula occludens-1 (ZO-1), claudin, and occludin protein expression as these proteins maintain tight junction making stable gut epithelial barrier to inhibits bacteria, their components and products from mixing with the circulation. This breach in the barrier was found in early development of inflammation and insulin resistance in humans and mice (Brun *et al.,* 2007; Cani *et al.,* 2008 ; Amar *et al.,* 2011; Burcelin *et al.,* 2012). Another mechanism through which the LPS is transported into circulation is with the help of chylomicrons protein (Wang *et al.,* 2009). Notably, the direct administration of LPS in animal models also induce systemic inflammation and insulin resistance (Csak *et al.,* 2011). An increase in LPS and LPS-binding protein in the serum of the obese individuals was recorded in studies on human subjects (Cox *et al.,* 2017; Lassenius *et al.,* 2011; Pussinen *et al.,* 2011)

LPS are detect by macrophages through TLR-4 receptor and result in switch of M2 from M1 phenotype, where there is up-regulation of pro-inflammatory cytokines such as IL-1 and TNF-α (Wang *et al.,* 2020). The LPS increase due to increased gut permeability causes endotoxemia characterised by the low grade inflammation in many metabolic diseases including obesity. In the obese mice model, use of antibiotics and intake of high fat diet had adverse effect on gut microflora which reduced *Lactobacillus*, *Bacteroides*/*Prevotella* and *Bifidobacterium* while increased abundance of *Enterobacter* which are endotoxin producers. This was also due to leaky gut where bacterial metabolites were allowed to cross the epithelial barrier, ultimately entering the blood stream (Bander *et al.,*, 2020). Such microbial products and LPS released by gut microflora interact with the TLRs on the peripheral blood monocytes to initiate the inflammation. Consequently, these metabolites result in establishing the local as well as systemic inflammation (Bäckhed *et al.,* 2005; Cani *et al.,* 2008; Turnbaugh *et al.,* 2006) Until now, majority of microbiome studies are exclusively pointing towards interaction of the bacterial components and metabolites with inflammation.

Predominantly studies examining the links between gut microbes and metabolic health have focused almost exclusively on the bacterial component of the microbiota (Sun *et al.,* 2001;Daniel *et al.,* 2014), irrespective of their phyla and species. Recently, the fungi colonizing gut have been identified in human and mice studies in which a huge fungal diversity is documented. In case of infections when antibiotics are used, there are effects on the bacterial diversity but fungal diversity is not effected (Dollive *et al.,* 2013; Heisel *et al.,* 2015; Iliev *et al.,* 2012; LaTuga *et al.,* 2011). Although the fungi comprises less than 1% of the microbes in the gut of humans (Balzola *et al.,* 2010; M *et al.,* 2011) still their importance and health effects can never be ignored. The fungal cells are 100 fold larger than the size of typical bacteria with unique capability of metabolic function and might be having more unique host microbe interactions. Moreover, fungal diversity also provides the collection of diverse fungal species which maintain gut integrity (Huffnagle & Noverr, 2013). Different studies identified 66 genera and 184 fungal species in human gut in which *Candida*, *Saccharomyces* and *Cladosporium* were the most dominant type. These fungi are also participate in the host immune responses (Hoffmann *et al.,* 2013; Huffnagle & Noverr, 2013; Underhill & Iliev, 2014). These intestinal fungal communities' products act as ligands for TLRs like TLR-2 recognizes the fungal phospholipomannan (PLM) (Jouault *et al.,* 2006; Uematsu &

Fujimoto, 2010) whereas O-linked mannans is agonist for TLR-4 (Netea *et al.,* 2006; Kawai & Akira, 2010). These immune receptors (TLR-2 and TLR-4) have crucial role in initiation of immune responses against *C. albicans* infections too. TLR-2 and TLR-4 have reduced expression in intestinal epithelial cells of a healthy gut linings but their expression is higher in crypt epithelial cells (Abreu *et al.,* 2001; Furrie *et al.,* 2005; Wells *et al.,* 2011). There is protective role of *TLR-2* against the colonisation and endogenous invasion by *C. albicans* (Prieto *et al.,* 2016).

These studies suggest a complex etiology of the obesity, where innate immune response is the vital player of initiating the pro-inflammatory cascades to develop low grade inflammation. A chronic state of disease develop may be due to interactions of resident gut microflora comprising of bacterial and fungal diversity. The type of diet intake and immunity also have intricate interactions resulting in low grade inflammation, a characteristic of obesity. Certain mutation and SNPs have been detected in *TLR-2* in various inflammatory diseases like cancers and lymphomas, which participate in progression and worsening of disease. A high *TLR-2* and *TLR-4* expression has been reported in obesity in peripheral mononuclear cells too.

There is lack of any published data on genetic polymorphism of the *TLRs* and their involvement in obesity. There is possibility of over expression of TLR-2 and TLR-4 due to mutations in their genes which can generate abnormal response with hyperactive signalling activation. These receptors are activated by various endogenous and exogenous factors as in case of obesity, the prominent ligands being microbial LPS, SCFA, PLM, O-linked mannans and mannan. Also, few studies on TLRs observed their association with dietary component. There is no work on relation of the risk factors of obesity with genetic polymorphisms of TLRs and gut microbiota, *TLR-2* or *TLR-4* polymorphisms in obese Pakistani population has not been investigated along with gut microbiome profile. Hence, there is need for the understanding of genetics of *TLR-2* and *TLR-4* gene and gut microbiome which will provide the molecular insight into the obesity associated inflammation. Such work would provide the basis to understand the pathophysiology of obesity to develop basis for devising the therapeutic interventions.

Toll Like Receptor being the versatile receptor of many ligands, can affect the outcome of disease as a result of possible SNPs in its critical domain involved in binding, recognition and activation. The gut microbial profiling of obese in our region would be helpful in designing the weight loss

regime depending upon the gut microbial profiling. Moreover, the association of behavioural, dietary and demographic factors would be helpful in defining the possible contributors to obesity increase. These findings would aid in obesity management and control programmes by medical experts to reduce the obesity burden in Pakistan.

CHAPTER 3: DEMOGRAPHICS, DIETARY HABITS AND LIFE STYLE CHARACTERISTICS OF OBESE INDIVIDUALS

3.1- INTRODUCTION

Obesity is a heterogeneous disorder in which multiple behavioural, environmental, biological and metabolic factors interact with each other with involvement of genetic and immunological factors (Calder, 2018). The etiology of obesity is complex and involve number of contributing traits, which are broadly grouped as: demographic features, behaviour, energy metabolism, hormone signalling, peripheral and central regulation of energy balance, adipose tissue biology, skeletal muscle biology and now even gut microbiome their genome as well as epigenome. Among demographic factors age, gender, ethnicity, marital status, occupation, physical activity and education level are important in obesity (Ghosh and Bouchard, 2017).

There has been drastic increase in obesity prevalence worldwide in recent times. The global estimates on obesity by WHO show an over 650 million population living with obesity (World Obesity Federation, 2022). According to world obesity atlas, the prevalence of obesity has been estimated to increase up to 1.0 billion worldwide by 2030 (Kelly *et al*., 2008).

The contributing factors for such drastic increase are changing dietary trends, sedentary lifestyle, rapid industrialization and urbanization, which are major reasons for the emerging epidemic of obesity worldwide. It is a well-established that countries sharing the highest burden of obesity, also share high burden of comorbidities (Campos *et al.,* 2006).

The association of obesity with non-communicable diseases makes it a priority health concern now. Obesity act as a risk factor for the development of other diseases like insulin resistance and type 2 diabetes, atherosclerosis, non-alcohol fatty liver disease (NAFLD) , infertility, dyslipidaemia, hypertension, immune system dysfunction, cardiovascular disorders and some cancers (Landsberg *et al.,* 2013; Djalalinia *et al.,* 2015; Al-ghamdi *et al.,* 2018; Kern *et al.,* 2018; Rada *et al.,* 2018) which impair the quality of life, also cause high mortality.

Various studies from different regions show different obesity rates based on various risk factors including socio-demographic, environmental, physical and dietary patterns, these discrepancies are still completely explaining the contribution of these risk factors in obesity. The socioeconomic

factors contribute to obesity on an individual leading to impact at community level (Anekwe *et al.,* 2020). The extensive studies on obesity have found that the socioeconomic strata has significant association specially in developing countries with higher SES, while an inverse relationship has been observed in developed countries with ethnicity (Sobal *et al.,* 1989; Araujo *et al.,* 2018).

In literature, high prevalence of smoking is also an indirect risk for obesity (Schroeder, 2007). Dietary pattern including healthy food choices, intake of more fast food and frequent visits to restaurants are also significantly associated with the increase in obesity among various communities (Ploeg *et al.,* 2011; Chen *et al.,* 2016; Cooksey-Stowers *et al.,* 2017). Increased commuting in urban areas is one of the factor for increase in obesity (Frank *et al.,* 2004). The shift in social and economic changes over the time has enhanced the chances of obesity (Faith *et al.,* 2006). According to a study on low to middle income countries, a trend of high prevalence of obesity in rural areas as compared to urban, such difference is up to 60% with an annual rise in BMIs (Bixby *et al.,* 2019). Other risk factors for obesity are personal life choices and life style including screen time, physical activity and sleep duration (Wu *et al.,* 2014; Chin *et al.,* 2016; Tambalis *et al.,* 2018).

Obesity was previously considered as a major health issue of developed countries only, but rapid transition of social, environmental and dietary habits plus choices, have increased burden of obesity three folds in middle and low income countries (Hossain *et al.,* 2007). In Pakistan, the disease burden is shift from more communicable diseases towards non-communicable diseases especially obesity. As per world obesity observatory report 2018, the prevalence of obesity among women (21.8%) is higher than the men (12.1%), which is more dominant in urban setting as compared to rural areas (Pakistan World Obesity Federation Global Obesity Observatory, 2018).

Pakistan, being the 5th most populated country in the world, with an estimated 39.1% urban population. Limited literature is available on the obesity prevalence nationwide and especially among adults. Also, the scarce available data on obesity is indicating that there is rise in obesity across various regions of the country including northern area, Malakand, and Multan (Shah *et al.,* 2004; Khan, 2008; Aslam *et al.,* 2010; Imran *et al.,* 2019). However, none of these studies has explored the comprehensively possible contributing risk factors above mentioned for possible contribution in obesity susceptibility as upward trend of obesity is seen across Pakistan. The

present work seeks to identify the potential socio-demographic, dietary pattern and lifestyle behaviours that could act as risk factors, if any association found with obesity in the local population. These findings would be helpful in devising the strategies against the control of increasing obesity situation among adults, which would be helpful in decreasing the health burden caused by obesity and campaigning for awareness health programs against the obesity nationwide.

3.2- MATERIAL AND METHODS

3.2.1- Type of Study and Data Collection

The current study is retrospective case control type and was conducted after the approval of Bio ethic committee of Quaid-I-Azam University (Islamabad) under assigned protocol number BEC-FBS-QAU 2020-237. During March 2019 to February 2022, the data with structured questionnaire and written consent was collected, consent form was as per declaration of Helsinki by World Medical Association from the study subjects. The questionnaire was filled by in person interview. (Appendix I). The anthropometric measurements were recorded carefully and then the BMI was calculated according to the WHO recommendation.

3.2.2- Inclusion and Exclusion Criteria of the Study

3.2.2.1- Inclusion Criteria

All the study participants were included on the basis of their age which was selected as greater than 18 years. The study subjects with BMI between ≥ 25-29.9 kg/m^2 were included and were categorized as pre-obese while those having BMI ≥30 kg/m^2 as obese. The healthy controls were included on the basis of BMI range between 18-24 kg/m^2.

3.2.2.2-Exclusion Criteria

Those individuals who were having any comorbid conditions, infection, metabolic disorder, cancers and immune disorder were excluded from this study. Individuals with steroid based therapy. All those with BMI range less than 18 kg/m^2 were also excluded.

3.2.3- Questionnaire Data and Variables

The questionnaire comprised of three sections: (1) Demographic data of obese and control including age, height, weight, BMI, socio-economic status (low, middle and high), residential area, education (2) Dietary habits and fluids intake including number of meals, number of snacks, preferred taste, preferred meal type, portion of fruits and vegetables, beef consumption, mutton consumption, chicken consumption, fluid intake, tea intake, sugar /cup, water intake, fresh juice consumption, whole milk, soda intake, green tea (3) Life style including sleeping hrs., sleeping pattern, gap between last meal and sleep, screen time, smoking history and exercise.

3.2.4- Statistical Data Analysis

For statistical analysis, Microsoft Excel and IBM SPSS (version 21) was used. Anthropometric measurement and socio-demographic factors were analysed by one-way ANOVA with Dunnett post hoc test to check the variation among groups. Multinomial regression analysis was carried out to predict these dietary factors, fluid intake and lifestyle as a risk factor for the obesity. For regression analysis obesity (obese, pre-obese) was taken as dependent variable. The association was taken significant when *p* value was equal to and less than 0.05.

3.3-RESULTS

3.3.1- Anthropometric Measurements of Groups

According to one-way ANOVA test, age ($F= 23.023$, p-value < 0.01), weight ($F=515.498$, p-value < 0.01) and BMI ($F=1141.381$, p-value < 0.01) were statistically significant among the groups. According to Dunnett post hoc test, no association of any anthropometric variable was found significant as p-value < 0.05 (Table 3.1a and 3.1b)

Table 3.1a: Comparison of anthropometric measurements among the individuals of obese, pre-obese and control group

Anthropometric variables		**Number (*n*)**	**Mean**	**Standard Deviation**	**Standard Error**
Age(yrs.)	Obese	245	37.53	11.75	0.75
	Pre-obese	86	37.20	11.53	1.24
	Controls	357	31.35	11.83	0.62
	Total	688	34.28	12.14	0.46
Weight (kg)	Obese	245	90.54	12.87	0.82
	Pre-obese	86	76.05	9.97	1.07
	Controls	357	60.13	10.70	0.56
	Total	688	72.97	18.10	0.69
Height (m)	Obese	245	1.60	0.10	0.006
	Pre-obese	86	1.63	0.08	0.009
	Controls	357	1.59	0.16	0.008
	Total	688	1.60	0.14	0.003
BMI (kg/m^2)	Obese	245	34.50	4.35	0.27
	Pre-obese	86	27.53	1.48	0.15
	Controls	357	22.57	1.97	0.10
	Total	688	27.45	6.25	0.23

Table 3.1b: Association of anthropometric measurements with obesity and pre-obese condition using One-way ANOVA with post hoc Test

Anthropometric variables	**ANOVA**		**Study group comparison**	**Post Hoc Test**	
	F-ratio	***p*-value**		**t-ratio**	***p*-value**
Age (yrs.)	23.02	0.000	Obese vs. controls	6.17	1.00
			Pre-obese vs. controls	5.85	1.00
Weight (kg)	515.49	0.000	Obese vs. controls	30.40	1.00
			Pre-obese vs. controls	15.92	1.00
Height (m)	2.28	0.103	Obese vs. controls	0.005	0.85
			Pre-obese vs. controls	0.035	0.99
BMI (kg/m^2)	1141.38	0.000	Obese vs. controls	11.93	1.00
			Pre-obese vs. controls	4.96	1.00

3.3.2- Distribution based on Socio-Demographic Characteristics

The descriptive analysis of sociodemographic characteristics of obese, pre-obese and controls is presented in Table 3.2. These factors were assessed for association with the obesity by using multinomial logistic regression. The control group was taken as reference group whereas the obese and pre-obese conditions were taken as dependent variable. The gender was not significantly associated with the obesity as coefficient was $\beta = 0.362$, *Wald*= 3.458, $p=0.063$, OR=1.436 (0.981-2.104) and also for pre-obesity *p* value was 0.76. In obesity, the marital status [coefficient $\beta= 0.928$, *Wald*= 19.919, $p= 0.000$, OR= 2.529(1.683-3.802)] and area of residence were found to have significant association as coefficient β was 0.737[*Wald*=11.708, $p= 0.001$, OR=2.090(1.370-3.189)]. In pre-obese group, marital status and area of residence were statistically significant too. Based on socioeconomic, lower strata [coefficient $\beta= 1.065$, *Wald*=9.267, $p=0.002$, OR= 2.900(1.461-5.757)] and middle strata had significant association with the obesity [coefficient $\beta=0.811$, *Wald*= 6.511, $p=0.011$, OR=2.250(1.207-4.193)]. In pre- obese group, no association was observed in term of socioeconomic strata. Based on education level, in higher education coefficient was $\beta= -1.260$, *Wald*=17.640, $p=0.000$, OR=0.284(0.157-0.511) which was significantly associated with the obesity (Appendix II).

Table 3.2: Descriptive analysis of obese, pre-obese and control group individuals on the basis of sociodemographic characteristics

Socio-demographic characteristics	**Risk Factors**	**Obese *n*=245 (%)**	**Pre-Obese *n*= 86 (%)**	**Controls *n*= 357 (%)**
Gender	**Female**	140 (57.14)	51(59.30)	138(38.65)
	Male	105 (42.85)	35(40.69)	219(61.34)
Marital status	**Single**	52 (21.22)	12(13.95)	173(48.45)
	Married	193(78.77)	74(86.04)	184(51.54)
Socioeconomic Status	**Lower strata**	87(35.51)	26(30.23)	89(24.92)
	Middle strata	141(57.55)	52(60.46)	204(57.14)
	Upper strata	17(6.93)	8(9.30)	64(17.92)
Area of residence	**Rural**	87(35.51)	20(23.25)	138(38.65)
	Urban	158(64.48)	66(76.74)	219(61.34)
Education	**Uneducated**	46(18.77)	22(25.58)	40(11.20)
	Secondary School level	92(37.55)	27(31.39)	57(15.96)
	Graduated	45(18.36)	12(13.95)	75(21.00)
	Masters	62(25.30)	25(29.06)	185(51.82)

3.3.3- Associations of Obesity and Pre-Obesity with the Eating Habits, Fluid Consumption and Lifestyle

To check the association, a multinomial logistic regression analysis was applied on various risk factors keeping obesity as dependent variable. According to the results of regression analysis, when the frequency of meal was kept 2 meals/day as reference, a significant high risk was with 4meals/day [coefficient β = 3.83, *Wald*=7.64, *p= 0*.006, OR= 46.45 (3.05-705.45)]. While 3meals/day was posing comparatively lower risk for obesity [coefficient β=2.09, *Wald*= 11.16, *p*=.001, OR=8.12 (2.37-27.78)]. The number of snack/day in study subjects where no snack intake as reference found that 2 snacks and 3 snacks/day had significant association with the obesity. Highest risk was with 3 snacks/day as coefficient β = 1.89, *Wald*= 5.38, *p=0*.020, OR= 6.61(1.34-32.67).

The use of daily raw fruits and vegetables was also computed in form of fruits/vegetable portion per day. By keeping no intake of raw fruits and vegetables/daily as reference, 3-4 portions of fruits/vegetables showed coefficient β=3.27, *Wald*=17.55, *p=0*.000, OR=26.54(5.72-123.06) and 1-2 portions of fruits/vegetables coefficient β was 2.17, *Wald*=12.47, *p=0*.000 and OR=8.76(2.62-29.22), interestingly more intake of vegetable and fruit was a risk factor.

The choice of flavour was checked by categorizing it as sweet, savour and both; these choices did not show any association with the obesity. The meal preferences were checked by keeping home cooked food as reference group while the restaurant food (*p=0*.000) and combination of restaurant based and home cooked (*p=0*.000), a strong association with the obesity as a risk was detected. Other factors were mutton, beef and chicken consumption but no association with the obesity was seen.

In pre-obese group, the association with the meal frequency was not significant, while the snacking number showed association keeping no snacking as reference. Three snacks/day were calculated to have coefficient β = -3.283, *Wald*=6.631, *p*=0.010 and OR= .038(.003-.456) and one snack/day coefficient β= 1.421, *Wald*=8.505, *p*=0.004, OR=4.141(1.594-10.761). Portion of fruits and vegetables had association upon regression analysis where values for 3-4 portion/day had coefficient of β= 1.945, *Wald*=8.470, *p*=0.004, OR=6.993(1.887-25.912) and for 1-2 portion/day coefficient β=2.196, *Wald*=13.575, *p*=0.000, OR=8.987(2.795-28.900). Preferred flavour was

also associated with the pre-obesity showing that sweet flavour had coefficient β= -4.418, *Wald*=45.920, *p*=0.000, OR=0.012(0.003-0.043), while for savoury flavour coefficient was β=-4.915, *Wald*=47.767, *p*=0.000, OR=0.007(0.002-0.030). Among meal preferences deep fried items showed significant association, while comparing mutton, beef, and chicken intake, only chicken intake had significant association with the pre-obesity with $p< 0.01$ (Appendix III).

In a daily routine consumption of different fluids was checked for association with the obesity. The consumption of two litres of liquid in different forms of fluids was found to have association with the obesity as risk factor where coefficient β value of 5.065, *Wald*=37.525, *p*=0.000, OR=158.405 (31.329-800.927) with litre/day as reference. Moderate to low intake of tea showed association with obesity, intake of 3 cup daily showed significant association coefficient β= 1.899, *Wald*= 5.691, *p*=.017, OR= 6.681 (1.403-31.809) and 2 cups/day was also associated as coefficient was β= 2.060, *Wald*=7.699, *p*=0.006, OR=7.847(1.831-33.630). Likewise, sugar uptake was also significantly associated with the obesity having *p* value less than 0.05. However, water intake as individual factor was not found to be risk of obesity. Also juice uptake did not show any association as *p* value was 0.067. Taking whole milk, soda intake and green tea once daily showed association as coefficient β was -1.592, *Wald*= 5.464, *p*=0.019, OR= 0.204(0.054 -0.773), coefficient β= -2.410, *Wald*= 7.578, *p*=0.006, OR=0.090 (0.016- 0.500) and coefficient β = 2.076, *Wald*=8.938, *p*=0.003, OR=7.976 (2.044 -31.115) respectively. In case of pre-obese group, the fluid consumption was major risk factor for increasing pre-obesity (Appendix IV).

Among the lifestyle variables, longer hrs of sleeping showed strong association as risk for obesity as >8 hrs of sleep coefficient was β was 8.477, *Wald*=20.813, *p*= 0.000, OR= 4804.214 (125.874-183361.167) and for 4-8 hrs. coefficient was β= 5.802, *Wald*=22.697, *p*= 0.000, OR= 330.844 (30.412-3599.181). Irregular sleep pattern also found as risk, when regular sleep pattern was reference in the model. Higher gap between meal timing and sleeping when it was ≥3 hrs [coefficient β= -2.696, *Wald*=9.653, *p*=0.002, OR=0.067(0.012-0.370)] was significantly associated with obesity when smaller gaps was used as reference. Increased hrs in front of screen like TV watching and mobile usage was also strongly associated with obesity. No association of exercise with the obesity was seen while smoking showed significant association with the obesity having coefficient β= -2.661, *Wald*= 10.814, *p*=0.001, OR=0.070 (0.014-0.341). In case of obesity

sleeping hrs and screen time was not associated with the pre-obesity while other patterns were associated as given in Appendix V.

3.4-DISCUSSION:

Obesity prevalence has been on rise from last few decades that has led to considerable interest in determining its causes and consequences. Various studies have been conducted to ascertain impact of demographic characteristics in a specific population but only few studied addressed the dietary and lifestyle behaviours affect. Sociodemographic and behavioural features vary greatly among populations, exploring such differences might have potential to point the risk factors for obesity. In Pakistan, studies on obesity and demographic characteristics are scarce, the demographic factors of other obese populations with different socioeconomic setting might not be applicable to our local population. Thus, this study was designed to study collectively various risk factors including sociodemographic factors, dietary habit and lifestyle for obesity in local Pakistani population.

It is evident by the results that obese individuals were statistically different from healthy controls based on their weight, and BMI. However, the post hoc test did not show any association of these anthropometric differences among these groups. In the present study, multiple logistic regression model was used to assess association, whether various risk factors were positively or negatively associated with obesity. The average age was 37 years and higher prevalence of obesity in females (57.1%) as compared to males (42.8%) in the current study. These findings are in line with the previous Pakistani studies, where obesity was also high prevalence in age range of 35-45 years and females (Haq *et al*., 2010; Asif *et al*., 2020). Similar results for gender and age wise association were found in similar kind of studies carried out in different part of Asian region (Biswas *et al*., 2017; Rawal *et al*., 2018). This higher trend of obesity among the females in the present study could be attributed to their early marriage age with many pregnancies where most of them considered household work as exercise. Majority of them had no or little physical activity and easy access to food all the time. Other factors could be that most of them had emotional stress, were snacking. Among the study participant, marital status was a 2.5 (1.683-3.802) fold risk for obesity with 60% increased odds for being obese in comparison to pre-obese. These findings were further supported by study conducted on Brazilian and Korean populations, where marital status acted as risk factor for obesity independent of their socioeconomic features (Friche *et al*., 2011;

Lee *et al*., 2020). This difference of obesity prevalence among married in comparison to unmarried, could be due to increased burden of responsibilities leading to negligence of self-care, children upbringing, hormonal changes, and lesser physical activity, a common observation in Pakistani females.

Area of residence can play a critical role in weight gain, also in the present study urban residency posed as risk (OR=2.090) for obesity. According to current study, 41% higher risk in obese individuals depending upon their residential area in comparison to pre-obese. Same trends for urbanization and obesity risk were observed in a study from South Africa (Sedibe *et al*., 2018) and Bangladesh (Hossain *et al*., 2021).

In the current study, the association of socioeconomic strata was observed with obesity where chances were calculated to be more in both (OR=2.2) middle and lower income strata (OR=2.9), this shows an increasing trend in the risk with decrease in the socioeconomic strata. Socioeconomic strata contribute to obesity at individuals and community level and it also an observation from other developing countries that an inverse relationship exist with obesity (McLaren *et al*, 2007;Newton *et al*., 2017). The results of this present work are opposite to studies from developing countries, it could be due to the fact that socioeconomic status was not the only risk factor for obesity, rather obesity was effected by many factor which also depend on the population and their settings (Ogden *et al*., 2018).

In present study, majority of the obese and pre-obese individuals were educated up to secondary level. While no association was seen with the secondary education level within the study subjects, however, the higher education up to master level had significant and inverse relation with the obesity. Same trends for education was observed in a survey from England, France, Australia, Canada and Korea, with obesity, less increase in obesity with education level (Devaux *et al*., 2011;Sánchez *et al*., 2017).

Moreover, among the participants of the current study those living in urban localities were high in numbers especially males, were more involved in exercise and walking than those living in rural localities, also the females were less active than men in both localities (Table 3.3). This could explain why females were high in number than males being obese in the current study. The difference in urban to rural and male to female percentage could be due to lack of awareness about

the health perspective in rural settings than in the urban settings. A survey based study from Iran has shown that staying at home as house wife acted as independent risk factor for obesity in females (Navadeh *et al*., 2011). Also obesity prevalence is high in females is supported by the study from US where 41% females and 37% males were obese (Brehm & D'Alessio, 2000; Brehm & D'Alessio, 2000;Garawi *et al*., 2014). These gender inequalities were further seen in the study participants with more females being obese with low education along with the lower income strata. Similar findings were also observed in Turkish population, where more women were tend to be more obese than men as were from low income strata, lower activity level and low education family (Islek *et al*., 2020)

Taking frequent small portion of meals are recommended for weight reduction. In current study highest meal frequency noted among study participants was 4times/day, posing 5.72 fold risk for obesity as compared to less meals. These results are in line with the study on United States adult population (Murakami & Livingstone, 2015), where high caloric diet was responsible for obesity. The frequent meals might be responsible for increasing chances of obesity in local population due to the improper meal timings, larger portion sizes and unhealthy fried food. These findings suggest that personalized plan for meals at proper timing with adequate frequency should be encourages for lowering obesity risk in such individuals in Pakistan (Ha & Song, 2019).

Snacks in between the meals lowers the appetite and also divides the calorie intake, while taking unhealthy snacks increases chances of obesity (Njike *et al*., 2016; Aljefree *et al*., 2022). In current study 1-2 snacks/day were found to increase risk of obesity with 27% higher odds in obese as compared to pre-obese, while taking 3 snacks were inversely proportional. Therefore, susceptibility to obesity decreases with increased number of snacks consumption by obese. This could be due to the fact that the snacks are smaller in portion which lowers the appetite and after eating them one could feel full for longer period of time. The snacking also reduces chance of having large meal portions (Ortinau *et al*., 2014).

Daily consumption of adequate amount of vegetables and fruits portion lowers the risk of chronic diseases (Blanck *et al*., 2008). But in the present study, obese group showed higher odds of 97% for the daily intake of 1-2 portions of fruit/vegetable in comparison with pre-obese. While obese consumes 3-4 portion of fruits/vegetables, 3.79 times more than the pre-obese. The risk of high

fruit/vegetable proportion was in case of obesity was very high (OR= 26.54). These results were further supported by other studies, where strong association with daily fruit and vegetable consumption acted as a risk for obesity, however week based moderate consumption of fruits/vegetables showed to lowers the risk of obesity (Wu *et al*., 2022; Liu *et al*., 2022). In other the cross sectional study where >3 times intake of fruits/vegetable on weekly basis increased obesity in university student of Tamale, Ghana (Mogre *et al*., 2015). This point towards that possibility that fruits, and vegetables consumption should have counted as portions per week basis because the study showed that lower portion of fruits/vegetables act to lowers the risk of obesity in individuals. While daily fruits/vegetable consumption increases overall calorie increase of an individual acting as risk of obesity.

The personal choice of taste for sweet or savoury food is an important risk of the weight gain, and in case of most obese persons preference is more sweet in their daily food choices as reported by Bartoshuk *et al*., (2006) after survey. In the current study, although high number of individuals preferred savoury food, but it was not associated with the obesity, however, inverse association was found with pre-obesity. The type of meal preferred in current study was home cooked meal that showed significant association with obesity. The strength of association was also obvious from the higher *Wald* ratio 78.276 as posing risk of obesity was OR=28.6. There 'was no association observed for fast food consumption in the present study, which is contrary to the previous meta-analysis (Qin *et al*., 2021). However, a study in Japan found a causal relationship of home cooked meal with unhealthy eating habits (Tani *et al*., 2019). The home cooked meal is a risk factor for obesity susceptibility as these meals are high in energy density due to wrong choice of meal, poor nutrient contents, unhealthy cooking procedure and use of processed material in cooking. While checking the type of meats consumption (mutton, beef, and chicken) as a risk for obesity, only type of meat showed association with obesity susceptibility (OR=3.60, p=0.016) that was 1-2 times/week intake of beef. The systemic reviews based on previous published data also showed that the obesity is associated with red meat consumption which is line with this finding of current study (Rouhani *et al*., 2014; Schlesinger *et al*., 2019). Similar kind of cohort study from UK. also strengthen this finding of red meat as increased risk of obesity especially in women (Kim *et al*., 2022).The chicken consumption showed inverse relation only in pre-obese individuals (coefficient

β, ≥3times/week =-3.54 and 1-2 times/week= -3.00), which is contrast to the previous studies (Kim *et al*., 2017; Schlesinger *et al*., 2019).

Fluid intake was positively associated and was posing 14.0 times risk for obesity in comparison to pre-obese. The direct relation (coefficient *β*) of fluid intake and obesity (OR=158.405, *p* value=0.000) was seen as 40% greater odds of being obese when 2L of fluid was consumed daily. Same trends were observed among pre-obese group for the fluid consumption. These findings are supported by cross sectional study on 7958 obese adults of Isfahan (Iran), where daily fluid consumption of more than 2L increased odds for obesity (Salari-Moghaddam *et al*., 2020). As the fluid intake in this study was considered 2 litres when it was water, tea, whole milk and juices, intake id tea and juices are sugar intense drinks, hence would have been contributing towards the weight gain. Other studies from USA and Europe point toward that lower water intake act as risk for obesity (Chang *et al*., 2016; Maffeis *et al*., 2016). Here, it can be inferred that not only volume of fluids should be considered but also type and nature of liquids consumed as to avoid extra calorie uptake in form of fluids.

In obese group taking tea 2cups/day (OR=7.847) had 1.2 times more risk than 3cups/day (OR= 6.681), same results were observed among pre-obese for 2cup/day intake of tea. According to these findings moderate level of tea uptake was showing direct relation with obesity susceptibility while odds for obesity were decreasing by 68% per increase of 1 cup. These results are supported by the experimental and epidemiological studies where increased use of tea acted as anti-obesity agent (Sirotkin & Kolesarova, 2021; Xu *et al*., 2021). In one study the polyphenol components of black tea were found to interfere with lipid and saccharide digestion causing reduction in obesity (Pan *et al*., 2016).

In the present work inverse relation with sugar usage/cup of tea was observed with significant association (p value ≤ 0.05). These findings are interesting as sugar in tea was not a risk for obesity. Many studies support this findings where sugar added in tea was inversely associated with the obesity (Gyntelberg *et al*., 2009;Rippe & Angelopoulos, 2016). The sugar intake not a risk for obesity is opposite to other available work, where weight gain with more sugar intake is recorded. The sugar intake in tea might be risk factor to increase weight gain depending upon the individuals' other life style behaviours too as reported in many other studies (Drewnowski *et al*, 1987;Siervo

et al., 2014; Stanhope *et al*., 2016). . Thus, it could be said that sugar consumption in small amount throughout the day in hot beverages may not pose risk for obesity, but its higher consumption can be a risk factors.

Water intake and fresh juice were not associated nor posing any risk of obesity according to the regression model in the current study. However, only whole milk intake showed association with the obese group only, but it was inverse relation (coefficient β = -1.592, *Wald*=5.464 and *p* value=0.019), it means that it is acting as protective factor (OR=0.2) for obesity. These results are in line with other studies where consumption of whole and low fat milk showed decrease in adiposity (Beck *et al*., 2017; Vanderhout *et al*., 2020; White *et al*., 2020; Vanderhout *et al*., 2020).

Green tea consumption was 2.91 times higher in obese in comparison with the pre-obese group and was posing risk (OR=7.976) for obesity. However, many research studies have indicated the beneficial effects of green tea usage and lowering of fat deposition in the body. Yet none of the study have recorded its exact dosage in case of obesity (Chacko *et al*., 2010; Dinh *et al*., 2019). Thus, these results suggest 1cup/day of green tea increases odds instead of lowering risk for obesity, this may be due to other diet related variables were contributing too.

In life style behaviours sleep duration showed association in obese group posing 14.56 times greater risk of obesity for those who sleep more than 8 hrs a day in comparison with those who sleeps less than 8 hrs. Studies have shown longer duration of sleep, a risk for obesity (Liu *et al*., 2019; Sunwoo *et al*., 2020). If sleep is more than 4 hrs, it was also risk which increased by 56% with duration, hence long sleep hrs should be avoided by obese. Sleep pattern showed inverse relation with both obese and pre-obese group (obese OR= 0.20, pre-obese OR= 0.34), indicating the irregular sleep pattern effects the weight gain too. In the current study irregular sleep was acting in favouring the obesity. Studies have shown short sleep durations and irregular sleep can promote weight gain (Zhou *et al*., 2018; Huang & Redline, 2019).

Gap between dinner timings and sleep time at night has inverse relation in both in obese -2.696 and pre-obese group -1.043. So, the gap between sleep and meal is an important factor, more than 3hr gap was not risk in study subjects but was rather protective factor. According to current findings, the 2-4 hrs spending in front of TV screen or using mobile phone was posing risk for

obesity 1.54 times more than spending 1-2 hrs screen usage. These results were supported by the findings of Baron *et al*., 2011 and Venkatapoorna *et al*., 2020.

According to current study, exercise alone could have beneficial effect in case of individuals with obesity, however, inverse was observed among pre-obese individuals. Thus, it could be said that exercise alone could have beneficial effect in case of individuals with BMI <30 but for obese people other factors like diet, life style etc. should be considered also. In case of smoking behaviour, inverse relationship (coefficient β= -2.661) with the obesity was found, many other studies also found obesity decreases by smoking (Gümüş *et al*., 2014; Dare *et al*., 2015). The smoking habit was found to be acting as protective factor instead of risk factor in this study. The obese individuals were comparatively less active as compared to the control also high number 27% of control were smokers while just 5.7% obese were smokers (Table 3.5). Studies found that less exercise and not smoking lead to gain weight even after quitting the smoking (Bouchard *et al*., 1993; Dare *et al*., 2015; Malatesta *et al*., 2022).

In conclusion, associations between obesity, socio-demographic and behavioural characteristics varied greatly in our population from other populations. There are many differences in the contributing risk factors after examine data from the obesity and pre-obesity group. Present study provides the overview of various socio demographic, dietary, fluid intake and lifestyle pattern that may be acting as risk or protective factor in local Pakistani population. In Pakistanis, for obesity possible high risk factors are being married, living in urban residency, and belonging to middle to lower socioeconomic strata. Also, high number of meal consumption along with more fruits and vegetable intake also led to obesity even with 1-2 times beef intake per week. While increased snacking showed protective relationship with the obesity susceptibility. Fluid intake in form of tea and green tea were risk for obesity in this study, while having one cup whole milk daily, one soda daily and up to 2 teaspoon sugar in tea were also protective, interesting were playing role in obesity reduction. More than 4 hr of sleep with increased screen timings were directly associated for obesity risk. While irregular sleep pattern, smoking profile and gap for meal more than 3 hrs before sleep was posing protective effects. Majority female especially from urban resident with lower socioeconomic strata and education were more obese in this study. Moreover, this increased obese female ratio was due to their dietary habits and sedentary lifestyle (Table 3.4).

This study is a snapshot of Pakistani population, which is comprised of different region, ethnicities and cultures. However, present work is significant for sensitizing authorities for initiating obesity awareness strategies, to alter masses to modifying their daily dietary routine and change lifestyle behaviours for controlling weight both at public as well as personal level. There is a need for conducting similar studies at National level to get the comprehensive estimates of obesity in both rural and urban setting. Also determine the complete record of food consumption, type of food, food groups and nutrition, lifestyle patterns and food culture in the Pakistani community. Such data will be beneficial for reduction of national obesity burden in the country by devising evidence based awareness guidelines suited to social, dietary and life style pattern.

Table 3.3: Gender based association of study subjects with the area of residence

Study variables				**Females**			**Males**		
				Obese	**Pre-obese**	**Control**	**Obese**	**Pre-obese**	**control**
Demography	**Rural**	**Exercise**	**NO**	31(22.1)	8(15.6)	12(8.6)	19(18.0)	2(5.7)	17(7.7)
			YES	27(17.1)	8(15.6)	54(39.1)	10(9.5)	2(5.7)	55(25.1)
	Urban		**NO**	52(37.1)	23(45.0)	23(16.6)	34(32.4)	15(42.8)	60(27.3)
			YES	30(21.4)	12(23.5)	49(35.5)	42(40.0)	16(45.7)	87(39.7)

Table 3.4: Gender wise distribution of study subjects based on socioeconomic strata and education level

Education level	Socioeconomic strata	Females			Males		
		Obese	Pre-obese	Controls	Obese	Pre-obese	Controls
Uneducated	Lower strata	14	4	12	4	1	6
	Middle strata	25	14	5	3	3	12
	Higher strata	0	0	2	0	0	3
School	Lower strata	24	12	11	11	5	8
	Middle strata	34	8	7	19	2	16
	Higher strata	1	0	3	3	0	12
College	Lower strata	8	1	5	5	1	6
	Middle strata	11	5	13	15	4	33
	Higher strata	2	0	4	4	1	14
University	Lower strata	9	1	24	12	1	17
	Middle strata	9	5	49	25	11	69
	Higher strata	3	1	3	4	6	23

CHAPTER 4: DETECTION AND ASSOCIATION OF *TLR-2* AND *TLR-4* GENOMIC VARIANTS IN OBESITY

4.1- INTRODUCTION:

TLRs play an important role in host defence mechanisms by being innate immune adaptors that also creates link with adaptive immunity. These are present as transmembrane proteins, which act as PRRs to detect antigens either endogenous or exogenous in nature. So far, 13 are known in mammals, out of which 11 members of the TLR family are found in humans. TLRs consist of 3 domains, namely, extracellular domain at N-terminus with LRR, a single helix transmembrane domain and a cytoplasmic toll-interleukin-1 receptor domain present at C-terminal. The LRR domain is mainly involved in ligand recognition and attachment. Many downstream signalling molecules like MyD88, TRAF6 and IRAKs are activated by the TIR domain (Frenkel *et al.*, 2010;Sameer & Nissar, 2021). Activation of MyD88 and other adaptor proteins leads to the stimulation of multiple cascades that ultimately results in up regulation of inflammatory mediators thus promoting inflammation.

TLRs initiate an immune response after recognition of specific ligands which can be either pathogen-associated molecular patterns derived from pathogens (Gram-positive and Gram-negative bacteria) or tissue damage and inflammation-induced non-microbe danger signals termed damage-associated molecular patterns. Many endogenous ligands including saturated fatty acids, endotoxin, heat shock proteins, modified low-density lipoproteins, high-mobility group box 1, extracellular matrix degradation products, and advanced glycation end-products are recognized by TLRs, especially TLR-2 or TLR-4, which trigger pro-inflammatory response (Kawai & Akira, 2010; Jialal & Kaur, 2012) . Various studies have reported elevated levels of TLR-2 and TLR-4 expression in adipose tissue of obese individuals and is found to be significantly correlated with inflammatory cytokines (Samuvel *et al.,* 2010).

Both TLR-2 and 4 are found to be highly expressed in peripheral blood mononuclear cell (PBMCs) in various inflammatory conditions (Go et al., 2014; Proença et al., 2015). Also, their expression levels have been recorded to be significantly high and directly proportional to increasing BMI as

TLR2 and *4* mRNA abundance in PBMCs is observed to be increased in case of obesity (Ahmad *et al.,* 2012). It has been reported that TLR-2 and TLR-4 also sense nutrients to regulate the metabolism in the adipose tissue and macrophages especially by detecting free fatty acids or other lipids (Könner & Brüning, 2011; Sun *et al.*, 2012). The loss of either *TLR-2* or *TLR-4* expression in mice fed on a high-fat diet reduced systemic inflammation in the adipose tissue and improved their insulin sensitivity (Himes & Smith, 2010; Jia *et al*., 2014). Studies changes with involvement of TLRs especially *TLR-2* and *TLR-4* was also associated with pro-inflammatory processes. However, their role in case of obesity is not yet well understood. There is no study on genomics of TLRs and its variants in case of obesity, despite previous studies have shown involvement of TLRs in inflammatory responses. Different genetic studies have proved that SNPs in various genes affect immune response especially inflammation in different diseases. To date, numerous studies have been conducted on various diseases to investigate the possible association of *TLR-2 and TLR-4* genes' mutations/SNPs as risk for sepsis, cancers, leprosy, diabetes, tuberculosis and cardiovascular disorder development (Malhotra *et al*., 2005; Thuong *et al.*, 2007; Zhu *et al.*, 2013; Gao *et al*., 2015; Sharma *et al*., 2016). No study so far has been conducted and reported on the mutations or single nucleotide polymorphisms in the *TLR-2 and TLR-4* in obesity inflammation. This study is an attempt to identify if any genetic alterations in gene sequence of *TLR-2* and *TLR-4* for their possible role in obesity. These findings will be helpful in providing the potential drug targets and novel insight into inflammation in obesity.

4.2- MATERIAL AND METHODS

4.2.1- Type of Study, Study Criteria and Data Collection

Previously described in chapter 3 (section 3.2).

4.2.2- Blood Samples Collection from Obese and Healthy Controls

Peripheral blood from study participants was collected in anticoagulant containing vials EDTA tubes by using sterile syringe. About 3-4mL of peripheral blood was drawn from obese, pre-obese and healthy participants by using sterile syringes (5.0 mL). The blood was then immediately transferred into pre-labelled EDTA tubes that were shifted to an ice box to maintain the temperature at 4°C. Also, blood samples were collected for LAL assay to detect LPS in the serum.

The collected samples were then transported to the Molecular Medicine Laboratory within 2-4 hrs after collection and were stored at 4°C in refrigerator until subjected to DNA Extraction.

4.2.3- Extraction of Genomic DNA by Using Phenol-Chloroform Method

The genomic DNA extraction from peripheral blood was done following protocol, which requires 48 hrs treatment time. It is based on organic to aqueous phase separation of DNA without damaging it`s integrity and is termed as phenol-chloroform extraction method. Four chemical solutions along with other reagents were used for extraction of DNA from the blood samples. These chemicals and reagents facilitated the degradation and removal of cellular debris but also played role in the effective precipitation of good quality genomic DNA from blood cells. Each solution had specific function like $MgCl_2$ was the most important component of Solution A as it protected DNA from other cell organelles' DNases and aided in the permeability of the cells. Solution B facilitated in the protection of DNA from dissolving in water and precipitated DNA. Phenol (solution C) removed proteins attached to the DNA. Chloroform iso-amyl alcohol (solution D) disrupted the cell membrane and separated aqueous and non-aqueous compounds. Sodium dodecyl sulphate (SDS) being a detergent helped in the solubilisation of lipids and proteins, thus played role in cell lysis. Isopropanol and sodium acetate together helped in the precipitation of DNA. Proteinase-K removed DNA associated proteins and other cellular contamination from nucleic acid. Ethanol purified DNA, preparations of stocks and working solutions are provided in Appendix VI and VII.

Day 1:

From freshly collected peripheral blood, 750µL was transferred to sterile 1.5mL Eppendorf and 500 µL of Solution 'A' was added followed by placing the Eppendorf in inverted position for about 15 minutes. Centrifugation was done at 13,000 rpm for 15 minutes to obtain the pellets by carefully removing the supernatant. The pellets were than dissolved into 500 µL of solution 'B', vortexed and followed by centrifugation at 10,000 rpm for 5 minutes. The supernatant was discarded, and 15 µL of 20%SDS, 4 µL proteinase-K and 400 µL of solution 'B' was added to the pellet followed by the overnight incubation at 37°C for the effective digestion of the proteins.

Day 2:

Equal amount of solution 'C' and solution 'D' were taken in falcon tube and placed at room temperature until the formation of two layers. A 500 µL of Solution C+D was taken from the lower layer and added into the overnight incubated pellets followed by centrifugation at 10,000 rpm for about 13 minutes. The supernatant was carefully shifted to the new Eppendorf and the pellets were discarded. An approximate equal amount to the supernatant obtained, solution D was added to Eppendorf tubes followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was again taken to the new Eppendorf into which 500µL of chilled isopropyl alcohol and 55 µL of sodium acetate were added, tubes were gently inverted several times to facilitate mixing. After the precipitation of the DNA, tubes were subjected to centrifugation at 10,000 rpm for 4 minutes to allow the DNA to settle down in the pellet. The pellets were than washed two time with 400 µL chilled ethanol at 10,000 rpm for 5 minutes. Supernatant was discarded and DNA in pellets were allowed to air dry at room temperature. The DNA was eluted and preserved in 100µL 1X Tris EDTA buffer and stored at -20°C

4.2.4-Visualization of The Extracted DNA using Horizontal Gel Electrophoresis

For qualitative and quantitative visualization of extracted DNA, the obtained DNA was subjected to horizontal gel electrophoresis. For this purpose, 1% agarose was prepared by adding 1.0 g of agarose in 100mL of Tris Boric EDTA buffer (TBE) and placed in microwave for 1-2 minutes or until transparent, then allowed to cool at room temperature. In order to pre-stain the gel, ethidium bromide (10mg/mL) was added and poured into pre-assembled gel casting tray. After pouring, the combs were inserted and left to solidify at room temperature. After solidification, combs were carefully removed, and the gel was shifted into 1X TBE containing gel tank. Freshly extracted 2.0 µL of DNA was mixed with bromophenol blue dye and loaded into the wells. The gel was then allowed to run at 90 volts for 30 minutes for quality check. After the electrophoresis, gel was visualized under UV light by using Trans-illuminator system (SYNGENE). After quality check the quantity was assessed by the nanodrop. The composition of all the reagents used in the gel electrophoresis is given in Appendix VIII and IX.

4.2.5-*TLR-2* and *4* gene SNPs/mutation Detection

4.2.5.1-Primer Designing:

In order to detect any change in *TLR-2* and *TLR-4* genes, amplification of these genes was carried out by using PCR approach. For PCR, multiple primer sets were designed to amplify the full *TLR-2* and *TLR-4* genes. Online tool used for primer designing was primer3 software, designed primer sets along with their band sizes are mentioned in Appendix X and XI.

4.2.5.2-Polymerase chain reaction for the amplification of various exons of *TLR-4* gene

Amplification of various exonic and intronic regions of TLR2 and *TLR-4* genes was carried out via PCR based approach by using designed primers, ready to use Dream Taq Green PCR Master Mix (2X) (Thermo Scientific, Massachusetts, USA) and freshly extracted DNA as template. For PCR based amplification of *TLR-2* and *TLR-4* genes, multiple thermocycler and reagent conditions were optimized. Multiple annealing temperatures were analysed (temperature range of 50°C to 62°C) and various annealing durations were also optimized (range of 30-45 sec). Various primer concentrations were also assessed ranging from 0.1 to 0.5µL. After optimization of multiple PCR conditions, extracted DNA of study subjects and controls were subjected for amplification. Optimized reagent concentration and thermocycler conditions and are mentioned in the Table 4.1 and 4.2 respectively.

Table 4.1: Optimized reaction mixture for the amplification of *TLR-2* and *-4* gene

Serial Number	**Reagents Stock (initial concentration)**	**Final Concentration**	**Initial Volume (µl)**	**Final Volume (10µl)**
1.	Master mix (2X) (Thermo Scientific)	1X	5.0	9µl
2.	Forward Primer (10 µM) (e-oligos)	0.1-1 µM	0.2-0.3	
3.	Reverse Primer (10 µM) (e-oligos)	0.1-1 µM	0.2-0.3	
4.	PCR Water (Thermo Scientific)	-	Up to 9.0	
5.	Extracted DNA	1µg	1.0	1µl

Table 4.2: Optimized conditions of PCR for amplification *TLR-2* and TLR-*4* gene

S.no.	**Stages**	**Temperature °C**	**Time**	**No. of Cycles**
1.	Initial Denaturation	95	5 minutes	1X
2.	Denaturation	95	45 seconds	35 X
3.	Annealing	51-57	45 seconds	
4.	Extension	72	45 seconds	
5.	Final Extension	72	7 minutes	1X

4.2.5.3-Visualization of the PCR Products Using Horizontal Gel Electrophoresis

After PCR based amplification of various regions of *TLR-2* and *TLR-4* gene, the PCR products were subjected to 2% horizontal agarose gel electrophoresis for their qualitative visualization. For 2% agarose gel, 2.0 g of agarose was dissolved in 100mL of 1X TBE and microwaved for 2 min to get the transparent solution. The solution was then allowed to cool at room temperature followed by the addition of ethidium bromide (10mg/mL). It was poured in pre-assembled gel casting tray and combs were inserted. After solidification, the combs were carefully removed, and the gel was placed into gel tank containing 1X TBE. About 3-4 µL of each of PCR product was directly loaded into each well. The gel tank was then connected to electrodes and was allowed to run at 90 Volts for 40 minutes. The gel was then visualized under SYNGENE Gel documentation system.

4.2.5.4-Single Stranded Chain Polymorphism

After amplification and visualization of *TLR-2* and *TLR-4* genes, amplicons were further subjected to SSCP. The basic principle of this technique is the selection of genetic variants of genes obtained from study participants based on the resolution of banding patterns of amplified PCR products. The resolution of banding pattern of PCR products with size <500 bp was checked on 6% polyacrylamide and for band size >500 bp at 8% polyacrylamide gel electrophoresis.

4.2.5.5-Polyacrylamide Gel Electrophoresis

For the separation of banding pattern and variation analysis, 6% and 8% polyacrylamide gel electrophoresis (PAGE) was prepared. It was run to detect if any variation in length of the amplified PCR products. The gel casting apparatus for PAGE comprised of differential sized two glass plates (one with larger size and the other smaller one), three plastic based spacers, 1.0 comb for each set of plate and various clamps to hold the plates together. All the plates, spacers and combs were rinse with 70% ethanol and were air dried. The two plates were assembled in such a way that these were held together and separated by spacers in between. The three side were sealed and one side remained accessible for insertion of comb in between the plates. Clamps were used to tighten the plates together especially from the edges to avoid any chance of leakage. After the apparatus was assembled, polyacrylamide gels were prepared. The composition of 6% and 8% gel

is mentioned in Table 4.3 and 4.4 and reagents used for its preparation are given in Appendix XII and XIII.

After the addition of TEMED (99% pure), which accelerate the polymerization reaction, the prepared gel solution was poured immediately with immense care to avoid any bubble formation. The combs were instantly placed in between the two plates for the formation of the wells. The gel was then allowed to solidify at room temperature for about for 45min-1.0 hour. After gel solidification, gel tanks were assembled. For running PAGE, two tanks were used, one was the vertical tank and the second was the horizontal tank. The vertical tank was placed inside the horizontal tank so that the two electrodes were facing the opposite directions, tanks were than filled with the 1.0 X TBE. After solidification of the gel, the clamps were removed along with the bottom spacer. The gel containing plates were than mounted on the vertical tank with the help of clamps in such a way that the smaller plate was toward the inner side of the tank filling the wells with buffer. After proper assembly and mounting of plates to the tanks, the denaturation of amplified PCR products was carried out by using formamide (99% pure). PCR products (8.0 µL) was added to 2.0 µL of formamide and tubes were then placed in thermocycler at 95° for 8.0 minutes. After the denaturation, these products were immediately shifted to ice to provide heat shock. About 5.0 µL of loading dye was then added into each tube of denatured product, and after mixing the whole mixture was loaded into the wells. The electrodes were connected to power supply and electrophoresis was carried out at 120 volts for 180 minutes.

Table 4.3: Composition of 6% Poly acrylamide gel

S.no.	Reagents	Amount	Final volume
1.	30% acryl-bisacrylamide solution	10 mL	50 µL
2.	10X-TBE	5 mL	
3.	10% ammonium per sulphate	350 µL	
4.	TEMED	25 µL	
5.	Distilled water (to adjust the final volume up to 50 mL)	34.6 mL	

Table 4.4: Composition of 8% Poly acrylamide gel

S.no.	**Reagents**	**Amount**	**Final volume**
1.	30% Acryl-Bisacrylamide solution	13.5mL	50 mL
2.	10X-TBE	5mL	
3.	10% Ammonium per sulphate	350µl	
4.	TEMED	25µl	
5	Distilled water (to adjust total volume up to 50mL)	31.1mL	

4.2.5.6-Staining and Visualization of Poly Acrylamide Gel Electrophoresis

After 180 minutes, the gel polyacrylamide gels sandwiched between the glass plates were removed carefully and shifted to the ethidium bromide staining solution (10mg/mL). These gels were allowed to stain for about 15-20 seconds and visualized under the UV-light in SYNGENE Gel documentation system. The variations in the banding patterns were analysed and samples with varied banding patterns were further selected for Sanger sequencing.

4.2.5.7- Purification of Amplified Products for Sequencing

The selected PCR products that showed varied banding pattern on PAGE were then re-amplified at already optimized conditions and subjected to purification before sequencing. For purification of PCR products, GeneJet PCR Purification kit (Thermo Fisher) was used. Equal amount of binding buffer with that of PCR products were added in the tube and colour of the mixture was assessed. The yellow colour indicated that the binding of buffer with the products while orange or purple colour was indication of no binding. About 10 µL of sodium acetate (3M) was added and at pH 5.2 colour was observed. Isopropanol was then added to the mixture based on the band size of the product. For band size > 500bp, 100% Isopropanol was added with 1:2 volume of the mixture. After proper mixing, the solution was shifted to purification column followed by 40-50 sec centrifugation. After centrifugation, the supernatant was discarded and the columns were washed with washing buffer, and centrifuged for 45-50 sec. The obtained supernatant was then again discarded, and centrifugation was again repeated to remove the residual buffer. The column was then placed in the sterile 1.5mL Eppendorf tubes and 40 µL of the elution buffer was added to the column membrane. The column containing Eppendorf tubes were then centrifuged for 50 sec. The column was then discarded, and the purified PCR products were sent for Sanger sequencing.

4.2.5.8- Sequencing of Selected Variants

For Sanger sequencing, 8 µL of purified PCR products and 4 µL of forward or reverse primer was added into barcoded tubes. These tubes were properly sealed and placed in the upright position in the box. After proper wrapping, these tubes were stored at -20°C or kept in ice overnight. For the

sequencing purpose, the purified samples were then forwarded for sequencing through Molecular Biology Products Inc.

4.2.5.9- Detection of Genetic Alterations in *TLR-2* and *TLR-4* Gene, their mRNA, and miRNA Regulation

The sequencing results were analysed using various bioinformatics tools. Bio Edit (version 7.2) software was used for the assessment of sequencing electropherograms and for the alignment of sequencing file with the reference gene for the analysis of possible variations. The detected variations were then further confirmed via mutational taster, NCBI, Ensemble database and Variant effect predictor (VEP). All the identified variants in *TLR-2* and *TLR-4* gene were predicted for any change in miRNA binding pattern. For this purpose, information and sequences of all the known miRNA previously reported in *TLR-2* and *TLR-4* genes were retrieved after accessing different databases 1) miRWalk (Sticht *et al.*, 2018) [Appendix XIV and XV], 2) miRDB(Yuhao Chen & Wang, 2020) [Appendix XVI and XVII], 3) miRTarBase (Hsu *et al.*, 2011)[Appendix XVIII and XIX]. The binding pattern was predicted with the database; miRBase(Griffiths-Jones *et al*., 2006)on site variation and also possible relation with regulation of genes of interest was assessed. This was a two-step process: (i) prediction of miRNA binding pattern with the reference sequence of *TLR-2* and *TLR-4* gene and (ii) alignment of variant possessing sequence of *TLR-2* and *TLR-4* gene with miRNAs. Also, *in silico* prediction was done on mRNA structure and change in Gibbs free energy. For this Visual Gene Developer software (version 1.9) was used to analyse any change in mRNA structure, Gibbs free energy that could ultimately result in altered interaction of these receptors in their intrinsic position was determined.

4.2.6- Statistical Data Analysis

For statistical analysis, Microsoft Excel and SPSS 21.0 were used. Odd ratios were calculated with 95% confidence interval. Allelic frequencies were calculated by gene counting method and the genotype frequencies by comparison among obese vs. controls and pre-obese vs. control using Chi-square test. *p* value less than 0.05 were considered statistically significant.

4.3-RESULTS

4.3.1-Anthropometric, Socio-Demographic, Dietary, Fluid Intake and Lifestyle of Study Subjects

Described previously in chapter 3 (section 3.3).

4.3.2- Detection of Polymorphisms in *TLR-2* and *TLR-4* Genes

The polymorphisms in both genes were checked after sequencing analysis. A total of four variants in *TLR-2* gene (reference: ENSG00000137462) were detected among obese individuals. These polymorphisms are identified for the first time among obese study participants in this case control study on obesity (Table 4.5). Among these identified variations, three were previously reported SNPs [153688371 T >C, 153702295 T >C, and 153703504 T >C] in other inflammatory conditions. While detected variant 153705074 C >A is novel variant and was identified for first time in obese individuals only. For the analysis of these variants, online available tools VEP and mutation taster were used (Table 4.5). All of the variants were synonymous-silent mutations in *TLR-2* gene. Among four identified variations, first variant 153688371 T >C was located in exon 3 located towards the 5`UTR region of the gene and is a protein coding region of the gene. The remaining three variants were detected in exon 4 of the *TLR-2* gene located towards the 3`UTR region of the gene. This region encodes various domains of the TLR-2 which are involved in binding, recognition, activation and signalling of TLR pathway. Among these three variations of exon 4, 153702295 T >C variation was located at coding region of signal peptide located in the extracellular domain. The second variant of exon 4 (153703504 T >C) was present in LRR of the extracellular domain. The third variant of exon 4 (153705074 C >A) was present in coding region of cytoplasmic domain.

In order to analyse the association of these detected variants with obesity, their genotypic and allelic frequency distribution were calculated (Table 4.6 and Appendix XX). The variant 153688371 T >C posed risk for obesity in comparison to the control group (Table 4.7), when taking TT genotype as reference, the odds ratio was 4.5 (3.25-6.49) for heterozygous TC and 5.5 (2.78-11.06) for homozygous CC genotype. It was observed that there was significant difference between the allele frequencies in individuals with obesity and controls showing strong association of C

allele with obesity (T vs. C: OR = 4.5; 95% CI= 2.87-7.04, *p* value 0.00001). The comparison of allelic frequency distribution and genotypes of variant 153702295 T >C with that of controls, revealed that the homozygous recessive genotype was more prevalent among the obese compared to controls. The odd ratios of heterozygous genotype CT was 0.6 (0.35-1.29) with *p* value of 0.4 and for homozygous CC odd ratios was 5.1 (3.00-8.86) with *p* value of 0.0001, while taking TT as reference.

The assessment of other variant 153703504 T >C as risk for obesity also had significant differences between the obese and control groups (Table 4.7). These results found dominant relation of homozygous CC [OR = 4.8 (2.57-9.18)] genotype for susceptibility to obesity as compared to heterozygous genotype CT [OR = 3.0 (1.25-7.41)] taking TT as reference genotype. In this variant, mutant allele C [OR= 4.3(2.90-6.52)3.57 (2.14-5.96), *p* value 0.00001] showed significant association with the obesity.

Risk of variant 153705074 C >A was also positively associated with obesity susceptibility (Table 4.7). These genotypic and allelic frequency analysis has showed an association between the variant 153705074 C >A and obesity. The odd of getting obesity due to homozygous AA [OR= 4.1 (1.99-8.54)] were higher than that of heterozygous CA [OR=3.6 (1.86-7.01)]. The allele A had OR=3.8 (2.31-6.37) and was posing higher risk, when CC genotype was taken as reference in this case.

An analysis for association of these variants with pre-obesity genotypic and allelic frequencies with the control group was done. Among the four identified variants in *TLR-2*, the variants 153688371T>C, 153702295 T >C and 153705074 C >A did not show any association with the pre-obesity. The only variant 153703504 T >C showed significant association with pre-obesity when comparing its genotypic and allelic frequency with controls. Taking TT as reference genotype, homozygous CC showed dominant relation [OR=4.0 (1.79-9.11) *p*=0.0003], while the heterozygous CT did not show any association. Significant association was found due to mutant allele C [OR=3.5 (2.06-5.95), *p*= < 0.00001] with pre-obesity.

The variants identified in various regions of the *TLR-2* gene were assessed for miRNAs binding, to predict binding pattern alteration in predicted mutant transcripts due presence of genetic change, both reference and variant sequence of the gene were analysed against miRNA reported in

miRBase. The only variant 153688371 T >C showed creation of new binding site in exon 3 for miRNA hsa-miR-4523 (ACAGCCGAGGCCCUCUCGGUC). The miRWalk, miRDB and miRTarBase databases for miRNAs and their respective target mRNA, did not have this miRNA documented for its ability to bind with *TLR-2* mRNA. Furthermore, *in silico* analysis of the variants 153688371 T >C and 153703504 T >C showed change in mRNA secondary structure and Gibbs free energy. While in variants 153702295 T >C and 153705074 C >A, change was observed only in Gibbs free energy. The predicted effects of these variants through *in silico* analysis are presented in Table 4.8.

Using reference sequence ENSG00000136869 of *TLR-4* gene, seven variants were detected in various coding and non-coding regions of the gene. All of these variants were identified for the first time in case of obesity in current case control study. The identified variants 117707870 G>A, 117708080 A>G, 117708780 G>A, 117713024 A>G, and 117715853 G>C were already reported in other various inflammatory conditions. The variants 117708777 C>G and 117715449 T>A were novel and are reported for the first time in this study on obesity. All of these identified mutations were synonymous-silent mutations except for the one 117713024 A>G which is non-synonymous missense mutation. The location of these variants 117707870 G>A and 117708080 A>G was in exon 2 which encodes the extracellular domain. The variants 117708777 C>G and 117708780 G>A also lie in the exon 3 which encode the lower end of polypeptide chain of extracellular domain. The variant 117713024 A>G lie in exon 4 coding for disulphide bond, 117715449 T>A and 117715853 G>C were also located in the 3`UTR region of exon 4. All of these mutations were analysed by online tools mutation taster and VEP (Table 4.9).

The identified variants in *TLR-4* gene were checked for their association with the obesity, for this purpose genotypic and allelic frequencies were calculated (Table 4.9 and Appendix XXI). The comparison of frequency distribution of alleles and genotypes in variants was done with controls, the recessive homozygous genotype was more prevalent in obese subjects. In the variant 117707870 G >A, odd ratios for heterozygous GA genotype was 0.9 (0.48 - 1.92) with *p*-value of 0.9 and for homozygous AA genotypes OR was 2.3 (1.07-5.09) with *p*-value 0.02, while taking GG genotype as reference (Table 4.10-4.11) (Appendix XXI-XXII).

The variant 117708080A>G posed risk for obesity, when taking reference AA genotype, odds ratio was 1.89 (0.91-3.93) for heterozygous AG and 1.63 (0.87-3.05) for homozygous GG genotype. Suggesting a possible dominant effect of this variant (117708080A>G) as mutant heterozygous AG genotype in obesity. There was significant difference between the allele frequencies in individuals with obesity and controls showing strong association of A allele with obesity (T vs. C: OR = 1.65; 95% CI= 1.07-2.53, *p* value 0.008).

The variant 117708777C>G, after analysis of genotypic and allelic frequencies showed that its association with obesity as a significant risk. The OR for heterozygous CG was found to be 10.21 (4.21-24.78) with *p*-value= <.00001, hence was found to pose dominant effect in comparison with the homozygous GG OR= 3.45 (1.68-7.10), *p*-value= 0.0003. There was significant difference of allele frequencies with OR 4.44 (2.87-6.88), *p*-value= <0.00001 in individuals with obesity in comparison with the controls showing strong association of G allele with obesity.

The variant 117708780G>A showed the dominant role of the heterozygous GA with the risk of obesity having odd ratios of 2.90 (1.65-5.10; *p*=.0001) in comparison with the homozygous AA genotype [OR= 0.95 (0.56-1.62)]. This allele also showed risk for obesity [OR=1.26 (0.93-1.73)]. In comparing the genotypic and allelic frequencies of variant 117713024A>G, keeping AA genotype as a reference, increased risk of obesity was observed due to homozygous GG genotype. While the heterozygous AG genotype was also associated with the obesity risk (OR=3.42, 1.95-5.98) in comparison of obese with the controls. The allelic frequency had OR= 4.00; 95%CI= 2.65-6.05 with *p*=0.00001 which had enhanced susceptibility to obesity with the mutant allele G.

In case of 117715449T>A, keeping TT as reference, the heterozygous TA was positively associated with the obesity OR= 1.92(1.08-3.42 *p*=0.02). While it's allelic frequency was not found to be associated with the risk of obesity. The genotypic and allelic frequencies of obese of variant 117715853G>C, an association with the obesity due to the homozygous CC genotype with OR= 2.00(1.04-3.84), *p*=0.03 was seen while keeping GG genotype in reference.

In case of pre-obese group, genotypic and allelic frequencies were computed and variant 117715449 T>A was not associated with the pre-obesity. The four variants 117707870 G>A, 117708080 A>G, 117713024 A>G and 117715853 G>C showed association with only

homozygous mutant genotypes. The variant 117707870 G>A showed that the homozygous AA genotype had OR= 11.5 (5.31-25.17) and association with the pre-obesity. Risk of mutant allele was observed in this case to be 6.5 at 95% CI (4.22-10.3; *p*= <0.00001). The comparison of genotypic and phenotypic frequencies of pre-obese group with control group, by keeping AA genotype as reference, variant 117708080 A>G also showed risk for homozygous GG genotype [OR= 2.24(1.03-4.86)] and mutant allele was also associated with OR=1.72; 95% CI= 1.02-2.91, *p* =0.013.

For the variant 117713024 A>G, homozygous GG genotype posed risk as OR was 5.49 (1.98-15.21) and the allelic frequency was associated with obesity (OR= 2.26; 95%CI=1.25-4.11, *p*=0.005). While in the variant 117715853 G>C, association with homozygous genotype was seen [OR= 3.47(1.57-7.63), *p*= 0.001] and the allelic frequency also showed risk (OR= 2.27; 95% CI= 1.58-3.85, *p*= 0.00004). The other two variants 117708777 C>G and 117708780 G>A had no association of mutant allele frequency with the risk for obesity (Table 4.11).

The variants detected in *TLR-4* gene were also assessed for miRNA binding pattern using *in silico* prediction tools. For this purpose, miRNA binding pattern of wild type sequence was compared with the mutated sequence in the miRBase. The exon 2 variant 117707870 G>A showed loss of binding site for the miRNAs hsa-miR-576-5p (AAAGACGUGGAGAAAUUAGAAU) and hsa-miR-122-3p (UAUUUAGUGUGAUAAUGG), which were previously involved in regulation of mRNA of the wild type sequence. In second variant of exon 2 (117708080 A>G), there was creation of new binding site for the hsa-miR-32-3p (AAUUUAGUGUGUGUGAUAUU). The variant 117708777 C>G did not show any change in miRNA binding pattern. The variant 117708780 G>A in the exon 3 showed loss of binding site for the miRNA, hsa-miR-215-3p (CUGUCAUUUCUUUA), and displacement of miRNA, hsa-miR-130b-3p (UGCCCUUUCAUCAUUGCACUG) in comparison to the wild type sequence.

The variant found in the exon 4 but did not affect the miRNA binding. The next variant 117715449 T>A at 3`UTR region had loss of binding site for miRNA; hsa-miR-1246(CCUGCUCCAAAAAUCCAU) within this mutated sequence. The exon 4 with change in 3`UTR (117715853 G>C) did not show any change in miRNA binding pattern. The novel binding

of sites for miRNA was checked within various databases (miRWalk, miRDB, and miRTarBase), all of these miRNAs predicted to bind to variants *TLR-4* were novel binding sites.

Moreover, *in silico* prediction of mRNA structure of these detected variants was performed by using Visual gene developer 1.9- Build 785. The variants 2, 3 (117708080 A>G, 117708780 G>A) and 7(117715449 T>A) showed change in mRNA structure with decreased Gibbs free energy which in turn was increasing stability of the mRNA structure. The variant 1 (117707870 G>A) and 5 (117715853 G>C) showed change in mRNA structure but increase in Gibbs free energy which might ultimately effect decrease in structure stability. The variant 4 did not show any change in Gibbs free energy and the structure. While the variant 6 (117708777 C>G) was found to have decrease in stability with increase in energy without affecting the structure of mRNA (Table 4.12).

Table 4.5: Detected variants in *TLR-2* gene among obese subjects and their predicted consequence using Variant Effect Predictor

Primer code	**Physical location of SNP**	**SNP site**	**Type of alteration**	**Most severe consequence by VEP**	**SNP status in current study**
Exon 3	Chr4:153688371 153688371 T >C	Start of Exon 3	Single base substitution	Non-coding transcript exon variant	Novel
Exon 4/ Exon 4B	Chr4:153702295 153702295 T >C	End of signal peptide	Single base substitution	5` UTR region	Novel
Exon 4/ Exon 4D	Chr4:153703504 153703504 T >C	LRR5 region	Single base substitution	Synonymous variant Coding sequence variant	Novel
Exon 4/Exon 4F	Chr4:153705074 153705074 C >A	Cytoplasmic domain	Single base substitution	Coding sequence variant	Novel*
*Novel= Not reported elsewhere Novel= reported in inflammatory diseases					

Table 4.6: Genotype and allele distribution of *TLR-2* gene polymorphisms in obese and healthy controls

Polymorphisms	**Genotype frequency**			**Allele frequency**		
	Obese		**Controls**	**Obese**		**Controls**
Chr4:153688371T>C	**TT**[a]	168(68.57%)	324(90.7%)			
	TC	24(9.79%)	14(3.9%)	**T**[a]	360(0.73%)	662(0.92)
	CC[b]	53(21.63%)	19(5.3%)	**C**[b]	130(0.26%)	52(0.07)
Chr4:153702295T>C	**TT**[a]	172(70.2%)	301(84.3%)			
	TC	14(5.7%)	36(10.0%)	**T**[a]	358(0.73%)	638(0.89)
	CC[b]	59(24.0%)	20(5.6%)	**C**[b]	132(0.26%)	76(0.10)
Chr4:153703504T>C	**TT**[a]	192(78.3%)	335(93.8%)			
	TC	14(5.7%)	8(2.2%)	**T**[a]	398(0.81%)	678(0.94)
	CC[b]	39(15.9%)	14(3.9%)	**C**[b]	92(0.18%)	36(0.05)
Chr4:153705074C>A	**CC**[a]	190(77.5%)	332(92.9%)			
	CA	29(11.8%)	14(3.9%)	**C**[a]	409(0.83%)	678(0.94)
	AA[b]	26(10.6%)	11(3.0%)	**A**[b]	81(0.16%)	36(0.05)

[a]Reference genotype/allele

[b]Mutant genotype/allele

Table 4.7: Association of genotypic and allele frequencies of *TLR-2* gene polymorphisms with obesity

Polymorphisms	Obese vs. controls(Genotypes)				Pre-obese vs. controls(Genotypes)			
	Odds ratio	**CI 95%**		***p* value**	**Odds ratio**	**CI 95%**		***p* value**
		L	**U**			**L**	**U**	
Chr4:153688371T>C								
TTvs.TC	4.5	3.25-6.49		0.00009*	1.7	0.64-4.65		0 .26
TTvs.CC	5.3	3.08-9.38		< 0.00001*	0.1	0.006-1.73		-
TTvs.CC+TC	4.5	2.87-7.04		< 0.00001*	0.7	0.29-1.81		0.50
Tvs.C	4.7	3.07-7.24		<0.001*	0.4	0.19-1.08		0.07
Chr4:153702295T>C								
TTvs.TC	0.6	0.35-1.29		0.24	0.04	0.002-0.78		-
TTvs.CC	5.1	3.00-8.86		<0.00001*	0.08	0.005-1.42		-
TTvs.CC+TC	2.2	1.53-3.38		<0.00003*	0.03	0.001-0.50		-
Tvs.C	3.0	2.26-4.22		<0.00001*	0.03	0.002-0.56		-
Chr4:153703504T>C								
TTvs.TC	3.0	1.25-7.41		0.009*	1.7	0.45-6.83		0.40
TTvs.CC	4.8	2.57-9.18		< 0.00001*	4.0	1.79-9.11		0.0003*
TTvs.CC+TC	4.2	2.47-7.12		<0.00001*	3.2	1.59-6.50		0.0006*
Tvs.C	4.3	2.90-6.52		<0.00001*	3.5	2.06-5.95		< 0.00001*
Chr4:153705074C>A								
CCvs.CA	3.6	1.86-7.01		0.00005*	1.2	0.38-3.79		0.6
CCvs.AA	4.1	1.99-8.54		0.00004*	1.5	0.48-4.99		0.4
CCvs.AA+CA	3.8	2.31-6.37		<0.00001*	1.3	0.59-3.13		0.4
Cvs.A	3.7	2.475.62		<0.00001*	1.4	0.71-2.77		0.31

*P-value<0.05 considered highly significant

L=lower limit, U=upper limit

Table 4.8: *In silico* analysis of mRNA structure change and miRNA binding pattern in identified variants of *TLR-2* among obese subjects

Physical location of SNP	**Splice site effect**	**Poly A signal**	**miRNA alteration**	**mRNA structure change**	**Gibbs free energy (kcal/mol)**		**Effecting mRNA structure**
					[reference]	**[variants]**	
Chr4:153688371 153688371 T >C	**Change**	Change	hsa-miR-4523 binding site created	Change	-13.4	-13.2	Increased stability
Chr4:153702295 153702295 T >C	**Change**	Change	No effect	No effect	-10.2	-8.2	Increased stability
Chr4:153703504 153703504 T >C	**Change**	Change	No effect	Change	-8.0	-9.1	Decreased stability
Chr4:153705074 153705074 C >A	**Change**	Change	No effect	No effect	-15.3	-15.9	Decreased stability

Table 4.9: Detected variant in *TLR-4* gene among obese subjects and their predicted consequence using Variant Effect Predictor

Primer code	Physical location of SNP	SNP site	Type of alteration	Most severe consequence by VEP	SNP status in current study
Exon 2	**Chr9:117707870 117707870 G>A**	Polypeptide chain in extracellular domain	Single base substitution	Intronic/ protein coding	Novel
Exon 2	**Chr9:117708080 117708080 A>G**	Polypeptide chain in extracellular domain	Single base substitution	Intronic/protein coding	Novel
Exon 3	**Chr9:117708777 117708777 C>G**	Polypeptide chain in extracellular domain	Single base substitution	Intronic/ protein coding	Novel*
Exon 3	**Chr9:117708780 117708780 G>A**	Polypeptide chain in extracellular domain	Single base substitution	Intronic/protein coding	Novel
Exon 4/Exon 4 P-2	**Chr9:117713024 117713024 A>G**	Disulphide bond	Single base substitution	Missense variant	Novel
Exon 4/Exon 4 P-7	**Chr9:117715449 117715449 T>A**	3` UTR	Single base substitution	3`UTR	Novel*
Exon 4/Exon 4 P-7	**Chr9:117715853 117715853 G>C**	3` UTR	Single base substitution	3`UTR	Novel
*Novel= Not reported elsewhere Novel= reported in other inflammatory diseases					

Table 4.10: Genotype and allele distribution of *TLR-4* gene polymorphisms in obese, and healthy controls

Polymorphisms	**Genotype frequency**			**Allele frequency**		
	Obese		**Controls**	**Obese**		**Controls**
Chr9:117707870 117707870 G>A	**GG[a]**	214(87.34%)	324(90.75%)			
	GA	14(5.71%)	22(6.162%)	**G[a]**	442(0.90)	670(0.93)
	AA[b]	17(6.93%)	11(3.08%)	**A[b]**	48(0.09)	44(0.06)
Chr9:117708080 117708080 A>G	**AA[a]**	206 (84.08%)	322(90.19%)			
	AG	17(6.93%)	14(3.92%)	**A[a]**	429(0.87)	658(0.92)
	GG[b]	22 (8.97%)	21(5.88%)	**G[b]**	61(0.12)	56(0.07)
Chr9:117708777 117708777 C>G	**CC[a]**	188(76.73%)	339(94.95%)			
	CG	34(13.87%)	6(1.68%)	**C[a]**	410(0.83)	684(0.95)
	GG[b]	23(9.38%)	12(3.36)	**G[b]**	80(0.16)	30(0.04)
Chr9:117708780 117708780 G>A	**GG[a]**	182(74.28%)	294(82.35%)			
	GA	38(15.51%)	21(5.88%)	**G[a]**	402(0.82)	609(0.85)
	AA[b]	25(10.20%)	42(11.76%)	**A[b]**	88(0.17)	105(0.14)
Chr9:117713024 117713024 A>G	**AA[a]**	183(74.69%)	329(92.15%)			
	AG	40(16.32%)	21(5.88%)	**A[a]**	406(0.82)	679(0.95)
	GG[b]	22(8.97%)	7(1.96%)	**G[b]**	84(0.17)	35(0.04)
Chr9:117715449 117715449 T>A	**TT[a]**	194(79.18%)	287(80.39%)			
	TA	30(12.24%)	23(6.44%)	**T[a]**	418(0.85)	597(0.83)
	AA[b]	21(8.57%)	47(13.16%)	**A[b]**	72(0.14)	117(0.16)
Chr9:117715853 117715853 G>C	**GG[a]**	206(84.08%)	305(85.43%)			
	GC	16(6.53%)	35(9.80%)	**G[a]**	428(0.87)	645(0.90)
	CC[b]	23(9.38%)	17(4.76%)	**C[b]**	62(0.12)	69(0.09)
[a]Reference genotype/allele						
[b]Mutant genotype/allele						

Table 4.11: Association of Genotypic and Allele Frequencies of *TLR-4* gene polymorphisms with obesity

Obese vs controls(Genotypes)				
Polymorphisms	**Odds ratio**	**CI 95%**		***p* value**
		L	**U**	
Chr9:117707870G>A				
GGvs.GA	0.9	0.48 - 1.92		0.91
GGvs.AA	2.3	1.07-5.09		0.02*
GGvs.AA+GA	1.4	0.84-2.39		< 0.00001*
Gvs.A	1.65	1.07-2.53		0.01*
Chr9:117708080A>G				
AAvs.AG	1.89	0.91-3.93		0.08
AAvs.GG	1.63	0.87-3.05		0.11
AAvs.GG+AG	1.74	1.06-2.83		0.02*
Avs.G	1.67	1.13-2.44		0.008*
Chr9:117708777C>G				
CCvs.CG	10.21	4.21-24.78		< 0.00001*
CCvs.GG	3.45	1.68-7.10		0.0003*
CCvs.GG+CG	5.71	3.26-9.98		< 0.00001*
Cvs.G	4.44	2.87-6.88		< 0.00001*
Chr9:117708780G>A				
GGvs.GA	2.90	1.65-5.10		0.0001*
GGvs.AA	1.20	0.56-1.62		0.88
GGvs.AA+GA	1.61	1.08-2.39		0.016*
Gvs.A	1.26	0.93-1.73		0.13
Chr9:117713024A>G				
AAvs.AG	3.42	1.95-5.98		0.00001*
AAvs.GG	5.65	2.36- 13.48		0.00001*

AAvs.GG+AG	3.98	2.45-6.44	0.00001*
Avs.G	4.00	2.65-6.05	0.00001*
Chr9:117715449T>A			
TTvs.TA	1.92	1.08-3.42	0.02*
TTvs.AA	0.66	0.38-1.14	2.23
TTvs.AA+TA	1.07	0.71-1.61	0.13
Tvs.A	0.87	0.63-1.20	0.62
Chr9:117715853G>C			
GGvs.GC	0.67	0.36-1.25	1.55
GGvs.CC	2.00	1.04-3.84	0.03*
GGvs.CC+GC	0.90	0.57-1.41	0.64
Gvs.C	1.35	0.94-1.94	0.10
*P-value<0.05 considered highly significant L=lower limit, U=upper limit			

Table 4.12: *In silico* analysis of mRNA structure change and miRNA binding pattern in identified variants of *TLR-4* among obese subjects

Physical location of SNP	**Splice site effect**	**Protein feature might affected**	**miRNA alteration**	**mRNA structure change**	**Gibbs free energy (kcal/mol)**		**Effecting mRNA structure**
					[reference]	**[variant]**	
Chr9:117707870 117707870 G>A	No change	No affect	hsa-miR-576-5p and hsa-miR-122-3p binding site lost	Change	-9.70	-11.70	Decreased stability
Chr9:117708080 117708080 A>G	Changed	Might lost function downstream of splice site	hsa-miR-32-3p binding site created	Change	-9.70	-8.90	Increased stability
Chr9:117708777 117708777 C>G	No change	Changed	No affect	Change	-12.90	-10.40	Increased stability
Chr9:117708780 117708780 G>A	Changed	Might lost function downstream of splice site	hsa-miR-215-3p binding site lost hsa-miR-130b-3p binding dislocated	No effect	-9.80	-9.80	No change
Chr9:117713024 117713024 A>G	No change	Amino acid change	No affect	Change	-7.00	-7.30	Decreased stability
Chr9:117715449 117715449 T>A	Changed	No affect	hsa-miR-1246 binding site lost	No affect	-11.30	-13.40	Decreased stability
Chr9:117715853	Changed	No affect	No affect	Change	-13.10	-12.90	Increased stability

117715853 G>C							

4.4- DISCUSION:

Obesity is categorized as meta-inflammation due to the involvement of multiple organs, cells and variety of immune molecules under the action of innate as well as adaptive immune modulators. The steady state of inflammation is maintained by innate immune receptors like TLRs, which play an important role due to their altered expression (Jialal *et al*., 2014). It has been found that *TLR-2* and *TLR-4* genes are highly expressed in peripheral blood mononuclear cells during inflammatory illnesses including obesity (Ahmad *et al*., 2012). In previous studies, high *TLR*-2 and *TLR-4* mRNA expression have been reported in obesity (Hardy *et al*., 2013). *TLR-2* and *TLR-4* gene are extensively reported for their polymorphic nature having number of variations in different regions across the gene length. An extensive literature is reported on the associated of these TLRs (*TLR-2* and *TLR-4*) with increased risk of inflammatory disorders (Zaki *et al*., 2018), such as inflammatory bowel disease (Török *et al*., 2017), *H. pylori* infection (Mirkamandar *et al*., 2018) pulmonary tuberculosis (Soeroto *et al*., 2018), and in several cancers (Gao *et al*., 2019). However, no study has been reported on association of obesity with *TLR-2* or *TLR-4* and its genetic variations. This case control study is first of its kind, in which association and identification of mutations or SNPs across *TLR-2* and *TLR-4* gene sequence in obese patients is carried out.

Detection of variations in this mutational analysis have revealed 4 variations across the length of *TLR-2* gene in the individuals. These variations were analysed using mutation taster and VEP for affects such as splice site and poly-A signal. The variant 153688371 T >C was located in exon 3 while the other three 153702295 T >C; 153703504 T >C; 153705074 C >A were identified in the exon 4. The first variant (153688371 T >C) is in the sequence of exon 3, the protein coding region. This is a novel mutation and found in only individuals with obesity, however, it has been previously detected in many inflammatory disorders as a risk for such diseases (Martín *et al*., 2019). There was a strong association of the mutant C allele of variant 153688371 T >C with obesity. This mutant allele C also showed association in both heterozygous and homozygous condition within obese individuals, therefore it could be inferred that it might be potentially

involved in increasing the susceptibility to obesity. While this mutant allele C did not show any association with the pre-obesity condition.

The rest of the 3 variations were located in exon 4, among these first two SNPs present in extracellular domain encoding part of TLR 2 protein while last one was present in region encoding the cytoplasmic domain of TLR-2. According to VEP database results, first variation 153702295 T >C in exon 4 lies in the end region of signal peptide , such change can disrupt the signal sequence of TLR protein and might interrupt the normal insertion process of this receptor by the help of proteolytic enzymes, on various cell surfaces (Henrick *et al*., 2016). The mutant C allele in this variant 153702295 T >C; showed significant association with obesity and homozygous CC genotype was found to be a dominant risk factor for obesity. While in case of pre-obese group the variation was absent.

The second variation (153703504 T >C) lied in the Leucine rich repeats (LRR5) of extracellular domain, these LRR5 makes the vital central part of the ligand binding motif that plays fundamental role in complex formation with various endogenous and exogenous antigens (Matsushima *et al*., 2007). In the current study, the heterozygous CT genotype of 153703504 T >C was more prevalent and C allele was significantly associated with obesity risk. In case of *TLR-2* gene none of the identified variants were significantly associated with pre-obesity except for the variant 153703504T>C that showed high prevalence of homozygous CC genotype and mutant C allele was significant risk for pre-obesity. In previous studies, these functional polymorphisms of TLRs were seen as strongly linked to NFκB-mediated inflammatory response in cancer and glucose metabolism, it can be assumed that such variation in *TLR-2* gene might have possible functional changes in TLR-2 dependent inflammation in obesity (Bank *et al*., 2014; Torres *et al*., 2019; Neamatallah *et al*., 2020).

The third variation 153705074 C >A in exon 4 of TLR-2 was predicted to be located in N-terminal region of cytoplasmic TIR domain, this intracellular domain plays vital role in downstream signal for activation and interaction with adaptors plus kinases (Botos *et al*., 2011). This is a novel variation and is reported for the first time in this current study. According to a structural and functional studies on *TLR-2* gene, any variation in TIR domain have potential to alter *TLR*

signalling and can impair cytokine production (Nagpal *et al*., 2009). After analysis, 153705074 C >A variant was observed to have dominant role as homozygous AA genotype [OR= 4.16 (1.69-10.2) where allele A OR=3.73 (2.2-6.2)] was a significant risk for obesity. This novel variation was not found in pre-obese group. Based on genotypic and allelic frequencies observed in *TLR-2* gene, it can be inferred that that these variations in the TLR 2 gene might have role in increasing susceptibility to obesity due to changes in various domain of the toll like receptor 2.

The second part of this work was *in silico* prediction of miRNA binding and mRNA structure change in the identified SNPs in *TLR-2* gene. To decipher possible role of these novel variants in obesity, impact of these genetic changes was determined by different bioinformatics tools. In case of *TLR-2* germ line variants, after analysis on the miRBase a miRNA (hsa-miR-4523) binding with 153688371 T >C variant of exon-3 was predicted, which was not observed in case of normal transcript. This predicted creation of novel binding site for hsa-miR-4523 on mRNA of the TLR 2, suggest a possible change in regulation of mRNAs by miRNA in obesity. Furthermore, this miRNA (hsa-miR-4523) which was previously reported in various cancers and inflammation by binding with chr17: 29390662-29390730 suggest that it might have role in inflammation too. While no database reported its binding with *TLR-2* which is located on Chromosome number 4 (Jones *et al*., 2006). The Kyoto Encyclopaedia of Genes and Genomes (KEGG) studies have revealed a significant association of hsa-miR-4523 with NF-kappa B signalling pathway too (Chen *et al*., 2017;Donato *et al*., 2018). The presence of novel binding site for hsa-miR-4523 in *TLR-2* mRNA might lead to its deregulation and expressional change of surface protein TLR-2 among subjects with obesity.

According to Visual gene developer results, these SNPs in *TLR-2* gene changed the predicted mRNA structures. The variants 153688371 T >C and 153703504 T >C showed change in mRNA structure, which could ultimately have effects on the interaction with translation machinery. Overall change in Gibbs free energy among the variants was found and was increased in 153688371 T >C and 153702295 T >C. While the variant 153703504 T >C and 153705074 C >A were found to have decreased structural stability of the mRNA secondary structure. Such changes in nucleotides of led to change in mRNA secondary structure such change can alter expression of protein by either overexpression or low expression due to varied translation efficiency (Mauger *et*

al., 2019). It can be inferred from current findings that these SNPs can affect the stability and half-life of mRNA of *TLR-2* and receptor expression in plasma membrane too.

In case of *TLR-4* gene seven SNPs are detected after current mutational analysis across the length of *TLR-4* in various intronic and exonic regions of this gene. According to mutation taster and VEP findings, all these variants (117707870 G>A, 117708080 A>G, 117708777 C>G, 117708780 G>A, 117715449 T>A and 117715853 G>C) were point mutations in nature except for 117713024 A>G which was missense in nature. These variants were also further assessed if any change in splice site with annotation of protein sequence. Among these, two of the variants were present in the initial exon 2 of the gene, which encodes for the polypeptide chain and portion of disulphide bond. The next two were present in exon 3 of the gene that encodes for first LRRs in the extracellular domain. The other three were present in exon 4 of the *TLR-4* gene that encodes for the remaining extracellular domain region (hypervariable region and LRRs 2-18), the single helical transmembrane domain and the embedded cytoplasmic domain (Vaure & Liu, 2014).

The variant 117707870 G>A was located in exon 2 for extracellular membrane region's disulphide bond involved in the coiling was novel for obesity, it is previously detected as risk of other inflammatory disorder too(Velez *et al*., 2009;Gardener *et al*., 2011). Upon analysis in the current study, a strong association of the mutant genotype AA and allele A with obesity was found which indicate a possible involvement in increasing the risk to obesity.

Other variation 117708080 A>G of exon 2 was also affecting the disulphide bond region, which is reported from US population in the online database, but for this SNP no association was found with any disorders or complication until now. In the present study, the mutant allele G was significantly associated with obesity and pre-obese condition, posing risk for development of obesity. This disulphide bond is crucial in post-translational modification of protein native structure formation, this bond in flexible part determine the folding pattern of protein (Klink *et al*., 2000; Krupa *et al*., 2017; Hutchison *et al*., 2017). In current study the variant 117708080 A>G was predicted to affect the protein structure of the_TLR-4, as disulphide bond required for the proper protein folding and stability for *in vivo* functioning is altered.

The SNPs 117708777 C>G and 117708780 G>A were located in the exon 3 of *TLR-4*. The variant 117708777 C>G was novel and detected for first time in current study in obesity. This variant encodes amino acid sequence of proximal end of the extracellular domain having ligand recognition and binding motif of this receptor like LPS. As ligand recognition is highly sensitive and intricately precise, any SNP in it can lead to differential outcome with possibility of disease. As, this variant is found in case of obesity, the *TLR-4* dependent LPS or nutritional ligand from fats recognition could ultimately be alter and can give varied inflammatory responses. The mutant allele G of variant 117708777 C>G was significantly associated with obesity, however, was not associated with pre-obese condition.

The fourth variant 117708780 G>A was only associated in heterozygous form as risk of obesity, without showing association with the mutant allele. The fifth variant 117713024 A>G in exon 4, was second disulphide bond effecting SNP. This variant has been extensively reported in many infectious and inflammatory disorders while for obesity it is reported for the first time in this study (Kim *et al*., 2020; Huang *et al*., 2020; Gao *et al*., 2021;Reilly *et al*., 2021). This variant causes the change of amino acid from aspartic (GAU/GAC) to glycine (GGU/GGC) by single base substitution (A to G). Aspartic acid is a polar amino acid with an acidic side chain while the glycine is a nonpolar amino acid having single hydrogen atom in its side chain (Plante *et al*., 2020;Eaaswarkhanth *et al*., 2020). The difference in basic nature of these two amino acids might affect the biological function of TLR. Such variant might potentially affect obesity due to its change in motif of cleft. The variant 117713024 A>G as both homozygous and heterozygous genotypes is strongly associated to obesity. While only its homozygous genotype and allele G was associated with the pre-obesity posing lower risk of obesity as compared to obese group.

The next two variants of exon 4 are located in 3`UTR region, where the number sixth variant 117715449 T>A was predicted to be disease causing according to mutation taster results. This is another novel mutation of the current study. It seen to be strongly associated with the obesity without showing any association with the pre-obese group. Thus, making it potential pathogenic in case of obesity as the genotypic and allelic frequencies as well as the mutational analysis prediction.

The seventh variant 117715853 G>C was also in exon 4 in the 3`UTR region of the gene. It has been previously seen to be involved in pro-inflammatory conditions (Wu *et al*., 2020;Zhu *et al*., 2020;Nath *et al*., 2020 ;Li *et al*., 2021). The untranslated 3`UTR region are very crucial for the gene expression as various regulatory sequences are present at the 3`UTR region having the potential to affect the translation machinery (Kuersten & Goodwin, 2003; Huang & Teeling, 2017). Thus, it could be inferred from these results that such variants in the TLRs' 3′ untranslated region might affect gene expression by regulating the binding of mRNA. Based on genotypic and allelic frequencies observed in *TLR-4* gene in case of obesity suggest that these variants may have role in increasing susceptibility to obesity.

The *in silico* analysis was carried for miRNA binding alteration and mRNA structural changes due to the presence of these identified variants in *TLR-4* gene. These analyses are helpful in finding consequences of these variants on *TLR-4* and its possible role in TLR 4 induced obesity inflammation. According to miRBase results, variant 117707870 G>A of exon 2 there was loss of miRNA hsa-miR-576-5p and hsa-miR-122-3p binding which are known to bind with the wild type sequence of *TLR-4* gene. This miRNA; hsa-miR-576-5p has previously been associated with the lower expression of estrogen in adipose tissues (Koźniewski *et al*., 2022). While the expression of hsa-miR-122-3p was reported to be associated with increased pathological progression of bone necrosis by increased chronic inflammation (Hutchison *et al*., 2017). Thus, the loss of binding of these two miRNA to identify variant might be involved in the abnormal *TLR-4* expression and signalling in inflammation.

Due to change 117708080 A>G of exon 2, there is now an additional site created for the binding of miRNA hsa-miR-32-3p, which was previously reported to bind with in the chromosome 9 NC_000009.12. *TLR-4* is on chromosome 4, in similar manner miRNA hsa-miR-32-3p might have role in obesity as in progression of cancers, metabolic and cardiovascular disorders (Hong *et al*., 2020;Fadaka *et al*., 2020). This predicted binding of miRNA with *TLR-4* transcript maybe altering TLR expression. The variant of exon 3, 117708777 C>G does not show any change in miRNA binding pattern with reference wild type sequence.

The next variant of exon 3 117708780 G>A showed loss of binding of native miRNA; hsa-miR-215-3p and miRNA; hsa-miR-130b-3p. The hsa-miR-215-3p have been associated with the differential expressional and regulation responses under the influence of dietary lipids and gut metabolites reported in other studies (Casas-Agustench *et al*., 2015;Gerhauser, 2018). The missense variant 117713024 A>G of exon 4, had no change miRNA binding. The next variant 117715449 T>A in exon 4 was predicted to be diseases causing in the current study as loss of site for binding of miRNA: hsa-miR-1246. This miRNA has been associated with many inflammatory and infectious conditions, as it is reliable indicator it is used as diagnostic biomarker for these diseases (Casas-Agustench *et al*., 2015;Wang *et al*., 2022; Zhao *et al*., 2022).

According to Visual gene developer findings, different variants had change of mRNA structure and change in Gibbs free energy too. The variants 117707870 G>A, 117708080 A>G, 117708777 C>G, 117713024 A>G and 117715853 G>C had change in the mRNA structure. While two variants 117708780 G>A and 117715449 T>A were predicted with no change secondary mRNA structure. The change in structure of mRNA can affect the post transcription cellular machinery for the protein translation. It has been observed that synonymous mutations can affect the translation rates too (Robert & Pelletier, 2018). On the basis of Gibbs free energy change among the identified SNPs, variant 117708780 G>A did not show any Gibbs free energy change while remaining other variants had altered Gibbs free energy. In variant 117707870 G>A, 117713024 A>G and 117715449 T>A increase in overall Gibbs free energy was observed. While variant 117708080 A>G, 117708777 C>G and 117715853 G>C had lower Gibbs free energy in comparison with the reference sequence. The increase in Gibbs free energy has been associated to increase the entropy increasing it stability which could reduce mRNA expression. Those variants with increased energy make these mRNA structure unstable in comparison with the reference structure that could again affects the interaction with cell translation machinery.

A number of known and novel variants have been identified in this study across the length of both *TLR-2* and *TLR-4* genes. The variants in crucial part of the extracellular region; LRRs and in the disulphide bond region could interfere in binding of the receptors with their ligands. The one in cytoplasmic domain could affect signal transduction. While the others in 5`UTR and 3`UTR region could directly interfere with regulatory machinery of theses gene. All the identified variants of the

TLR-2 and *TLR-4* gene after *in silico* prediction point towards their pathogenic nature in case of obesity. All these variants were significantly associated with the obesity and could directly or indirectly change the obesity outcome. They may play role in gene regulation, translation and structural manipulations. Further expressional investigation is required for understanding the post transcriptional as well as post translational events of these variants. These mRNA and miRNA could further be explored for their potential as diagnostic biomarkers and therapeutic targets.

CHAPTER 5: STUDY OF CULTUREABLE GUT FUNGAL DIVERSITY AND ITS PATHOGENIC POTENTIAL IN OBESITY

5.1- INTRODUCTION

The pathogenic mechanism of obesity is difficult to understand completely due to involvement of multiple factors that play role in constructing complex metabolic system and sustaining a steady state of low grade inflammation (Mraz & Haluzik, 2014). Obesity is a state of inflammation, which involves various causative factors that creates chronic milieu leading towards complications and comorbidities. Obesity is affecting millions of people around the world and becoming prominent cause of complication with increasing mortality rate (Ataey *et al*., 2020). Gut microbiome studies has opened a new era of investigation that shows mutualistic relationship between the resident microbes and their metabolic products which shape host immune system and could be involved in inflammatory events. These studies indicate significant association between the dysbiosis and loss of indigenous organisms in various inflammatory diseases like cardiovascular, bowel syndrome including obesity (Hawrelak & Myers, 2004; Blaser & Falkow, 2009;Hand *et al*., 2016; Lynch & Pedersen, 2016). Beside bacteria, gastrointestinal tract (GIT) also harbour fungi which constitute less than 1% of overall gut flora and are essential part of the GIT which is still under explored (Scanlan & Marchesi, 200; Arumugam *et al*., 2011;Huffnagle & Noverr, 2013). Recently, field of mycology is flourishing with more advancement in it and hence making possible to understand role of fungi in different inflammatory complications including obesity (Mukherjee *et al*., 2015; Richard *et al*., 2015). The genus *Thermomyces* and *Saccharomyces* were found to be strongly associated with metabolic disturbance and weight gain in a study by Mims *et al*., (2021). Different kind of studies on animal model in which high fat diets were fed to mice have shown association of changing gut fungal diversity with obesity (Heisel *et al*., 2017). A study individuals reported that there was shift in mycobiota in obesity, this shift was increased yeast form in obese individuals while more filamentous fungi in healthy controls (Borges *et al*., 2018).

Among all fungi, *Candida* specie causes 96% of the infections in comparison to other fungi extending from mild cutaneous to life threatening systemic infections. It is known now that fungi colonize abundantly various body parts as a commensal including mucosal lining, oral cavity, vaginal tract and gastrointestinal tract (Hiippala *et al*., 2018; Henriques & Silva, 2021). The

presence of these commensals on the body especially in the gut play an important role in keeping homeostatic conditions by maintaining gut permeability, endotoxemia and gut lining evasion (Hiippala *et al*., 2018; Henriques & Silva, 2021). A shift in microbial diversity causes the GIT dysbiosis, which leads towards the disturbance in homeostasis (Toor *et al*., 2019). There are about 200 *Candida* species known up till now, but limited number of strains are pathogenic to human which cause candidemia in immunological and physiological compromised state. The medically significant fungal specie are *C. albicans* (50-60%); *C. glabrata* (15-20%); *C. parapsilosis* (10-20%); *C.tropicalis* (6-12%) ; *Candida lusitaniae* (<5%); *C. kefyr* (<5%); *Candida guilliermondi* (<5%) and *T. krusei* (1-3%)(Abi-Said *et al*., 1997 ;Moran *et al*., 2014).

Candida is a versatile yeast with its capabilities to survive and thrive in extraneous environment inside the host body by adapting to wide pH range, environmental changes and stresses. To aid in transition from commensal to pathogenic phase, virulence traits present in *Candida* species which are yeast to hypha form transition, adhesins expression, cell invasion, thigmotropism, slime production, phenotypic switching and the secretion of hydrolytic enzymes play important role (Staniszewska, 2019). Moreover, biofilm formation in *C. albicans* help these yeast to resist traditional antifungals (azoles) and are considered as virulence attribute in *Candida* contributing to its pathogenesis (Masur *et al*., 1977; Mukherjee & Chandra, 2004; Sardi *et al*., 2013; Nobile & Johnson, 2015 Staniszewska, 2019; Mba & Nweze, 2020; D'Enfert *et al*., 2021). Some studies recent studies on obese animal model studies have focused on gut mycobiome dysbiosis (Borges *et al*., 2018;Mims *et al*., 2021), however, still there is no study on cultureable gut fungi. This is the first attempt to profile fungal diversity and their possible potential pathogenicity in three group comprising of pre-obese, obese and healthy individuals for profiling of gut fungi and their pathogenicity in obesity.

5.2- MATERIAL AND METHODS

5.2.1-Study Type and Subjects

Previously described in chapter 3 (section 3.4)

5.2.2-Inclusion Criteria for Sampling

The faecal samples were also collected from 26 pre-obese,75 obese and 50 healthy controls on the basis of inclusion criteria which was individuals who have not used probiotics and antibiotics in last three months. Faecal samples were collected according to designed protocol of International Human Microbiome Standards (IHMS) project (Wu *et al*., 2019).

5.2.3-Isolation of Fungi from Faecal Samples

All the faecal samples were processed aseptically and serially diluted upto10^6 followed by fungal isolation by inoculating 0.1mL of each dilution on Sabouraud`s Dextrose agar (SDA) plates. For SDA preparation 65.0 g of SDA was added to 1000 mL distilled water and sterilized by autoclaving at 121º C and 15 psi for 15 min. The abundance of *Candida* and filamentous fungi was recorded after 48 hrs of incubation by colony forming unit (CFU)/Gram of faeces. Colony morphology, colour size, textures were noted, and different colonies were re-streaked to purify on SDA for 48hrs.

5.2.4-Identification of Fungal Species

5.2.4.1-Methylene Blue Staining:

Microscopic identification was carried out by staining filamentous and non-filamentous fungi isolates with methylene blue stain. Sterile glass slides were prepared as recommended by the microbiology lab manual. By using sterile loop fungal colonies were picked and mixed with methylene blue drop on the slide surface. While tear mount method was adopted to prepare slides for filamentous fungi by using sterile needle; culture mycelium was placed, teased and spread on the drop. The bubble formation was avoided, and glass cover slip was mounted on the prepared slide. For microscopic examination, 10X and 40X lens (Nikon YS-100) were used. Methylene blue stain stock was prepared by adding 1.5g methylene blue $C_{13}H_{18}CIN_3S.3H_2O$ powder (DAEJUNG) in 100mL of 95% ethyl alcohol. Working solution was prepared by mixing 30mL of stock solution in 100mL of distilled water and 0.1mL of 10% KOH.

5.2.4.2-Germ Tube Test

Germ tube test was performed to screen *C. albicans* from the other yeast. A small portion of yeast colonies were picked after cooling red hot loop to maintain sterility and inoculated in a glass test

tubes containing 0.5 mL serum. It was incubated for 2-3 hrs at 37°C aerobically. The source of serum was human blood, which was collected aseptically in yellow top tube and kept at room temperature for 15-30 min to clot. After clotting, these tubes were centrifuged at 10,000 rpm for 15 min. This collected serum was collected in a separate sterile tube to proceed with the germ tube test. After incubation period of 2-3hrs, it was observed under the light microscope (Nikon YS-100) by placing a small drop of inoculated serum and covered with cover slip. *C. albicans* showed germ tube positive results while in case of *C. dubliniensis* it was negative. All other *Candida* species were negative for germ tube.

5.2.4.3-Nitrate reduction test

This test was used to determine nitrate assimilation by yeast (*Candida* species) by checking presence of end product nitrite produced enzyme nitrate reductase. For this test, yeast colonies were inoculated in semisolid medium of nitrate in test tubes and nitrite presence was checked after incubation at 37°C for 24-48 hrs aerobically. Nitrate broth (M439S, HIMEDIA) was prepared by completely dissolving 39.0g of powdered media in 1000 mL of distilled water and autoclaving for 15min at 121°C (15 psi). The media in test tube after incubation was checked for nitrite presence by adding few drops of alpha naphthylamine and sulfanilic acid. These indicators formed red colour indicating positive nitrate reduction. No colour formation was further confirmed by adding a small amount of zinc in nitrate broth tubes. Appearance of red colour after zinc indicated negative nitrate reduction test while no change in colour indicated positive reduction test.

5.2.4.4-Growth of Yeast at 45°

Growth at high temperature (45°C) differentiate between *C. albicans* and *C. dubliniensis*. Both species give positive germ tube test, hence, to differentiate between these germ tube test positive *C. albicans* and *C. dubliniensis* were grown at 45˚C. Only *C. albicans* survive and grow at this temperature while *C. dubliniensis* cannot grow at 45˚C. This test was performed by inoculating from 48hrs old cultures on plates into SDA media plates and incubating at 45˚C for 10 days under aerobic conditions.

5.2.4.5-Characterization of Fungi by Using Differential Agar

Identification of different yeasts up to specie level was assessed by pigment production on *Chromatic Candida* agar; a differential and selective media for yeast isolation plus differentiation based on specific coloured colonies formation. Yeast colonies from 48 hrs old cultures were streaked on *Candida* Differential Agar (CDA-M1297A) and incubated for 24-48 hrs at 37°C. The media was prepared by adding 42.7g CDA in 1000mL distilled water. The media was prepared by boiling below 100°C on hot plate until it dissolved and poured into the petri plates under sterile conditions. After incubation, results were interpreted for specie identification according to manufacturer's guideline which were *C. albicans:* light green coloured smooth colonies, *T. krusei:* purple fuzzy colonies, *C. Kefyr:* cream to white with slight purple centre, *C. utilis:* pale pink to pinkish purple, *C. tripocalis:* blue to metallic blue coloured raised colonies, *C. dubliensis:* pale green, *C. glabrata:* cream to white smooth colonies *C. parapsilosis:* white to cream, *C. membranifaciens:* white to cream colonies.

5.2.5-Virulence Profiling of Identified *Candida* Species

For screening of virulence capabilities of *Candida* specie isolated from faecal samples. Different enzyme assays were performed and biofilm production along with their resistance pattern to antifungals was determined.

5.2.5.1-Preparation of Yeast Suspension for Different Enzymatic Activities

Colonies from 48 hrs old cultures of *Candida* on SDA were used to prepare inoculum in Phosphate buffer saline (PBS) and kept at 37°C for 24 hrs. Standard yeast suspensions of density 1.0×10^6 cells/mL were prepared after matching with 0.5 McFarland. The turbidity was also adjusted to optical density (OD) of 0.38 at 520 nm using spectrophotometer (Multiskan Go, Thermoscientific) (Deepa *et al*., 2015).

5.2.5.2-Extracellular enzymatic activities

All of the enzymatic activities were measured by EAI, which is a ratio of colony diameter to the zone of precipitation plus colony diameter, except for coagulase activity which was checked by ability to form clump. These activities were categorized into 4 groups as 1) high activity 0.59; 2) medium activity 0.6–0.79; 3) low activity 0.8–0.99; 4) no activity=1.

a. Phospholipase activity

Phospholipase activity **(**Phz) of *Candida* isolates was assessed on Egg yolk agar media by following protocol of Price *et al*. The media consisted of 65g SDA, 58.4g NaCl, 5.5g $CaCl_2$ and 80mL sterile egg yolk (centrifugation at 3000 x g for 15min). Media was prepared in 920mL water, which was after autoclaving cooled at 45°C before adding egg yolk. The yeast suspensions (10^6 yeast cells/mL) having turbidity matched with 0.5 McFarland were spot inoculated on egg yolk agar media in duplicates. These plates were dried in air and incubated for 4 days at 37°C. Standard formula was used for measuring zone of precipitation in mm (Williamson *et al*., 1986).

b. Esterase activity

Esterase activity (Ez) of *Candida* isolates was measured in duplicate by using Tween-80 opacity medium. It was prepared by 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2$, 15 g agar in 1000 mL water. After autoclaving, 5mL sterile tween 80 was added (pH 6.8). Ten µl of yeast suspensions (10^6 yeast cells/ml) were spot inoculated followed by incubation under aerobic conditions at 37°C for 10 days. The precipitation halo zone formation was denoted as positive result. The activity was pervious to light and measured as described previously (Slifkin, 2000).

c. Haemolysin activity

Haemolytic activity (Hz) for *Candida* isolates was evaluated by inoculating cultures in duplicate on blood agar plates by following method prescribed earlier (Manns *et al*., 1994). Blood agar plates were prepared by supplemented SDA with 3% (w/v) glucose and 7mL of sheep blood in 100mL of media (pH 5.6). Standard yeast suspension (10^6 yeast cells /ml) of each isolate was prepared in phosphate buffer saline and 10µl of the suspension was inoculated on the plates followed by incubation at 37°C aerobically for 48hrs. The haemolytic activity was measured by observing clear zone of haemolysis (mm) around inoculated *Candida*.

d. Proteinase activity

Proteolytic activity (Prz) of *Candida* isolates was evaluated on medium containing 2.0 g bovine serum albumin as a protein source,1.0 g KH_2PO_4, 0.1 g yeast extract, 0.5 g $MgSO_4$ and 10 g glucose in 100 ml distilled water (pH 5.0). The sterilization of solution was

achieved by syringe filtration and then mixed with sterilized agar solution (20 g agar/900 mL distilled water), which was autoclaved at 121°C for 15 minutes at 15psi. The yeast suspensions as described in previous assays were spot inoculated in duplicates and incubated for seven days at 37°C. The proteolysis of bovine serum albumin was visualized as a clear halo around the inoculum(Rüchel *et al*., 1982).

e. Caseinolytic activity

Caseinolytic activity (Cz) for the production of protease enzyme against casein was checked on minimal medium (MM agar) supplemented with 0.6% skim milk at pH 4.0 (Botelho *et al*., 2012). The medium was prepared by adding 55 µmol/L Glucose, 11 µmol/L KH_2PO_4, 6 µmol/L KCl, 2 µmol/L $MgSO_4.7H_2O$, 65 µmol/L $FeSO_4$, 61 µmol/L$ZnSO_4$) in 1000mL of distilled water. Casein degradation was recorded as halo zone appearance in mm (Botelho *et al*., 2012).

f. **Coagulase activity**

Coagulase activities (Cg) of isolates were evaluated by classical tube method using sheep blood plasma from EDTA tubes. All of the *Candida* strains were inoculated in Sabourauds dextrose broth and incubated at 37°C for 18-24 hrs. aerobically. After incubation, 0.1mL of each overnight culture was then transferred into test tubes already filled with 0.5 mL of plasma. All of the tubes were immediately transferred to water bath for incubation at 37°C and were checked at different time intervals from (2, 4, 6 and 24 hrs). The coagulase activity was considered positive if platelet clump as pallet remained intact even after gentle shaking. Whereas negative activity was the absence of platelet clumping. The assay was performed in a duplicate (Sasani *et al*., 2021).

5.2.5.3- Screening of Biofilm Formation

a. Biofilm formation capability by Congo red based qualitative assay

Congo red assay (CRA) was used for qualitative screening of biofilm formation. *Candida* isolates were inoculated on brain heart infusion medium supplemented with sucrose (50g/L) mixed with Congo red dye (0.8 g/L), separately prepared and autoclaved. Later incubated at 37°C for 24 to 48 hrs in aerobic conditions. Colony colour appearance on media indicated the biofilm producers and non-producers. Appearance of dark red to black colony with crystalline appearance indicated strong biofilm formation. The red colour formation categorized isolates for biofilm forming ability

as moderate biofilm producers, while pink colour as weak biofilm formers. While white colonies were considered to be negative biofilm formers.

b. Biofilm Formation Capability by Crystal Violet Based Quantitative Assay

Microliter plate assay (MTP) was used for quantitative screening of biofilm formation. Inoculum of *Candida* strains were prepared by mixing 48 hrs old culture in Sabouraud`s dextrose broth (SDB supplemented with 10% glucose) and incubated at 37°C for 24 hrs in shaking incubator at 200 rpm. After 24 hrs, suspension was centrifuged at 5000 rpm for 10 minutes to get the pellet. After discarding the supernatant, pellets were washed twice with sterile phosphate buffer solution and re-suspended the pellets in 1 mL SDB. To prepare yeast suspensions 10^6 cells/mL, turbidity was matched with 0.5 McFarland. The biofilm formation was assessed by adding 200 µL of prepared yeast suspension in 96 well microliter plate. The negative control was sterile SDB without the yeast culture in the last column of the microliter plate. These plates were then covered in aluminium foil keeping moisture intact to avoid evaporation of inoculum from wells following incubation at 37°C for 24 hrs in aerobic conditions.

On third day the media were carefully removed without disturbing the biofilm and each well was washed thrice with PBS (pH 7.2) in order to remove the non-adherent cell from the well. After washing with PBS 95%, ethanol was added to each well for fixing the adherent cells by keeping the plate at room temperature for 30 minutes. After incubation time, ethanol was carefully removed without disturbing the biofilm and stained with 1% w/v crystal violet then incubated again at room temperature for 15 minutes. Washing was performed with deionized water for removing extra crystal violet dye from the well. The plates were air dried after washing and 33% acetic acid was added to treat the adherent cells to solubilize the crystal violet with adherent cell and incubated the plates for 15 minutes at room temperature. Using the microliter plate reader, OD was measured at 492nm. All the isolates were checked in triplicates in microliter plate. The analysis was performed by comparing optical density of isolates with cut off optical density value. Isolates were categorized for the biofilm formation on the basis of values of optical density.

ODc =Average OD of negative control + 3(standard deviation of negative control)

Classification of biofilm former *Candida*

OD ≤ ODc = Negative for biofilm, ODc < OD ≤ 2 x ODc = Weak biofilm former, 2 x ODc < OD ≤ 4 x ODc = Moderate biofilm former, OD > 4 x ODc = strong biofilm former

5.2.5.4-Resistance Against Antifungals Azoles

Disc diffusion method was used for anti-fungal susceptibility recommended by Clinical and laboratory Standard Institute (CLSI). As per CLSI guideline, antifungal discs of voriconazole (01 µg) and fluconazole (25 µg) were used for susceptibility testing. For anti-fungal susceptibility testing, suspension of yeasts was made in 1 mL of normal saline. The fungal colonies from 18-24 hrs old cultures on SDA plate were used to prepare suspension. The suspensions were matched for their turbidity with 0.5 McFarland Standard. Muller Hinton (MH) media supplemented with glucose and methylene blue was prepared by adding glucose 10.0 g, 38.0 g MHA, and methylene blue 70 microliter in 1000 mL distilled water and autoclaved at 121 °C (15psi) for 15 minutes. Suspensions were inoculated on the plates and the antifungal discs were placed on the media plates which were incubated for 24 hrs at 37°C in aerobic condition. Reference zone of inhibition of fluconazole was ≤14 mm and voriconazole ≤13 mm as per CLSI M27- A3 and CLSI M44-A (Espinel-Ingroff, 2007; Fothergill, 2012).

5.3-Statistical Data Analysis

For statistical analysis, IBM SPSS version 21 was used. Mean and standard deviation (SD) was measured for descriptive data and percentages were calculated for categorical data. ANOVA and post hoc test was used to assess difference among study groups for anthropometric variables. Correlation analysis was done for significant association among the virulence factors. Odds ratio (OR) was calculated for risk assessment. Chi-square (χ^2) test and Fisher's exact test (where count was less than 5), were used to determine significant associations of obesity with different variables, where level of significance was described by p value <0.05.

5.4-RESULTS:

5.4.1-Characteristics of Study Subjects:

In the present study, mean age of 75 obese subjects were 35.1±9.9 years where 39 were females (52.10%) in this group. In pre-obese group of 26 individuals; 15(57.69%) were females and 11(42.30%) males with mean age of 36.40±12.27 years. The mean age of 50 subjects of control

group was 32.45 ±11.5 years. Mean height, weight and BMI with Standard deviation (SD) are given in Table 5.1 and 5.2.

Table 5.1: Distribution of obese, pre-obese and healthy controls on the basis of demographic attributes

Anthropometric variables		**Number(n)**	**Mean**	**SD**	**Std. Error**
Age (years)	Obese	75	35.147	10.017	1.16
	Pre-obese	26	36.407	12.273	2.36
	Control	50	32.451	11.512	
	Total	151			
Weight (kg)	Obese	75	84.613	11.9117	1.37
	Pre-obese	26	72.259	9.4119	
	Control	50	59.392	10.7165	
	Total	151			
Height (m)	Obese	75	5.36	0.390	0.04
	Pre-obese	26	26.885	1.4948	
	Control	50	5.30	0.501	
	Total	151			
BMI (kg/m^2)	Obese	75	32.405	2.1568	0.24
	Pre-obese	26	5.33	0.375	
	Control	50	22.427	1.9918	
	Total	151			

Table 5.2: Comparison of anthropometric variables among the study subjects from obese, pre-obese and healthy controls by ANOVA and post hoc test

Anthropometric variables	**ANOVA**		**Study group comparison**	**Post Hoc Test**	
	F-ratio	***p*-value**		**t-ratio**	***p*-value**
Age (years)	1.599	0.206	Obese vs control	3.4262	0.215
			Pre-obese vs control	3.9564	0.257
Weight (kg)	63.763	0.000	Obese vs control	25.2921*	0.000
			Pre-obese vs control	12.8671*	0.000
Height (m)	0.033	0.968	Obese vs control	0.019	0.967
			Pre-obese vs control	0.023	0.969
BMI (kg/m^2)	347.449	0.000	Obese vs control	10.3673*	0.000
			Pre-obese vs control	4.4577*	0.000

5.4.2- Abundance of Fungi in Faecal Samples

Serial dilutions (10^{-1}-10^{-6}) of all faecal samples were prepared and fungal CFU were counted. Only dilutions where there were colonies from 30-300 colonies per plate were considered for CFU/mL calculation. All those plates with more than 300 colonies denoted as "too numerous to count" (TNTC) and those which had less than 30 were denoted as "too few to count". The average abundance of fungi in faecal samples for obese were $1.32x10^6$ CFU/mL, pre-obese $1.36x10^6$ CFU/mL and controls $7.72x10^5$ CFU/mL. Also, isolation and purification of the suspected *Candida* was done and isolates were stored in 50% glycerol stock solution in Eppendorf tubes.

5.4.3- Prevalence of Culture-Able Fungal Diversity in Gut of Obese, Pre-Obese and Control Subjects

After incubation, colonies were identified through observation of morphological characteristics and through microscopic examination. All the filamentous fungi were identified up to genus level while all the yeast samples were identified at specie level.

In present study, out of 75 obese subjects' faecal samples, 6(8%) samples were positive for *Aspergillus niger* and 3 (4%) positive for *Aspergillus flavus.* From the pre-obese faecal samples, 4(3.8%) were positive for each *A. niger* and *A. flavus.* In control group individual`s sample, 3/50 faecal samples yielded mycelial growth, which were identified as *A. niger* and 1/50 was identified as *A. flavus.*

In total faecal samples from obese subjects, only 47(64.3%) were positive for *Candida*, in 26 pre-obese faecal samples 11(42.30%) were *Candida* culture positive whereas in control group 22(30%) faecal samples had culture-able *Candida* growth. There were total 104 isolates purified from 47 culture positive obese samples, 22 isolates purified from 11 culture positive pre-obese and 62 isolates from 22 culture positive control samples (Table 5.3).

In obese group, out of 104 isolates different *Candida* spp. Were further identified to be *C.albicans* 30(28.8%), *C.kefyr* 47(45.1%), *C.utilis* 3.0 (2.8%), *C. glabrata* 1.0 (0.9%), *C. tropicalis* 1.0 (0.9%), *C. dubliensis* 2.0 (1.9%), *C. parapsilosis* 3.0 (2.88%) and *T. krusei* 17(16.3%). In pre-obese group *C.albicans* 12(54.54%), *C. kefyr* 5.0 (22.72%), *C. utilis* 4.0 (18.18%) and *T.krusei* 1.0

(4.54%) were confirmed. In the control group, the strains identified were *C.albicans* 20 (32.2%), *C. kefyr* 23(37.0%), *C. utilis* 4.0 (6.4%), *C.tropicalis* 2.0 (3.25%), *C.membranifaciens* 4.0 (6.4%) and *T. krusei* 9.0 (14.5%).

Based on cultureable diversity, *C. kefyr, C.albicans, T.krusei,* and *C.utilis* were recovered from individuals of all three study groups. *C. tropicalis* was common in two groups: obese and controls. *C. glabrata, C.dubliensis* and *C. parapsilosis* were isolated from only faecal samples of obese individuals. While *C.membranifaciens* was present only in control subject faecal samples.

Table 5.3. Percentage positivity of faecal samples for various *Candida* species among obese, pre-obese and healthy controls

Identified *Candida*		**Obese *n* (%)**	**Pre-obese *n* (%)**	**Healthy *n* (%)**
Candida spp	Yes	47(62.7)	11(42.3)	22(44.0)
	No	28(37.3)	15(57.7)	28(56.0)
Candida kefyr	Yes	25(53.2)	5(19.2)	13(59.1)
	No	22(46.8)	21(80.8)	9(40.9)
Candida albicans	Yes	22(46.8)	8(30.8	11(50.0)
	No	25(53.2)	18(69.2	11(50.0)
Teunomyces krusei	Yes	15(31.9)	1(3.8)	7(31.9)
	No	32(68.1)	25(96.1)	15(68.1)
Candida utilis	Yes	2(4.3)	1(3.8)	2(9.1)
	No	45(95.7)	25(96.2)	20(90.9)
Candida tropicalis	Yes	1(2.1%)	-	2(9.1)
	No	46(97.9)	-	20(90.9)
Candida glabrata	Yes	1(0.9)	-	-
	No	103(99.0)		
Candida dubliensis	Yes	2(11.5)	-	-
	No	102(98.7)		
Candida parapsilosis	Yes	3(2.88)	-	-
	No	101(97.1)		
Candida membranifaciens	Yes	-	-	4(6.4)
	No			58(93.5)

5.4.4- Pathogenic potential of *Candida* species

a) Extracellular enzyme profiling:

On the basis of enzymatic capabilities, pathogenic potential of various strains was checked and further their association with the obesity was determined. Various enzymes production in identified strains was assessed as risk factor by calculating odd ratios. In *C. kefyr* isolates 4 enzymes; phospholipase, esterase, haemolysin and coagulase were found to pose risk for obesity. While significant association was observed for phospholipase [*p* value=<0.01 (21.20), OR=13.45 (CI=4.04-44.82)] and coagulase [*p* value=<0.01 (10.5), OR= (11.2) (CI=0.02-16.46)] with obesity as risk factor. In obese group, phospholipase [*p* value=<0.01(23.5), OR= 29.4 (CI=6.21-139.7] and coagulase [*p* value= 0.008 (7.41) OR= (6.98) (CI=1.46-37.59)] activities of *C.albicans* were with obesity. In case of *T. krusei,* only phospholipase [*p* value = <0.01, OR=26.25 (11.33) (CI=3.04-226.6)] showed significant association with obesity. All other enzymes were risk for obesity, but no significant association was found. *C. utilis* isolates' phospholipase and coagulase production was significant with *p* value less than 0.05 and OR= 63.0 (CI=0.99-4042.3) and 63.0 (CI=0.98-4042.37) respectively, while none of the isolate showed proteinase and haemolysin activity.

In pre-obese group esterase, proteinase, coagulase and caseinolytic production by *C.albicans* was significantly associated it. The *C.ultilis* for this group was greater risk as *p* value= 0.02, OR=81.0 (CI= 1.30-5046.71), While *T.krusei and C.kefyr* did not have any association with pre-obesity (Table 5.4-5.6).

Table 5.4: Various virulence factors and their percentage production by cultureable *Candida* species colonizing the gut of the obese, pre-obese and control subjects

Enzymes	**Categories**	**% production of overall gut *Candida* isolates**		
		Obese *n* (%)	**Pre-obese *n* (%)**	**Control *n* (%)**
Phospholipase activity	Yes	88 (84.6)	13 (59.0)	12 (19.3)
	No	16 (15.3)	9 (40.9)	50 (80.6)
Esterase activity	Yes	98 (94.2)	18 (81.8)	62 (100)
	No	6 (5.7)	4 (18.1)	-
Proteinase activity	Yes	29 (27.8)	2 (9.0)	16 (25.8)
	No	75 (72.1)	20 (90.9)	46 (74.1)
Hemolysin activity	Yes	17 (16.3)	7 (31.8)	12 (11.5)
	No	87 (83.6)	15 (68.1)	50 (80.6)
Caseinolytic activity	Yes	37 (35.5)	8 (36.3)	4 (6.4)
	No	67 (64.4)	14 (63.6)	58 (93.5)
Coagulase activity	Yes	52 (50.0)	7 (31.8)	7 (11.2)
	No	52 (50.0)	15 (68.1)	55 (88.7)
Biofilm forming activity	Yes	99 (95.2)	22 (100.0)	60 (96.8)
	No	5 (4.8)	0 (0)	2 (3.2)
Fluconazole resistance	Resistant	33 (31.7)	4 (18.1)	3 (4.8)
	Sensitive	71 (98.3)	18 (81.8)	59 (95.2)
Voriconazole resistance	Resistant	2 (1.9)	0 (0)	0 (0)
	Sensitive	102 (98.1)	221 (100)	62 (100)

Table 5.5: Extracellular enzyme production by various cultureable *Candida* species recovered from gut of obese, pre-obese and control subjects

***Candida* species**	**Virulence factor**		**Obese *n*(%)**	**Pre-obese *n*(%)**	**Control *n*(%)**
Candida kefyr	Phospholipase	Yes	40(83.3)	2(40.0)	6(26.0)
		No	8(16.6)	3(60.0)	17(73.9)
	Esterase	Yes	48(100)	4(80.0)	23(100)
		No	0(0)	1(20.0)	0(0)
	Proteinase	Yes	5(10.4)	0(0)	5(21.7)
		No	43(89.5)	5(100)	18(78.2)
	Hemolysin	Yes	1(2.0)	0(0)	0(0)
		No	47(97.9)	5(100)	23(100)
	Coagulase	Yes	24(50)	1(20.0)	2(8.6)
		No	24(50)	4(80.0)	21(91.3)
	Caseinolytic	Yes	11 (22.9)	1(20.0)	1 (4.3)
		No	37 (77.0)	4(80.0)	22 (95.6)
Candida albicans	Phospholipase	Yes	25(83.3)	7(58.3)	3(15)
		No	5(16.6)	5(41.6)	17(85)
	Esterase	Yes	25 (83.3)	9(75.0)	20(100)
		No	5(16.6)	3(25.0)	0(0.0)
	Proteinase	Yes	22(73.3)	2(16.6)	11(55)
		No	8(26.6)	10(83.3)	9(45)
	Hemolysin	Yes	12(40.0)	7(58.3)	9(45)
		No	18(60.0)	5(41.6)	11(55)
	Coagulase	Yes	14(46.6)	6(50.0)	2(10)
		No	16(53.6)	6(50.0)	18(90)
	Caseinolytic	Yes	13(43.3)	7(58.3)	1 (4.7)
		No	17(56.6%)	5(41.6)	19 (90.4)
Teunomyces krusei	Phospholipase	Yes	14(87.5)	0(0.0)	2(22.2)
		No	2(12.5)	1(0.0)	7(77.7)
	Esterase	Yes	16(100)	1(100)	9(100)
		No	0	0(0.0)	0(0.0)
	Proteinase	Yes	1(6.2)	0(0.0)	0(0.0)
		No	15(93.7)	1(100)	9(100)
	Hemolysin	Yes	4(25.0)	0(0.0)	2(22.2)
		No	12(75.0)	1(100)	7(77.7)
	Coagulase	Yes	8(50.0)	0(0.0)	3(33.3)
		No	8(50.0)	1(100)	6(66.6)
	Caseinolytic	Yes	6 (37.5)	0(0.0)	1 (11.1)

		No	10 (62.5)	1(100)	8 (88.8)
Candida utilis	Phospholipase	Yes	3(100)	4(100)	0(0.0)
		No	0(0.0)	0(0.0)	4(100)
	Esterase	Yes	3(100)	4(100)	4(100)
		No	0(0.0)	0(0.0)	0(0.0)
	Proteinase	Yes	0(0.0)	0(0.0)	0(0.0)
		No	3(100)	4(100)	4(100)
	Hemolysin	Yes	0(0.0)	0(0.0)	0(0.0)
		No	3(100)	4(100)	4(100)
	Coagulase	Yes	3(100)	0(0.0)	0(0.0)
		No	0(0.0)	4(100)	4(100)
	Caseinolytic	Yes	0(0.0)	0(0.0)	0(0.0)
		No	3(100)	4(100)	4(100)
Candida tropicalis	Phospholipase	Yes	1 (100)	-	0(0.0)
		No	0(0.0)	-	2(100)
	Esterase	Yes	0(0.0)	-	2(100
		No	1(100)	-	0(0.0)
	Proteinase	Yes	0(0.0)	-	0(0.0)
		No	1(100)	-	2(100)
	Hemolysin	Yes	0(0.0)	-	0(0.0)
		No	1(100)	-	2(100)
	Coagulase	Yes	1(100)	-	0(0.0)
		No	0(0.0)	-	2(100)
	Caseinolytic	Yes	0(0.0)	-	1(50)
		No	1(100)	-	1(50)

Table 5.6: Association of extracellular enzyme production ability of cultureable *Candida* species with the obesity

***Candida* species**	**Virulence factor**	**Obese vs control**		**Pre-obese vs control**	
		***p* value**	**Odd ratio**	***p* value**	**Odd ratio**
Candida kefyr	Phospholipase	<0.01[b*]	13.45	0.53[b]	1.88
		(21.20)	(4.04-44.82)	(0.389)	(0.25-14.19)
	Esterase	1[a]	1.97	0.17[a]	0.06
			(0.03-102.89)		(.00-1.82)
	Proteinase	0.22[b]	0.43	-	-
		(1.46)	(0.11-1.70)		
	Hemolysin	1[a]	1.54	-	-
			(0.06-39.5)		
	Coagulase	<0.01[b*]	10.5	0.45[b]	2.62
		(11.2)	(0.02-16.46)	(0.54)	(0.18-36.34)
	Caseinolytic	3.46[b]	6.11	0.21[b]	5.50
		(.062)	(0.73-51.06)	(1.51)	(0.28-107.15)
Candida albicans	Phospholipase	<.01[b*]	29.4	0.10[b]	7.93
		(23.5)	(6.21-139.7)	(6.55)	(1.47-42.58)
	Esterase	1[a]	0.1	0.044[b*]	0.06
			(0.006-2.24)		(0.003-1.41)
	Proteinase	0.2 [b]	2.0	0.032[b*]	0.16
		(1.35)	(0.61-6.46)	(4.56)	(0.02-0.94)
	Hemolysin	0.65[b]	0.7	0.46[b]	1.71
		(0.198)	(0.24-2.89)	(0.53)	(0.40-7.27)
	Coagulase	0.008[b*]	7.41	0.01[b*]	9.00
		(6.98)	(1.46-37.59	(6.4)	(1.41-57.11)
	Caseinolytic	8.32[b]	13.7	0.0007[b*]	26.60
		(.0039)	(1.62-115.89)	(11.37)	(2.62-269.42)
Teunomyces krusei	Phospholipase	<0.01[b*]	26.25	-	-
		(11.33)	(3.04-226.6)		
	Esterase	1[a]	1.84	1[a]	0.15
			(0.03-100.45)		(0.00-11.42)
	Proteinase	1[a]	1.72	-	-
			(0.06-46.77)		
	Hemolysin	0.94[b]	1.07	-	-
		(0.005)	(0.15-7.42)		
	Coagulase	0.50[b]	1.77	-	-
		(0.45)	(0.33-9.55)		
	Caseinolytic	1.749[b]	4.36	-	-

		(.1859)	(0.435-43.72)		
Candida utilis	Phospholipase	0.02[a*]	63.0	0.02[a]*	81.00
			(0.99-4042.3)		(1.30-5046.71)
	Esterase	1[a]	0.77	1[a]	1.00
			(0.01-49.90)		(0.01-62.30)
	Proteinase	-	-	-	-
	Hemolysin	-	-	-	-
	Coagulase	0.02[a*]	63.0	-	-
			(0.98-4042.37)		
	Caseinolytic	-	-	-	-
Candida tropicalis	Phospholipase	0.33[a]	15.0	-	-
			(0.18-1236.2)		
	Esterase	0.33[a]	0.066	-	-
			(0.0008-5.4946)		
	Proteinase	-	-	-	-
	Hemolysin	-	-	-	-
	Coagulase	0.33[a]	15.00	-	-
			(0.182-1236.2)		
	Caseinolytic	1[a]	0.33	-	-
			(0.006-6.80)		
[a] association with Fisher exact test					
[b] association with Pearson Chi square (χ^2) test					
***p*-value <0.05 shows significant association.**					

b) Biofilm Forming Ability

The qualitative assay for biofilm forming ability testing was CRA, in which 101 isolates from total (104) from obese individuals were biofilm former. Among these, 68 (65.3%) isolates were strong biofilm formers, 33 (31.7%) moderate biofilm former while none of them was weak biofilm former. Among them, 3.0 (2.8%) were recorded to be negative with white colour colonies on the CRA. The quantitative biofilm formation results by MTP method showed that 100/104 were biofilm formers, among them 49 (47.1%) were strong biofilm formers, 45(43.2%) with moderate biofilm formation, 5 (4.80 %) were weak biofilm formers and 4 (3.84%) were non-biofilm formers. In the pre-obese group, all isolates showed biofilm formation with 81.8% being strong former and 18.1% moderate biofilm formers on CRA. While upon MTP analysis, 68.1% were strong formers and 31.8% showed moderate biofilm forming ability. The biofilm forming ability of control group isolates on CRAwas 100% biofilm production, among these 50(80.6%) were strong biofilm former and 12(11.5%) moderate biofilm former. However, none of these isolates had weak biofilm forming ability. On the base of MTP results, there were 60 (96.7%) isolates positive for biofilm forming ability and 2.0 (3.2%) were negative for biofilm formation. Among these 60 positive isolates, 22(35.4%) were strong biofilm formers, 29(46.7%) moderate biofilm former and 9(14.5%) were weak biofilm former (Table 5.7).

Table 5.7: Qualitative and quantitative analysis for biofilm forming ability in various cultureable *Candida* species from obese, pre-obese and control subjects

Virulence Factor		**Obese *n*(%)**	**Pre-obese *n*(%)**	**Control *n*(%)**
Congo Red assay	**Categories**			
	YES	101(97.1)	22(100)	62(100)
	NO	3(2.8)	0	0
	weak	-	-	-
	Moderate	33(31.7)	4(18.1)	12(19.3)
	strong	68(65.3)	18(81.8)	50(80.6)
Microliter plate assay	**YES**	99(95.1)	22(100)	60(96.7)
	NO	5(4.8)	0	2(3.2)
	Weak	5(4.8)	-	9(14.5)
	Moderate	45(43.2)	7(31.8)	29(46.7)
	strong	49(47.1)	15(68.1)	22(35.4)

The ability of biofilm production was observed in majority of the isolates recovered from all three groups (obese, pre-obese and control samples). Among obese samples 96.6% *C.albicans* isolates showed biofilm production while in pre-obese and control samples all the *C.albicans* isolates were biofilm former. Among *C. kefyr* isolates, 95.7% were biofilm producers in obese and control group while all (5) isolates of pre-obese group also showed biofilm formation. In obese samples 66.6%, in pre-obese 80.0% and in control sample 31.8% isolates were strong biofilm formers.

C.utilis was not found to be risk of obesity, its number of biofilm former within a group was higher for control group and pre-obese group (100%) as compared to obese group samples (66.6%). In all of the study group samples (obese, pre-obese and controls), all of the *C. utilis* isolates were strong to moderate biofilm producers. The isolates of *C. tropicalis* were lower in numbers as compared to other species and were not found to be a risk for obesity. Among obese individuals, moderate biofilm formation activity and in isolates from controls strong biofilm formation was seen.

The *Teunomyces krusei* isolates were found to have increased risk for obesity susceptibility [OR= 1.8750 (CI= 0.1030 to 34. 1324)] as 93.7% in obese group in comparison to control group (88.9%) and pre-obese group where only one isolate was recovered. Majority of these isolates showed moderate biofilm forming activity. Other three species *C.glabrata* (n=1)*, C.dubliensis* (n=2) and *C. parapsilosis* (n=3) were recovered only from obese samples and were positive for the biofilm production. Both *C.glabrata* and *C. dubliensis* were strong biofilm producers while three of the *C. parapsilosis* isolated from obese samples in this study showed weak, moderate and strong category of biofilm production. In case of 4 *C. membranifaciens* isolates that were present only in control individuals, moderate ability was in 3(75%) of the isolates while strong ability in 1(25%) of the isolate (Table 5.8-5.9).

Table 5.8: Biofilm forming abilities of various cultureable *Candida* species isolated from gut of obese, pre-obese and control subjects

***Candida* species**	**Categories**	**Obese n (%)**	**Pre obese n (%)**	**Controls n (%)**
Candida kefyr	**Yes**	46(95.8)	5(100)	22(95.7)
	No	2(4.1)	0	1(4.3)
	Weak	5(11.1)	0	2(9.1)
	Moderate	13(28.9)	1(20.0)	13(59.1)
	Strong	30(66.6)	4(80.0)	7(31.8)
Candida albicans	**Yes**	29(96.6)	12(100)	20(100)
	No	1(3.4)	0	0(0)
	Weak	4(13.4)	0	5(25)
	Moderate	12(40)	2(16.6)	8(40)
	Strong	13(43.3)	10(83.3)	7(35)
Teunomyces krusei	**Yes**	15(93.7)	1(100)	8(88.9)
	No	1(6.3)	0	1(11.1)
	Weak	2(13.4)	0	2(25)
	Moderate	9(60)	1(100)	3(37.5)
	Strong	4(26.6)	0	3(37.5)
Candida utilis	**Yes**	2(66.6)	4(100)	4(100)
	No	1(33.4)	0	0(0)
	Weak	0(0)	0	0(0)
	Moderate	1(33.3)	3(75.0)	2(50)
	Strong	1(33.3)	1(25.0)	2((50)
Candida tropicalis	**Yes**	1(100)	-	2(100)
	No	0(0)	-	0(0)
	Weak	0(0)		0(0)
	Moderate	1(100)		0(0)
	Strong	0(0)		2(100)

Table 5.9: Association of Biofilm forming abilities of various cultureable *Candida* species with the obesity

	Obese vs control		**Pre-obese vs control**	
***Candida* species**	***p* value**	**Odd ratio (95% Cl)**	***p* value**	**Odd ratio (95% Cl)**
Candida kefyr	1 [b]	1.0227	1[a]	0.733
		(0.0879 to 11.899)		(0.0262 - 20.563)
Candida albicans	1[a]	0.4797	1[a]	0.609
		(0.0186 to 12.3698)		(0.0114 - 32.717)
Teunomyces krusei	1 [b]	1.8750	1[a]	0.529
		(0.1030 to 34. 1324)		0.0139 - 20.1876
Candida utilis	0.4286[a]	0.1852	1[a]	1.000
		(0.0053 to 6.4761)		0.0161 - 62.3051
Candida tropicalis	1 [a]	0.6000	-	-
		(0.0073 to 49.4513)		
a association with Fisher exact test				
b association with Pearson Chi square(χ2) test				
**p*-value <0.05 shows significant association.				

c) Resistance to antifungals

Relatively high antifungal activity was observed among obese samples isolates as compared to isolates from the pre-obese and control group samples. Majority of study isolates were resistant to fluconazole in comparison to voriconazole, only 2.0 isolates showed resistance to it which were recovered from obese samples only.

C. kefyr showed significant association with fluconazole resistance [*p* value = 0.0035 OR= 6.9565 (CI= 1.81 to 26.60)] and voriconazole resistance [p value= 0.5524, OR= 2.5824 (CI= 0.11 to 56.01)] posing high risk of obesity. The pre-obese group also had significant association [*p*= 0.020(5.30); OR=10.0 (CI=1.151-86.879)] with resistant *C. kefyr* isolates. These were resistant to both drugs among obese individuals while pre-obese and control sample isolates showed only fluconazole resistance. *C.utilis* do not show any significant association [p value 0.4286] but was risk for obesity as the odd ratios against fluconazole was OR= 5.400 (CI=0.15 to 188.84) and voriconazole OR= 1.2857 (0.020 to 82.49]. *T.krusei* showed resistance to only fluconazole drug and had higher odds of obesity susceptibility [OR=11.76 (CI= 0.58 to 237.9)] with significant association (p value= 0.0571). *C.parapsilosis, C.glabrata, C.tropicalis* and *C. membranifaciens* did not show any resistance against both the drugs in the current study. While *C.dubliensis* showed resistance against both fluconazole and voriconazole and was also risk for obesity as odd ratios was 1.000 (CI=0.010 to 92.42) and OR= 2.000 (CI= 0.001 to 28.47) respectively. In *C. membranifaciens* from control group, no resistance to antifungal drug was detected (Table 5.10 and 5.11).

Table 5.10: Antimicrobial resistance pattern displayed by various cultureable *Candida* species recovered from gut of obese, pre-obese and control subjects

***Candida* species**	**Antifungals**	**Obese**		**Pre-obese**		**Control**	
		Resistant	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**
Candida kefyr	FCA	24(50%)	24(50%)	3(60%)	2(40%)	3(13.0%)	20(86.9%)
	VOR	2(4.16%)	46(95.8%)	0	5(100%)	0	23(100%)
Candida albicans	FCA	1(3.33)	29(96.6%)	1(8.3%)	11(91.6%)	0	20(100%)
	VOR	0	30(100%)	0	12(100%)	0	20(100%)
Teunomyces krusei	FCA	6(37.5%)	10(62.5%)	0	4(100%)	0	9(100%)
	VOR	0	16(100%)	0	4(100%)	0	9(100%)
Candida utilis	FCA	1(33.3%)	2(66.6%)	0	1(100%)	0	4(100%)
	VOR	0	3(100%)	0	1(100%)	0	4(100%)
Candida tropicalis	FCA	0	1(100%)	-	-	0	2(100%)
	VOR	0	1(100%)	-	-	0	2(100%)

Table 5.11: Association of Antimicrobial resistance of various cultureable *Candida* species with obesity

***Candida* species**	**Antifungals**	**Obese vs control**		**Pre-obese vs control**	
		***p* value**	**Odd ratio(95% Cl)**	***p* value**	**Odd ratio(95% Cl)**
Candida kefyr	FCA	0.0035[b*]	6.956 (1.81-26.60)	0.020(5.30)	10.0 (1.151-86.879)
	VOR	0.5524[a]	2.582 (0.11-56.01)	-	-
Candida albicans	FCA	1[a]	2.084 (0.08-53.76)	0.375[a]	5.34 (0.2010-42.281)
	VOR	-	-	-	-
Teunomyces krusei	FCA	0.0571[a*]	11.760(0.58-237.9)	-	-
	VOR	-	-	-	-
Candida utilis	FCA	0.4286 [a]	5.400(0.15-188.84)	-	-
	VOR	-	-	-	-
Candida tropicalis	FCA	-	-	-	-
	VOR	-	-	-	-

5.5-DISCUSSION:

Obesity is complex physiological condition in which various factors including microorganisms contribute to its initiation, development and maintenance (Vettor & Conci, 2019;(Turnbaugh et al., 2006). There has been complex cross talk between microflora, metabolite and immune cells which is controlled by resident microbes under homeostatic condition. Any disturbance in colonization could shift commensal relationship of these resident fungus, especially *Candida* which make it opportunistic pathogen (Wilbrink *et al*., 2020;Portincasa *et al*., 2022). Current study was carried out to observe cultureable fungi colonizing the human gut fungi through faecal analysis. Also to detect if any change in its diversity in the obesity. Furthermore, various virulence factors were assessed for their pathogenic capability as possible indicator in obesity prognosis.

In the current study, abundance was assessed by the percentage prevalence of particular specie present in the samples of the study groups. These findings showed the most abundant species among all three groups were *C.kefyr* (40.42%) followed by *C.albicans* (32.97%) and *Teunomyces krusei* (13.82%). Although the trend remained same for most abundant specie in all study groups, however, the percentage abundance of *C.kefyr* (44.2%) and *Teunomyces krusei* (16.3%) was higher in obese group. Several studies have also reported that their identification where more species of candida like C*.albicans*, *C.glabrata, C.parapsilosis, C.krusei, C.dubliensis* and *Candida kefyr* are the common in both healthy and disease persons. Some of these *Candida* species also posses pathogenic nature due to their abilities to form various virulence factors like change from yeast to filamentous form, secretion of hydrolases, interaction with mucosal linings vio biofilm and resistance to antimicrobials (Haynes, 2001;Yang, 2003;Mayer *et al*., 2013). There was low abundance of *C.albicans* in faecal samples of study subject, it may be due to isolation of other species even filamentous form.

Based on types of *Candida* species colonization among the study group, 4.0 out of 9.0 *Candida* species were common in all the three groups. While obese and control group shared one additional specie. In pre-obese and control samples, *Candida* diversity was comparatively low as compared to obese group, however, *C.membranifaciens* was the only present distinctively in control samples which is a known commensal. In contrast to the other group, *Candida* diversity was rich in obese group as three different *Candida* species including *C.dubliensis*, *C.parapsilosis* and *C. glabrata*,

which were identified in addition to five common *Candida* species. Previously studies have also found increased richness of *Candida* species to be present in the inflammatory conditions like in inflammatory bowel disease, Crohn`s disease and hepatitis B (Ott *et al*., 2008; Chen *et al*., 2011; Li *et al*., 2014). These findings are also supported by the study on obese individuals in which higher diversity of yeast was present when it was compared with healthy individuals' diversity (Borges *et al*., 2018). Among all of the identified *Candida* species, different studies on human have shown involvement of these three *Candida* species namely *C.dubliensis, C.parapsilosis and C.glabrata* in blood stream infections, invasive candidiasis and dermal yeast infections (Trofa *et al*., 2008; Z. Khan *et al*., 2012; Silva *et al*., 2012; Hassan *et al*., 2021). These 3 species which were found in the obese individuals are normally commensal in the healthy human body but if the immunity is compromised or any alteration in physiology, then these species become opportunistic pathogens. Then, they act as pathogens by producing large number of hydrolases which helps them to adhere, penetrate, invade, and acquire nutrients to aid in dissemination to other body parts to cause systemic infections.

In the current study, six different hydrolytic enzymes production, biofilm forming ability and resistance to azoles by all of the identified isolates was checked to assess if they have pathogenic potential. All of the isolates that were in common among all three groups were compared for their pathogenic capabilities on the basis of presence of virulence characteristics. The remaining isolates distinctively isolated from either obese or from the control group samples have been given in the Table 5.12. The comparison of all identified species have shown that *C.Kefyr* isolates from the obese individuals were displaying potent virulence factors as were positive for 9.0 virulence factors activities. *T.krusei* and *C.albicans* showed positive activity of the 8.0 virulence factors (Table 5.13). All of the isolates were biofilm producers; the strong biofilm formers were further checked for their level of extracellular enzyme activities. The obese study subjects' isolates showed significant correlation with virulence factors, however, in these isolates phospholipase and esterase formation were inversely proportional to one another (χ^2= -0.307, *p* value = 0.002). The caseinolytic and haemolysin activities were positively correlated (χ^2= 0.229, *p* value =0.019) showing that invasion of membranes and haem acquisition ability might be present at same time in fungi which might be making these fungal isolate more potent for tissue evasion(Merino *et al.,* 1992).

The haemolysin activity of the isolates was inversely related to fluconazole resistance (χ^2= -0.226, *p* value=0.021), which indicates that haemolysin could be effective in absence of resistance. While the fluconazole resistance showed positive correlation with the voriconazole resistance (χ^2=0.225, *p* value= 0.022) this means that if resistance to both antifungal is present in strains, they may be more virulent irrespective if any other virulence factors is present or absent (Table 5.14). The strong biofilm former isolates in the obese individuals displayed strong esterase activity, strong to moderate coagulase production, moderate level of phospholipase and proteinase activity; while moderate caseinolytic activity and less haemolysin activity. While antimicrobial resistance among identified strong biofilm formers (*C.albicans, C.kefyr, C.utilis* and *Teunomyces krusei*) from obese subjects was highest in *C.kefyr* followed by *T.krusei* isolates only (Table 5.15). Furthermore, isolates with strong esterase activity, moderate to strong coagulase and low to moderate proteinase activity had co-prevalence of antifungal resistance too. As these isolates had moderate to strong biofilm formation ability, it points toward possibility of biofilm formation as contributing factor to increase resistance (Table 5.16).

Phospholipase enzyme is potent and one of the commonly involved hydrolytic enzyme in host cell invasion, penetration, and lysis such as mucosal linings. It breaks ester linkage of phospholipids and release free fatty acids sand lipid compounds (Sachin *et al*., 2012; K *et al*., 2015; Kaur, 2018). In different studies on various clinical samples, phospholipase production was recorded high by various *Candida* species showing up to 80 to 100% production (Tsang *et al*., 2007; Jasim *et al*., 2016). In current study, high phospholipase activity of isolates from obese group was in line with the previous studies on clinical *Candida* isolates, pre-obese and control groups *Candida* had low phospholipase production. This activity was highest among *Teunomyces krusei* (88.2%) followed by *C.albicans* (83.8%) and *C.kefyr* (82.6%) from obese study subjects. The difference in study groups for phospholipase production may be due to difference in the survival strategies depending on the environmental factors like pH, inhibitory substances, nutrients acquisition medium and colonization surfaces. Also, behavioural responses of the same species can be varied from person to person and in different sites within the same gut lining as there were individuals with different BMI.

Esterase hydrolyse the ester bond of the cell membranes and is very important in invasion of host tissues (Borst & Fluit, 2003). In current study, only *C.kefyr* isolates from obese group showed high esterase production. In pre-obese and control group, all *Candida* isolates had 80%-100% esterase producers. This could be due to fact that health status differs with gut health also, it may have been cause for difference in survival strategies of different *Candida* species to internal environment. There is difference in esterase activity displayed by *Candida* species from previous publications, where non- *C.albicans* species were being reported to have high esterase production in comparison with the *C.albicans* (Seifi & Mahmoudabadi, 2014; Fatahinia *et al*., 2015).

Proteinase enzymes are involved in breakdown of proteins inside the host(Zaugg et al., 2001). In case of obese individual's higher proteinase production was observed in *C.albicans* isolates (70.96%) as compared to pre obese (9.0%) and control group (55%). This *C.albicans* being high producer of proteinase has been substantiated by the previous data in which *C.albicans* in comparison with the non-*C. albicans* showed high proteinase production. The *C.albicans* is the most common colonizer of the gut lining and various studies have pointed that it has a opportunistic pathogenic nature by release of proteinases including aspartyl proteinase which increase colonization and enhance its potential to impart negative health outcomes by invading different body sites and host tissues (Zaugg *et al*., 2001; Naglik *et al*., 2003; Meenambiga *et al*., 2018).

Haemolysin is helpful in acquisition of iron from host blood which is required for survival and adaptation of *Candida* pathogen in the host. Among various identified *Candida* species, *C. albicans* isolates showed higher haemolysin production in comparison with the other *C.* species. These findings are in line with the study of Hussein *et al.,* (2019), in which *C.albicans* were found to have higher haemolysin production than the other species. Haemolysin production was observed high among *Candida albicans* in all three groups of present study. This capability enables them to be a potential pathogen capable to survive in the blood stream.

Protease enzymes act against the casein protein and causes degradation resulting in the penetration and dissemination of the pathogens. Another, hydrolytic enzyme assessed was caseinolytic enzyme, where isolates with high caseinolytic in all three groups were *C. albicans* specie followed by *T. krusei* and *C. kefyr.* However, the isolates recovered from pre-obese and control group had

very low caseinolytic activity. Previously protease activity against the casein has been observed in environmental strains, while no literature on involvement of clinical *Candida* isolates for protease production against casein is available (Phaff *et al*., 1994; Braga *et al*., 1998; Ozturkoglu-Budak *et al*., 2016; Ramos *et al*., 2017). The protease involvement has been reported to cause tissue necrosis that is caused by casein breakdown. Such ability of isolates in the study might have role in damaging the gut lining and compromising gut penetrability (Phaff *et al*., 1994).

Coagulase enzyme act against the plasma protein and causes conversion of fibrinogens into fibrins, prothrombin to thrombin and are believed to be enhancer of virulence in *Candida*. In current study, coagulase production was detected higher in *C.kefyr* (50%) followed by *Teunomyces krusei* and *C.albicans* among the obese group. These results were in contrast with previously reported studies that showed *C.albicans* as potent coagulase enzymes producers (Rodrigues *et al*., 2003; Yigit *et al*., 2008;Yigit *et al*., 2011).

Presence of biofilm makes the cell resilient to environmental effects and they become more resistant towards various environmental stresses including antimicrobial agents. In the current study, first generation (fluconazole) and second generation (voriconazole) azoles were used to check their ability to survive adverse condition by observing their resistance pattern. These antimicrobials are among the most common treatment regime used against various *Candida* infections and are taken orally. In the current study, most of the *Candida* isolates from obese group were found to be resistant to fluconazole drug as compared to voriconazole. In other studies, depending on the site of infection, different species like *C.glabrata, C.parapsilosis, C.krusei* and *C.tropicalis* were commonly observed to be resistant to different azoles. In the current study, highest resistance was observed in *C.kefyr* isolates from all three groups. The resistance in isolates of *C.kefyr* was followed by *Teunomyces krusei* and *C.albicans* from obese group, in case of pre-obese it was only *C.albicans*, while in controls resistance was seen only in *C.kefyr* isolates.

The resistance to antifungal was also assessed for its association with obesity where fluconazole resistance in *C.kefyr* was a risk for it with odd ratio of 6.9565 (CI=1.81 to 26.60) and *p* value=0.003. While no association with voriconazole resistance was seen [*p* value=0.55 OR=2.5824 (CI=0.11 to 56.01)]. *T.krusei* strains also showed high resistance against fluconazole, was posing risk (odd ratios OR= 11.76; CI= 0.58 to 237.9) and was significantly associated (*p*-

value 0.0571) with obesity. Here, although fluconazole resistance was most common but was not found in all detected *Candida* species. Earlier reports cited that *C.albican*s has high resistance which is in contrast with the current study as high resistance in *C.kefyr* was detected followed by *T.krusei* (Bhattacharjee, 2016;Maheronnaghsh *et al*., 2020).

Different studies have reported the change in diversity and abundance of gut fungal diversity or dysbiosis as possible risk factor for other inflammatory disorders. Thus, there is possibility that the dysbiosis in cultureable *Candida* species in the current study among obese subject might be playing role in triggering adverse outcome like inflammation by producing PAMPs or by releasing their metabolites. In the present study, *C.kefyr* and *T. krusei* were also significantly associated with the obesity. *C.kefyr* is being recognise as emerging potent pathogen among immunocompromised individuals in many studies. The other recognized fungal genus was *T. krusei* in the current study and these isolate also displayed various virulence factors. There is already an evidence of *Candida* has association with immune evasion and degradation of mucin layer of the GIT lining by releasing various types of hydrolases which degrade epithelial keratin, dermal collagen, albumin, haemoglobin and immunoglobulin A, also indicate may be these fungi are involved in obesity by using their virulence factor when weight is gained. In the present study involving obese individuals, *Candida* species (*C.albicans, C.kefyr* and *T. krusei*) producing hydrolytic enzymes were major colonizers which might have role in obesity development or maintenance. Also, presences of high hydrolytic enzymes points towards chances of compromised gut lining which might have promoted abundant colonization of these pathogenic *Candida* species in obesity. These strains have the ability to further disseminate up to epithelial and endothelial linings with the help of enzymes that could complicate the condition especially obesity.

In conclusion, there was difference in the abundance and diversity of *Candida* species colonizing the obese gut indicate that there is cultureable fungal diversity dysbiosis in this study. This data is first of its kind in which pathogenic potential of commensal *C.albicans, C.kefyr* and *Teunomyces krusei* isolates was detected through production of various pathogenic enzymes in case of obese individuals. *C.kefyr* and *T. krusei* are seen as emerging potential potent pathogen in case of obesity. In isolates from obese individuals, expression of various hydrolytic enzymes, high biofilm forming potential and non-susceptibility to common inhibitory substance particularly fluconazole indicates

that there is possible shift from non-pathogenic behaviour towards pathogenic. Moreover, virulence profiling of *Candida* species has shown that not a single factor but combination of several virulence factors like phospholipase, esterase, proteinase, haemolysin, coagulase, caseinolytic, biofilm formation and resistance to antimicrobials might be involved in increasing the pathogenic potential of these isolated species in case of obesity. Pathogenic *Candida* colonization in the GIT tract of the obese individuals might have some role in obesity susceptibility.

Table 5.12: Comparison of virulence factors activity of cultureable *Candida* species of obese and healthy subjects

Virulence factors	Categories	Obese faecal sample`s isolates			Control faecal sample`s isolates
		C.dubliensis	*C.glabrata*	*C.parapsilosis*	*C.membranifaciens*
Phospholipase	Strong	0	1	0	0
	Moderate	1	0	2	1
	Weak	1	0	0	0
	No activity	0	0	1	3
Esterase	Strong	2	1	3	4
Coagulase	Strong	1	0	2	0
	Moderate	1	1	1	4
Haemolysin	Strong	0	0	0	1
	No activity	2	1	3	3
Proteinase	Moderate	1	0	0	0
	No activity	0	0	0	4
Caseinolytic	Strong	0	0	2	0
	Moderate	2	1	1	0
Biofilm former	Strong	1	1	1	1
	Moderate	1	0	1	3
	Weak	0	0	1	0
Fluconazole resistance	Resistant	1	0	0	0
	Sensitive	1	1	3	4
Voriconazole resistance	Sensitive	2	1	3	4

Table 5.13: Potential pathogenic potential of cultureable *Candida* species on the basis of observed virulence factors with obesity

Virulence factors		Obese					Pre-obese				Control				
		C.kefyr	*C. albicans*	*T. krusei*	*C. tropicalis*	*C. utilis*	*C.kefyr*	*C. albicans*	*T. krusei*	*C. utilis*	*C. kefyr*	*C. albicans*	*T. krusei*	*C. tropicalis*	*C. utilis*
Pathogenicity intensity		+++ +++ +++	+++ +++ ++	+++ +++ ++	+++	+++ +++	+++ +++	+++ +++ ++	+ +	++ +	+++ +	+++ +++	+++	++	++
Phospholipase (Pz)		0.795	0.661	0.77	0.933	0.797	0.78	0.68	-	0.86	0.738	0.784	0.826	_	_
Esterase (Ez)		0.325	0.341	0.384	0	0.322	0.26	0.32	0.43	0.39	0.375	0.373	0.348	0.35	0.357
Hemolysine (Hz)		0.72	0.55	0.507	0	_	-	0.53	-	-	_	0.52	_	_	_
Proteinase (Prz)		0.65	0.65	0.6	0	_		0.7			0.578	0.628	_	_	_
Caseinolytic (Csz)		0.668	0.683	0.737	0	0.75	0.77	0.67			_	_	_	_	_
Coagulase		+	+	+	+	+	+	+	-	-	+	+	+	-	-
Biofilm (OD)		1.02	0.66	0.81	0.75	0.17	0.82	0.83	0.72	0.68	0.92	0.38	0.51	1.28	0.87
Antifungal resistance	FCA	50 %	3.3 %	37.5 %	-	33.3 %	60 %	8.3 %	-	-	3	-	-	-	-
	VOR	4.15 %	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5.14: Pearson Correlation analysis among various virulence factors of cultureable *Candida* species in obese study subjects

Virulence factors		**Phospholipase activity**	**Esterase activity**	**Coagulase activity**	**Hemolysin activity**	**Proteinase activity**	**Caseinolytic activity**	**Biofilm former**	**Fluconazole resistance**	**Voriconazole resistance**
Phospholipase activity	Pearson Correlation	1	-.307**	-.123	.167	.013	.049	.023	-.156	.071
	Sig. (2-tailed)		.002	.216	.090	.943	.620	.816	.113	.473
Esterase activity	Pearson Correlation	-.307**	1	.085	-.094	.016	.090	.051	.154	.035
	Sig. (2-tailed)	.002		.391	.341	.932	.361	.607	.119	.727
Coagulase activity	Pearson Correlation	-.123	.085	1	-.136	-.203	.041	.133	.145	-.001
	Sig. (2-tailed)	.216	.391		.171	.273	.679	.180	.144	.989
Hemolysin activity	Pearson Correlation	.167	-.094	-.136	1	.286	.229*	-.157	-.226*	-.060
	Sig. (2-tailed)	.090	.341	.171		.118	.019	.111	.021	.543
Proteinase activity	Pearson Correlation	.013	.016	-.203	.286	1	-.114	-.258	.126	.[c]
	Sig. (2-tailed)	.943	.932	.273	.118		.543	.161	.499	.000
Caseinolytic activity	Pearson Correlation	.049	.090	.041	.229*	-.114	1	.137	-.071	-.097
	Sig. (2-tailed)	.620	.361	.679	.019	.543		.167	.472	.325
Biofilm former	Pearson Correlation	.023	.051	.133	-.157	-.258	.137	1	-.013	.031
	Sig. (2-tailed)	.816	.607	.180	.111	.161	.167		.894	.753
Fluconazole	Pearson Correlation	-.156	.154	.145	-.226*	.126	-.071	-.013	1	.225*

resistance	Sig. (2-tailed)	.113	.119	.144	.021	.499	.472	.894		.022
Voriconazole resistance	Pearson Correlation	.071	.035	-.001	-.060	.[c]	-.097	.031	.225*	1
	Sig. (2-tailed)	.473	.727	.989	.543	.000	.325	.753	.022	
**. Correlation is significant at the 0.01 level (2-tailed).										
*. Correlation is significant at the 0.05 level (2-tailed).										
c. Cannot be computed because at least one of the variables is constant.										

Table 5.15: Hydrolase production and resistance among strong biofilm forming cultureable *Candida* species in obese study subjects

Virulence factors	categories	*Candida albicans n*=12 (40%)	*Candida kefyr n*=29 (60.41%)	*Candida utilis n*=1(25%)	*Teunomyces krusei n*=4(25%)
Esterase	Strong	11(91.6%)	29(100%)	1	4(100%)
	No activity	1(8.33%)	0	0	0
Coagulase	Strong	8(66.6%)	15(51.7%)	1	3(75%)
	Moderate	4(33.3%)	13(44.8%)	0	1(25%)
Phospholipase	Strong	2(16.6%)	3(7.69%)	0	0
	Moderate	8(66.6%)	13(44.8%)	0	1(25%)
	Weak	1(8.3%)	9(31.0%)	1	1(25%)
	No activity	1(8.3%)	4(13.7%)	0	2(50%)
Proteinase	Strong	2(16.6%)	1(3.4%)	0	0
	Moderate	7(58.3%)	2(6.8%)	0	1(25%)
	Weak	3(25.0%)	0	0	0
	No activity	0	0	0	3(75%)
Caseinolytic	Strong	1(8.3%)	3(10.3%)	0	0
	Moderate	4(33.3%)	2(6.8%)	1	2(50%)
	Weak	1(8.3%)	1(3.4%)	0	0
	No activity	6(50%)	23(79.3%)	0	2(50%)
Hemolysin	Strong	2(16.6%)	0	0	0
	Moderate	0	1(3.4%)	0	1(25%)
	Weak	1(8.3%)	0	0	0
	No activity	9(75%)	28(96.5%)	1	3(75%)
Fluconazole	Resistant	0	9(31.0%)	0	1(25%)
	Sensitive	12(100%)	20(68.9%)	1	3(75%)
Voriconazole	Resistant	0	1(3.4%)	0	0
	Sensitive	12(100%)	28(96.5%)	1	4(100%)

Table 5.16: Hydrolase production and biofilm forming ability of antifungal resistant isolates cultureable *Candida* species in obese study subjects

		C.albicans	***C.dubliensis***	***C.kefyr***	**C.utilis**	**T.krusei**
Phospholipase activity	Strong	0	0	3	0	0
	Moderate	0	1	5	0	1
	Weak	1	0	7	1	6
	No activity	0	0	4	0	0
Esterase activity	Strong	1	1	19	1	7
Coagulase activity	Strong	1	1	11	1	4
	Moderate	0	0	8	0	3
Hemolysin activity	Moderate	0	0	1	0	0
	No activity	1	1	18	1	7
Proteinase activity	Strong	0	0	1	0	0
	Moderate	0	1	1	0	0
Caseinolytic activity	Strong	0	0	1	0	0
	Moderate	0	1	3	0	1
	Weak	0	0	1	1	2
	No activity	1	0	14	0	4
Biofilm formers	Strong	0	1	9	0	1
	Moderate	1	0	10	1	5
	No activity	0	0	0	0	1

CHAPTER 6: STUDY OF CULTUREABLE AND UNCULTUREABLE GUT BACTERIAL DIVERSITY IN OBESITY

6.1-INTRODUCTION:

Obesity is a complex disease that results from the interaction of multiple environmental and genetic factors (Musso *et al*., 2010). Over this decade, research on the gut microbiota has implicated microbes as one of the contributing factor for the development of inflammatory and metabolic disorders particularly obesity (Nguyen *et al*., 2015; Dabke *et al*., 2019). The host and the gut microbiota has been coevolving over centuries by maintaining a symbiotic relationship, but still can be influenced by multiple other host's factors including genetics, gender, age, immune status and gut motility (Andoh *et al*., 2016). Human GIT harbour about trillions of microorganisms with concentration up to 10^9 -10^{12} CFU/mL(Whitman *et al*., 1998; Baquero & Nombela, 2012). The evident role of microbiome in energy harvesting and metabolism has been found in many studies where they play crucial role in weight gain (Okeke *et al*., 2014; Mullin, 2015; Vandeputte *et al*., 2016). The gut microbiota are thought to play role in inflammatory disease like obesity through modulating the host immune system, where endogenous and environmental like diet act as triggers to induce inflammation (Ferreira *et al*., 2011; Khan *et al*., 2014). The commensal microbiota of the human intestinal tract helps in maintaining the intestinal integrity, protect from colonization of pathogens, in digestion and assimilation of nutrition while producing many useful metabolites (Turnbaugh *et al*., 2006; Turner, 2009; Hooper and MacPherson 2010; Tremaroli & Bäckhed, 2012). On the basis of 16SrRNA sequencing against V4 region, significant difference among proportion of gut microflora in obese and control individuals has been observed. The marked difference was among Bacteroidetes and Firmicutes which together comprise 90% of the microflora associated with the obesity. As increased abundance of Firmicutes and reduction in Bacteroidetes in case of obesity and weight gain is seen in some earlier work (Sun *et al*., 2001; Balamurugan *et al*., 2010).

The change in the bacterial diversity is reported in many population survey studies which are primarily based on clinical data, epidemiological surveys, in addition there are also some gnotobiotic mice studies too. Early data on involvement of microflora in obesity come from the population based studies involving risk groups like breastfed vs. formula fed and caesarean vs.

vaginal birth , which was focused on obesity in special condition (Koenig *et al*., 2011; Maynard *et al*., 2012). But now, with advancement of research on obesity the data suggest for general population a strong relation of dietary habits, hygienic practices and drug usage with changes in the gut microbiota composition. Such studies, also indicated that use of antibiotic at the early age has devastating effects on the gut microflora development which could expose child to obesity in later years of their life (Turta & Rautava, 2016). There is distinct effects of probiotics on the improvement of gut microflora as it alters microbial composition resulting in differential functionality of microbes residing the gut (Carmody *et al*., 2015; Fontané *et al*., 2018). The change of gut microflora in obesity when compared with the healthy controls, was found to be associated with the body weight loss or gain too (Cox *et al*., 2015). Such changes in microbial diversity associated to obesity was also causing metabolic complications (Rastelli *et al*., 2018). The metabolites formed by the microbial communities in the gut such as short-chain fatty acids and membrane proteins can in turn regulate appetite, lipogenesis, gluconeogenesis, inflammation, and other functions (Sierra *et al*., 2019).

Furthermore, obesity and related metabolic disorders effect the gut epithelium leading to impaired gut barrier (Guzman *et al*., 2013). A dysfunctional gut barrier allows the contact of bacterial components with epithelium cells and tissues where there infiltration occurs that leads to local or systemic inflammation (Liang *et al*., 2013). Metabolic endotoxemia characterized by the presence of plasma lipopolysaccharide which are also observed in obesity in low concentration (Boutagy *et al*., 2016). These bacterial components are recognized by innate immune PRRs like TLR-4 which upon binding initiate downstream cell signalling pathways leading to the secretion of various pro-inflammatory cytokines like IL-6, TNF-α *etc.* (Saad *et al.*, 2016; Al-Assal *et al*., 2018). High fat diet has been shown to alter microbial diversity that brings metabolic modifications via production of bacterial products (Singh *et al*., 2017). In obesity, gut microbial diversity changes which is termed as dysbiosis (Zuluaga *et al.*, 2018; Vallianou *et al*., 2019). In the dysbiosis, metabolic changes take place under the influence of many bacterial metabolites especially LPS by Gram negative which simultaneously is found to be associated to Bacteroidetes reduction in a gut (Amabebe *et al*., 2020 ; Aoun *et al*., 2020). In case of obesity high level of SCFAs are formed (Schwiertz *et al*., 2010) along with high diversity of bacteria which are capable of energy

extraction from indigestible dietary fibres such bscteria are *Bacteroides, Roseburia, Bifidobacterium, Fecalibacterium*, and *Enterobacteria* (Al-Lahham *et al*., 2010).

The role of gut microbiota is reported in many studies, where dysbiosis of gut microbiome is detected with increase in number of SCFA-producing bacteria. This change in extra energy harvesting bacterial diversity might be involved in increasing adiposity and weight gain. Gram-negative LPS producer are also recognised to increase gut permeability and trigger endotoxemia with low-grade systemic inflammation. The tendency for obesity might be increasing due secreted microbial products, which act as PAMPs for activation TLRs which can initiate and sustain inflammation. Thus, the gut microbiota especially bacteriome is critical to decipher for its contribution towards the development of obesity. The studies focused on dietary factors and their impact on the bacteriome has been done on the European populations but such studies in our region are very limited. In Pakistan, only two studies on gut microbiome have been conducted in previous years one on the healthy controls and the other on type-2 diabetic comorbid obese individuals (Batool *et al*., 2018; Ahmad *et al*., 2019). The study by Batool *et al*., was focused on gut profiling of normal weight individuals and association of their dietary habits with gut microflora. While Aftab *et al*., have compared type 2 diabetic individuals having obesity with the healthy control without no focus dietary factors.

Up till now, there is no study on obesity in our region that could highlight the differences of gut microflora of obese verse controls with data on their dietary habits to assess contribution of the factors on the gut microbiota composition among Pakistani individuals. This study was designed to understand the bacterial diversity via both culture independent and culture dependent approaches through faecal samples using 16S rRNA gene sequencing and standard microbiological procedures. The purpose of current study is to detect gut microbial diversity among obese Pakistani population. This study would be helpful in bacterial gut profiling of local Pakistani population, which will set basis for future studies in this domain to understand holistically bacterial diversity among obese population in Pakistan. Furthermore, it would be beneficial for devising obesity treatment strategies based on microbial gut profiling, which is still majorly unknown for local population.

6.2- MATERIAL AND METHODS

6.2.1- Study Design and Settings

Described previously in chapter 3 (section 3.2).

6.2.2- Study Population and Sampling Technique

The subset of samples from total samples were selected for microbiome study based on the age criteria with age < 40 years individuals. The further details are presented previously in chapter 5 (section 5.2.2).

6.2.3- Part I: Study of Gut Bacterial Diversity by Culture Dependent Approach

6.2.3.1- Isolation of Cultureable Bacteria from Stool Samples

From the fresh stool samples collected, 1 g of faecal material was homogenized and serially diluted by using normal saline 0.9%. Samples were serially diluted making different dilutions (10^{-1}-10^{-6}). The isolation of aerobic bacteria was done on nutrient agar plates (Oxoid, England). It is a general-purpose media for the growth of bacteria and was prepared by dissolving 28g into 1L distilled water followed by autoclaving at 121°C for 15 minutes. From each dilution 10µl was inoculated on nutrient agar plates in duplicate with help of pipette which was spread on plate using sterile glass spreader. The inoculated plates were than incubated at 37℃ for 18-24 hrs in aerobic incubator.

For the isolation and optimization of growth requirements of cultureable facultative anaerobic bacteria, blood agar, thioglycolate agar and broth were used. For blood agar, 14.0 g of blood base agar (Oxoid, England) was dissolved in 1L distilled water followed by autoclaving at 121°C (15lbs pressure) for 15 minutes. The blood agar was supplemented with 5% (v/v sterile de-fibrinated) sheep blood and were streaked after sterility checking overnight. Each blood agar plate was inoculated with 10µL of diluted stool suspension via spread plate technique.

For the preparation of thioglycolate broth, 29.75 g of media was dissolved in 1L distilled water and autoclaved at 121°C for 15 minutes. The thioglycolate broth after transferring into tubes were inoculated with various dilutions of each stool sample. After the inoculation, blood agar plates and

thioglycolate broth tubes were placed in anaerobic glass jars supplied with CO_2Gen Anaerobic sachets (Oxoid, England) and instantly sealed with wax. After 24-48 hrs of incubation, broth cultures were re-inoculated on to the thioglycolates agar plates.

The thioglycolate agar was made by adding 14.25 technical agar to thioglycolate broth solution (19.75g broth in 1L distilled water) followed by autoclaving as mentioned above. After the sterility check the plates were prepared by spreading 10µL of broth culture. These plates were anaerobically incubated in airtight anaerobic glass jars supplied with CO_2 sachets and were placed in static conditions in incubator (37ºC) for 24-48 hrs. After incubation, the distinctive colonies were further streaked onto nutrient agar plates and blood agar plates for aerobic and anaerobic bacteria respectively to obtain pure cultures.

6.2.3.2- Determination of Bacterial Abundance

Serial dilution (10^{-1}-10^{-6}) of stool samples were prepared and inoculated on nutrient agar media. For aerobic bacteria, the plates were incubated for 18-24 hr while for facultative anaerobic bacteria, these plates were incubated in glass jars supplied with CO_2 for 24-48 hrs at 37°C. The number of colonies formed on plates were counted and plates with 30-300 colonies were considered for CFU/mL while >300 colonies were considered as too numerous to count (TNTC) and less than 30 as too few to count (TFTC).

6.2.3.3-Differentiation Based on Gram Staining

After incubation, the purified bacterial colonies were differentiated based on cell-wall composition. Gram staining was done on isolated bacterial colonies. The bacterial smear was treated with iodine followed by ethanol (95%) was as decolourizer. Later on, smear was counter stained with safranin (secondary stain). The air-dried smear was then observed under bright field microscope (Micros-Austria) by using 40X and 100X magnification lens. It was repeated for all the isolated bacteria from all the samples.

6.2.3.4-Identification of Gram negative and Gram-positive bacteria:

After Gram staining, the identified Gram-negative and Gram-positive bacterial colonies were sub-cultured on selective and differential agar plates. For Gram-negative bacteria, MacConkey agar

media was used that differentiate on basis of lactose monohydrate fermentation by different bacteria. This media also contains neutral red indicator which upon lactose fermentation it turns pink. For preparation of media, 51.53g of MacConkey agar media (Sigma) was weighed and dissolved in 1L distilled water. All the suspected Gram-negative bacteria were streaked on it. On the other hand, the suspected Gram-positive bacteria were sub-cultured on Mannitol salt agar (MSA) that differentiate mannitol fermenter fron non fermenter. MSA (sigma) was prepared by dissolving 55.5 g of media component into 1L distilled water. All the suspected Gram-positive bacterial colonies were streaked on the plates and incubated for 24 hrs at 37ºC, after incubation colony morphology was noted.

6.2.3.4.1-Biochemical Identification Scheme for Gram negative Bacterial Species

6.2.3.4.1.1-Oxidase Test:

For this test, filter paper strips were soaked into the Kovac's reagent (tetramethyl-p-phenylenediamine dihydrochloride). A 24 hrs, fresh single isolated colony was picked by the help of sterile toothpick and rubbed to the Kovac's reagent-soaked filter paper. Change in the colour from colourless to purple indicated the positive test while no change was interpreted as negative.

6.2.3.4.1.2-Triple Sugar Iron Test

The slants and butt were inoculated by using overnight bacterial culture plates. A single bacterial colony was picked by the help of sterile inoculating needle and pierced into the butt followed by streaking the slant surface as well. After inoculation, the test tubes were capped and placed in the incubator for 18-24 hrs. For facultative anaerobic bacteria, the slants were incubated for about 24-48 hrs under wax sealed airtight glass jar with gas generation sachet at 37°C.

6.2.3.4.1.3-Indole Test

Overnight fresh cultures were taken and inoculated with isolated single colony of bacteria into peptone water, which was aerobically incubated for 24 hrs. The facultative anaerobic bacteria were inoculated in peptone water test tubes and incubated in airtight glass jars which supplemented with CO_2 sachets for about 24 hrs at 37°C. For aerobic bacteria, after incubation 0.5mL of Kovac's

reagent was added into each tube, while for facultative anaerobic bacteria 0.5mL of Ehrlich's reagent. The colour change from yellow to cherry red indicated a positive indole test while no colour change indicated bacteria as indole negative.

6.2.3.4.1.4-Citrate utilization test:

Slants were prepared with sterile Simmons citrate medium and inoculated with suspected colonies. The results were recorded from all suspected isolates and were further analysed.

6.2.3.4.1.5-Catalase Test

This test was performed by using 3% H_2O_2 solution, glass slide and overnight bacterial culture plates. A drop of 3% H_2O_2 was poured in the middle of labelled slide. Single bacterial colony was then picked from the overnight culture plate by the help of sterile toothpick. The colony was then mixed with the H_2O_2 solution. The immediate bubbles formation was indication of strong positive test while no bubble production was considered as negative.

6.2.3.4.1.6-Salmonella Shigella Agar:

Salmonella Shigella agar (SS agar) selectively allow the isolation of *Salmonella* and some *Shigella* species by inhibiting the growth of other Gram positive and even Gram-negative bacteria like coliforms and Proteus species. For the preparation of media plates, 60g of SS agar was dissolved in 1L of distilled water and autoclaved (121ºC for 15 minutes). The suspected bacterial colonies were inoculated on the plates via streak plate method followed by incubation at 37℃ for 18-24 hrs.

6.2.3.4.1.7-Eosin Methylene Blue Agar Test

Eosin methylene blue (EMB) agar is a selective and differential media for the isolation and identification of Gram-negative bacilli like enteric and non-enteric bacilli. It further helps in the differentiation between lactose fermenter and non-fermenter organisms. The acid production was detected by dark purple complex formation with the colony having green metallic sheen. Organisms with slower fermentation rate produced colonies as light pink and non-fermenter produced slight pink or opaque colour. For aerobic bacteria, plates were incubated in aerobic

incubator at 37°C while for facultative anaerobic bacteria, plates were incubated after placing in wax sealed glass jars supplemented with CO_2 sachets for 24 hrs at 37°C. After incubation, culture was checked carefully for any change in the colour of the colonies.

6.2.3.4.2-Biochemical Identification Scheme for Gram-Positive Bacterial Species

6.2.3.4.2.1-Catalase Test

The catalase test was performed to distinguish between various Gram-positive isolates obtained. This test differentiated between *Staphylococcus* spp and *Micrococcus* spp (Catalase test was procedure as mentioned in section 6.2.3.4.1.5.

6.2.3.4.2.2-Oxidase Test

This test was used to distinguish between *Neisseria gonorrhoeae* (oxidase-positive) from *Staphylococcus* species which are oxidase negative. Oxidase test was performed as mentioned in section (6.2.3.4.1.1).

6.2.3.4.2.3-Coagulase Test

This test differentiated between *Staphylococcus aureus* which are coagulase-positive bacteria, from *S. epidermis* and *S. saprophyticus* which are coagulase negative. For this test, bacteria were allowed to grow in 0.5 mL of nutrient broth (Oxoid, England) test tube at 37°C for 24 hrs. Nutrient broth was prepared by adding 13.0 g of media in 1000 mL of distilled water and autoclaved. After incubation, 2-3 drops of plasma were added in test tube and again incubated for about 4-5 hrs. Presence of coagulation after incubation confirmed the presence of *S. aureus*.

6.2.3.4.2.4-Hemolysis Test on Blood Agar

The test was used to test the ability of the bacteria to produce haemolysin enzyme that act on red blood cells and lyse erythrocytes. Blood agar media plates supplemented with 5% (v/v) sheep blood was used to assess the degree of haemolytic capability of the bacteria. It was used to distinguish between *Staphylococcus*, *Streptococcus* and *Enterococcus* bacteria which have three types of haemolytic ability, namely α, β, and γ.

Blood agar media plates were prepared as previously mentioned (Chapter 5, section 5.2.5.2). Fresh bacterial cultures were used for inoculation, a single isolated colony of the bacteria to be tested was picked from the culture plate by using sterile loop and streaked on the blood agar plate via streak plate method. The inoculated plates were incubated at 37 ℃ for 24 hrs. After 24 hrs, α, β, γ haemolysis was checked and the results were interpreted.

6.2.4- Part II. Study of Bacterial Diversity by Culture Independent Approach

6.2.4.1- Study population and Sample Collection

For gut microbiome detection, variable region (V4 region) of bacterial 16S rRNA gene was used for sequencing, a total of 36 obese stool samples were selected from the total faecal samples as described in Appendix III. The stool samples used for analysis were equal number from both gender, age ≤ 40 years and BMI ≥30 kg/m^2.

6.2.4.2- Bacterial DNA Extraction from Stool, Sequencing and Bioinformatics Analysis

The DNA extraction from stool samples for 16S rRNA gene sequencing was carried out by using standard phenol/chloroform method with some modifications (Neumann *et al*. 1992; Hassanzadeh & Sciences, 2019).

DNA Extraction Procedure:

1. A fresh stool sample (1.0 g) was weighed and suspended in 1 mL of normal saline solution in the Eppendorf followed by centrifugation at 8000g for 2 min.
2. After centrifugation, supernatant was discarded and to the pellet 200 µl TE buffer, 5 µl of proteinase K and 30 µl of sodium dodecyl sulphate was added and the pellet was incubated in water bath for 1 hr at 37℃.
3. After incubation, CTAB buffer (100 µl) and NaCl (80 µl) were added to the tubes and allowed to incubate further for 10 minutes in water bath at 65℃.
4. After incubation, 500 µl of phenol-chloroform isoamyl alcohol (PCI) was added to the tubes followed by centrifugation at 10,000 rpm for 20 minutes at room temperature.
5. After centrifugation, two layer were forms based on density gradient. Supernatant was carefully shifted into fresh Eppendorf tubes without touching the lower layer. The Step 4

was addition of PCI and step 5 was separation of supernatant to fresh tube. It was repeated twice for effective removal of proteins.

6. The supernatant was treated with 500 µl of Isopropanol and 300 µl of Sodium acetate solution to precipitate out the DNA. These tubes were incubated overnight at -4℃ for effective precipitation.
7. These tubes from -4℃ was taken and centrifuged at 10,000 rpm for 5minutes.
8. After centrifugation, supernatant was discarded while pellets were treated with 200 µl of Ethanol and washing step was repeated twice.
9. After washing, pellets were allowed to air dried at room temperature for 30 minutes.
10. After drying, the pellets were re-suspended into 50µL of TE buffer and store at -20℃.
11. For qualitative and quantitative assessment of DNA, gel electrophoresis and nano-drop were used. The qualitative analysis was done on 1% agarose gel as mentioned previously in Chapter (4) section (4.2). The DNA quantification was done via A260/A280 ratio by using nanodrop spectrophotometer (Titertek Berthhod, Germany).

Sequencing and Bioinformatics Quality Control Analysis

The V4 region of 16S rRNA gene was amplified in each sample using universal primers 515F 5'GTGCCAGCMGCCGCGGTAA-3' 806R 5'GGACTACHVHHHTWTCTAAT-3' containing common adaptor sequences, and then the Illumina flow cell adaptors and dual indices were added in a secondary amplification as described by Gohl *et al*., (2016). The resulting amplicons were subsequently subjected to high-throughput sequencing using the Illumina MiSeq platform. Sequencing was performed using Illumina MiSeq platform at University of Minnesota (College of Veterinary Medicine).

Bioinformatics quality control process

1. The data sequenced by Illumina Miseq are called raw reads a double-ended sequencing data in FASTQ format. The obese and the control subjects DNA were distinguished based on barcodes.

2. The bioinformatic workflow was carried out in SHAMAN that follows the metataxonomic pipeline based on Galaxy platform (Jalili *et al*., 2021).
3. Optional filtering of reads was done based on alignment with the host genome and the PhiX174 genome (used as a control in Illumina Mi seq). This step was performed with Bowtie2 v2.2.6 (Langmead *et al*., 2009).
4. Quality of reads trimming off contaminant sequences and clipping was done with Alien Trimmer v0.4.0 (Criscuolo & Brisse, 2013).
5. Paired-end reads are then merged with Pear v0.9.10.1 (J. Zhang *et al*., 2014).
6. OTU picking, taxonomic annotation and OTU quantification are performed using Vsearch v2.3.4.0 (Rognes *et al*., 2016), that has been considered as both accurate and efficient software. The whole procedure consists of five steps: dereplication, singleton removal, chimera detection, clustering and alignment. A contingency table was constructed by input amplicons aligned against the set of detected OTUs that create table with number of amplicons assigned to each OTU.
7. The taxonomic annotation of OTUs was performed based on SILVA(Pruesse *et al*., 2007) and Greengenes (DeSantis *et al*., 2006).
8. OTU annotations were filtered according to their identity with the reference (Yarza *et al*., 2014). Phylum annotations were identified after ≥ 75% similarity with the reference sequence, for class ≥ 78.5%, for order ≥ 82%, for family ≥ 86.5%, for genus ≥94.5% and for specie ≥ 98%. The taxonomic interpretation was carried out by naive Bayesian approach by RDP classifier (Q. Wang *et al*., 2007) v2.12.
9. The outcomes of the overall workflow were in the form of BIOM file (per reference database) as well as a summary file.

6.2.4.3-Metanalysis of 16S rRNA Gene Data of Obese vs. Control Subjects

The microbiome analysis was carried out by comparing data from obese 16S rRNA gene sequencing data of current study with 16S rRNA gene sequencing data of healthy controls from previous Pakistani study by Batool *et al*., (2020) . That data was of 32 healthy individuals, it was used to compare the DNA analysis data from 36 obese study subjects from current study, with 16S rRNA gene sequencing data to compare gut diversity. These samples were analysed along with

the control samples through an online software Microbiome Analyst assessed on 28 November, 2022. As taxonomic nomenclature has been recently updated thus to avoid any confusion the nomenclature was kept similar to the results interpreted by the software Microbiome analyst for clarification of the statement in relation with the presented graphs and heatmaps. These were further checked for correlation of dietary and demographic factors with the gut microbial composition with obesity.

6.2.4.4-Chromogenic Limulus Amebocyte Lysate assay

The quantitative analysis for measurement of serum endotoxin level among obese samples which were also sequenced with control samples to check relation of gut dysbiosis with reference to LPS formation was done via Limulus Amebocyte Lysate assay (LAL assay). As the serum endotoxemia (presence of LPS), trigger TLR-4 mediated low grade inflammation in obesity. The experiment was carried out using Pierce™ Chromogenic Endotoxin Quant Kit. The LAL assay produces a yellow colour (chromogenic) by causing the activation of limulus clotting factor C by an enzymatic action of bacterial endotoxin if present in the serum sample. The assay was carried out as per manufacturers protocol for quantification of bacterial toxin as endotoxin units (EU) per mL and OD at 405nm using a spectrophotometer (MultiScan Go.)

6.3- RESULTS

6.3.1- Part I. Isolation and Identification of Aerobic Cultureable Bacterial Isolates

All of the faecal samples from obese, pre-obese and controls were inoculated on nutrient agar for 24 hr at 37ºC. After incubation, colonies with different colony morphology were picked with sterile loop and streaked on the plate then incubated for 24 hrs at 37 ºC. Among obese group, in 75 samples 370 isolates were recovered. In case of 26 pre-obese stool samples 137 isolates and 255 isolates were obtained from healthy controls' samples.

6.3.1.2- Abundance of Bacteria Using CFU/mL Method

All faecal samples were serially diluted up to 10^{-6} and bacterial count was found in range o10^{-1}-10^{-4} CFU/mL. The average abundance of bacteria in faecal samples for obese were $4.42x10^{6}$

CFU/mL, pre-obese 5.88x10^6 CFU/mL and controls 6.16 x10^6 CFU/mL. All the bacterial isolates were stored as 40% glycerol stock solution in Eppendorf tubes.

6.3.1.3- Gram Staining

All of the isolates were Gram stained. In 370 isolates from obese individuals, 36(9.7%) were Gram-positive cocci, and rest 335(90.5%) were Gram negative rods. In pre-obese group out of 136 isolates, 8 (5.8%) were Gram positive cocci, and 128 (94.1%) were Gram negative rods. In control group samples out of 255 isolates, 18(7.05%) were Gram positive cocci, and majority 237 (92.9%) isolates were Gram negative rods.

6.3.1.4- Identification of Aerobic Gram-Negative Gut Bacteria

6.3.1.4.1- Growth on MacConkey Agar

All of the isolated Gram-negatives from obese, pre-obese and controls were checked for lactose fermentation that showed out of 335 Gram negative rods, 234 (71.3%) produced pink colour colonies on MacConkey agar indicating the lactose fermentation. In pre-obese stool isolates, 95/136 isolates showed lactose fermentation. While in control group isolates, 163/232 isolate were lactose fermenter.

6.3.1.4.2-Oxidase Test

In obese group, 71 isolate were oxidase positive and were 19.1% of all Gram negative isolates, whereas 19 (13.9%) were oxidase positive in pre-obese group. Among controls, 43 (18.5%) were oxidase positive.

6.3.1.4.3-Triple Sugar Iron Test

In obese group 242 isolates produced reactions like acidic slant and acidic butt (A/A) with positive gas production. Sixty-four isolates produced alkaline-slant and alkaline-butt (K/K) with no gas and H_2S production, which were also negative for lactose fermentation and oxidase. Among all, about 26 isolates showed alkaline-slant and acidic-butt (K/A) with no gas or H_2S production. There were four isolates that showed alkaline-slant and acidic-butt (K/A) with gas and H_2S production.

In pre-obese group, there were total of 163 isolates that produced acidic-slant and acidic-butt (A/A) with positive gas production. Nineteen non-lactose former and oxidase negative isolates were positive for alkaline-slant with alkaline-butt (K/K) but there was no gas or H_2S production. Nine isolates showed alkaline-slant and acidic-butt (K/A) results with no gas or H_2S production.

In controls group isolates, acidic-slant and acidic-butt (A/A) with positive gas production was observed in 163 isolates. The oxidase negative and non-lactose former isolates (*n*=43) showed alkaline-slant and alkaline-butt (K/K) with no gas or H_2S production. Fourteen isolates with alkaline-slant and acidic-butt (K/A) with no gas or H_2S production were observed. About twelve isolates produced alkaline-slant and acidic-butt (K/A) with gas and H_2S production

6.3.1.4.4-Indole Test

Further confirmation of the isolates was made on basis of indole production; 161 obese individuals' stool sample isolates were indole positive rest 167 negative. In pre-obese indole positive were 68 indole positive. In controls group isolates, 132 were indole positive and 100 indole negative.

6.3.1.4.5-Citrate Utilization Test

In case of obese 144 were citrate positive and 184 were citrate negative whereas in pre-obese 50 isolates showed citrate positive results and 75 negative. In case of control group, 84 isolates were citrate positive and 148 were citrate negative.

6.3.1.4.6-Catalase Test

Catalase for identification of *Enterobacteriaceae* family member along with other biochemical tests was performed in which positive catalase activity by forming bubbles in reaction to addition of H_2O_2 was checked. In case of obese group 214 were positive for catalase activity, in case of pre-obese all the isolates were catalase positive while in control group 245 isolate were positive.

6.3.1.4.7-Salmonella Shigella Agar

In case of isolates from obese individuals, 1 *Salmonella* and 21 *Shigella* isolates were confirmed after observing colourless colonies with black centre for *Salmonella* and colourless colonies for

the *Shigella* isolates. In pre-obese group 7 isolates were confirmed to be *Shigella* and 12 isolates to be *Salmonella*.

6.3.1.4.8-Eosin Methylene Blue Agar Test

Further confirmation of the Gram negative rods was carried out on the Eosin methylene blue agar. In case of obese group isolates, 80 isolates produced purple colour colonies with green metallic sheen while 73 isolates produced dark purple colonies and 53 isolates produced light purple colonies. Among pre-obese, 37 isolate produced dark purple colonies with green metallic sheen, 29 produced dark purple colonies and fourteen produced light purple colonies. In case of control group isolates 51 isolates produced purple colour colonies with green metallic sheen while 53 isolates produced dark purple colonies and 28 were light purple in colour. The isolates that were producing dark purple colour and green metallic sheen were identified to be *E. coli*. The light purple colour producing colonies were identified to be *Klebsiella pneumoniae*.

6.3.1.4.9-Identified Gram Negative Bacterial Isolates from Pre-obese, obese and Control Subjects

Afet series of biochemical test, the identified different Gram negative species as shown in Table 6.1(Appendix XXIII). Among them *Escherichia coli* was the most prevalent specie followed by the *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter cloacae* in all three groups. The less common species in obese gut were *Salmonella typhi* and *Proteus vulgaris*.

Table 6.1: Identified Gram negative bacterial isolates from gut of obese and healthy control subjects

Isolates Identified	Obese *n* (%)	Control *n* (%)	*p* value	OR (CI-95%)
Escherichia coli	153 (45.6)	107 (45.1)	0.9	1.02 (0.73-1.42)
Pseudomonas aeruginosa	64 (19.1)	43 (18.1)	0.7	1.06 (0.62-1.63)
Klebsiella pneumoniae	53 (15.8)	28 (11.8)	0.1	1.40(0.85-2.29)
Enterobacter cloacae	19 (5.6)	15 (6.3)	0.7	0.88(0.44-1.78)
Shigella spp	21 (6.2)	11 (4.6)	0.4	1.37(0.64-2.90)
Enterobacter aerogens	9 (2.6)	13 (5.4)	0.08	0.47(0.19-1.13)
Campylobacter specie	7 (2.0)	5 (2.1)	0.9	0.99(0.31-3.15)
Acinetobacter	5 (1.4)	2 (0.8)	0.4	1.78(0.34-9.25)
Salmonella typhi	1 (0.2)	13 (5.4)	>0.01*	0.05(0.006-0.39)
Proteus vulgaris	3 (0.8)	-		
*Significant association at *p* value <0.05				

6.3.1.5- Identification of Aerobic Gram-Positive Gut Bacteria from Pre-obese, obese and Control Subjects

6.3.1.5.1-Catalase Test

All the Gram positive isolates among obese samples were subjected to catalase test. These results showed 36 catalase positive (*Staphylococcus*) while remaining 8 as catalase negative (*Streptococcus*) isolates among obese group. Among pre-obese group all the isolates (11) were catalase positive, and none was catalase negative. In control group all the isolates (23) were catalase positive suspected to be *Staphylococcus*.

6.3.1.5.2-Oxidase Test

All of the suspected *Staphylococci* were checked by the oxidases test, all of the suspected *Staphylococcus* isolates from all three group samples showed oxidase positive results.

6.3.1.5.3-Coagulase Test

All the suspected *Staphylococcus* isolates were confirmed as *Staphylococcus* aureus, which were were in obese 27 (64.2%), pre obese 8(72.7%) and controls 18(78.2%). There were also coagulase positive *Staphylococcus* aureus isolates.

6.3.1.5.4-Hemolysis Test on Blood Agar:

Haemolysis on the blood agar by isolates from obese subjects showed alpha haemolysis by 8 isolates which were negative for catalase production, hence were confirmed to *Streptococcus* specie. While 27 isolates showed beta haemolysis that were confirmed as *Staphylococcus* aureus.

6.3.1.5.5-Identification of Gram Positive Gut Bacteria from Pre-obese, obese and Control Subjects

All biochemical tests confirm the identification of Gram positive which are shown in Table 6.2 (AppendixIV). Among these isolates, *S. aureus* were identified among isolates of all the three study groups. *Streptococcus* specie was identified in obese group samples but it was only one isolate.

Table 6.2: Identified Gram positive bacterial isolates from gut of obese and healthy control subjects

Type of bacteria	**Isolates**	**Obese *n* (%)**	**Control *n* (%)**	***p* value**	**OR (CI-95%)**
Gram positive cocci (aerobes)	*Staphylococcus aureus*	27 (64.2%)	18 (78.2%)	0.08 *	0.04 (0.004-1.60)
	Streptococcus specie	8 (19.0 %)			
*Significant association at *p* value <0.05					

6.3.2-Part II. Isolation and Identification of Gut Anaerobic Cultureable Bacteria from Pre-obese, obese and Control Subjects

Abundance of Bacteria Using CFU/mL Method

All faecal samples were serially diluted up to 10^{-6} and checked on blood base agar and thioglycolate agar for their growth. The bacterial count was 10^{-1}-10^{-3} CFU/mL. The average abundance of bacteria in faecal samples for obese were $1.3x10^5$ CFU/mL, pre-obese $1.7 x10^5$ CFU/mL and controls $1.5x x10^5$ CFU/mL. All the bacterial isolates were stored in 40% glycerol stock solution in Eppendorf tubes.

The facultative anaerobes isolated from the initial 75 samples of obese showed 88 isolates, from 26 pre-obese samples 47 isolates were recovered while from 50 control samples 97 isolates were obtained. All the isolates were Gram negative rods that were recovered as presented in Table: 6.3(Appendix XXV). These were identified as described in the previous section (6.3.1.4). The conditions provided by airtight jars with gas generation system has allowed the isolation of only facultative anaerobes among all three study groups samples. To assess further details about the bacterial gut diversity cultureable independent techniques were performed following PCR based 16S rRNA gene sequencing.

6.3.2.1-Identification of Cultureable Gut Facultative Anaerobic Bacteria from Pre-obese, obese and Control Subjects

The highly prevalent facultative anaerobic bacteria identified were *E.coli* followed by the *K. pneumonia* and *P. aeruginosa* among study subject samples. In obese group, *E. aerogens* was absent which was present only in the control group samples. While facultative anaerobic *S. typhi* was present only in obese samples in comparison with other two study subject groups. Among Gram positives were only 3 isolates recovered from obese group and were identified as *Streptococcus* specie.

Table 6.3: Identified Gram negative anaerobic bacteria among obese, and healthy control subjects

Isolates	**Obese n (%)**	**Controls n (%)**	***p* value**	**OR (CI-95%)**
E.coli	37 (42.0)	33(34.0)	0.2	1.40(0.77-2.55)
K. pneumonia	24 (27.2)	23(23.7)	0.5	1.20(0.62-2.34)
P. aeruginosa	12(3.6)	12(12.3)	0.8	1.07(0.45-2.54)
E cloacae	3 (3.4)	12(12.3)	0.02*	0.24(0.06-0.88)
Shigella species	3 (3.4)	11(11.3)	0.03*	0.26(0.07-0.98)
E aerogenes	-	6(6.1)	-	-
S. typhi	9 (10.3)	-	-	-
*Significant association at *p* value <0.05				

6.3.3- Part III. Gut Microbial Diversity by Culture Independent Approach

6.3.3.1- Sequencing characteristics:

After 16S RNA based sequencing, the taxonomic based classification obtained 4,329,278 reads in case of 36 obese samples with minimum=31954 and maximum= 162070 reads. While in case of the control samples taxonomy 5,580,520 reads were obtained with minimum = 7,847 and maximum = 353,529 reads.

6.3.3.2- Taxonomic Analysis

The relative abundance of order *Oscillospirales p*=0.000847, Clostridia_UCG_01 *p*= 43.00e11, Clostridia_vadinBB60_group *p*=1.37e10, *Christensenellales p*= 7.97e14, *Clostridiales p*= 3.08e12 and *Peptostreptococcales_Tissierellales p*= 2.79e13 significantly increased in the obese individuals while the order *Bacteroidales p*=0.065917 decreased in the obese person (Table 6.4.). The relative abundance of class Bacilli *p*= 2.41e09, *Negativicutes p*= 7.97e14, *Gammaproteobacteria p*= 7.97e14, *Alphaproteobacteria* p= 9.77e12, *Lentisphaeria p*= 3.08e12, *Vampirivibrionia p*= 4.74e07, *Desulfovibrionia p*= 8.43e08 and *Saccharimonadia p*=9.77e12 significantly increased in obese individuals while the class *Bacteroidia p*= 0.065917 significantly decreased in obese. The relative abundance of phyla *Firmicutes p*= 2.66e03, *Actinobacteriota p*= 3.86e01, *Proteobacteria p*= 7.97e14, *Verrucomicrobiota p*= 3.08e12, *Cyanobacteria p*=4.74e07, *Desulfobacterota p*= 8.43e08 and *Patescibacteria p*=9.77e12 significantly increased in obese group members. While the relative abundance of *Bacteroidota* significantly decreased in this group.

Heat map was generated using relative abundance of various taxa for each sample at the Phylum, class and order level (Figure 6.1-6.3). Red colour in Figure 6.4 represents relatively higher abundance of phyla and yellow represents relatively less abundant phyla followed by purple and orange.

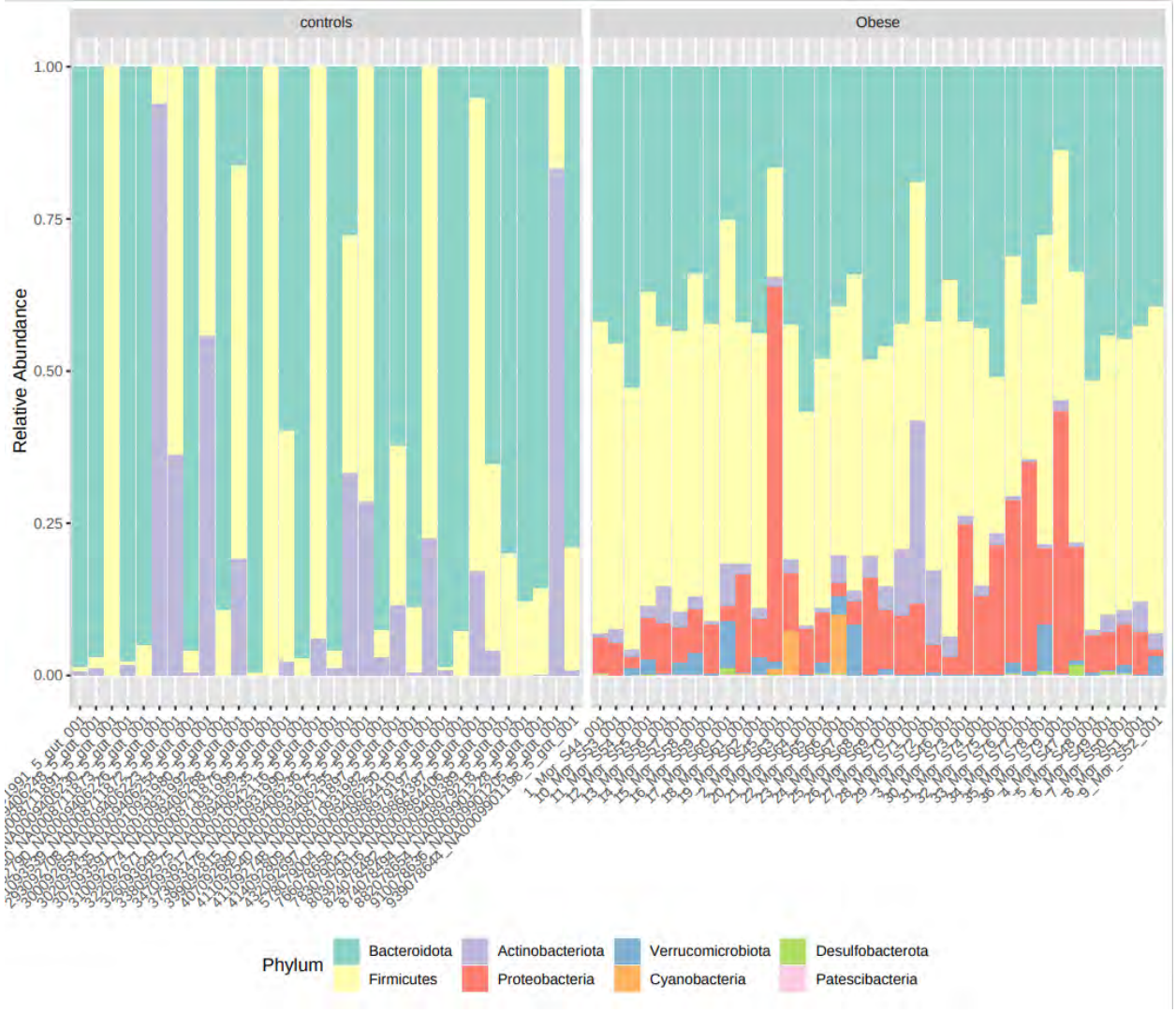


Figure 6.1: Relative Abundance of different phyla identified in gut microbiome of obese and control subjects

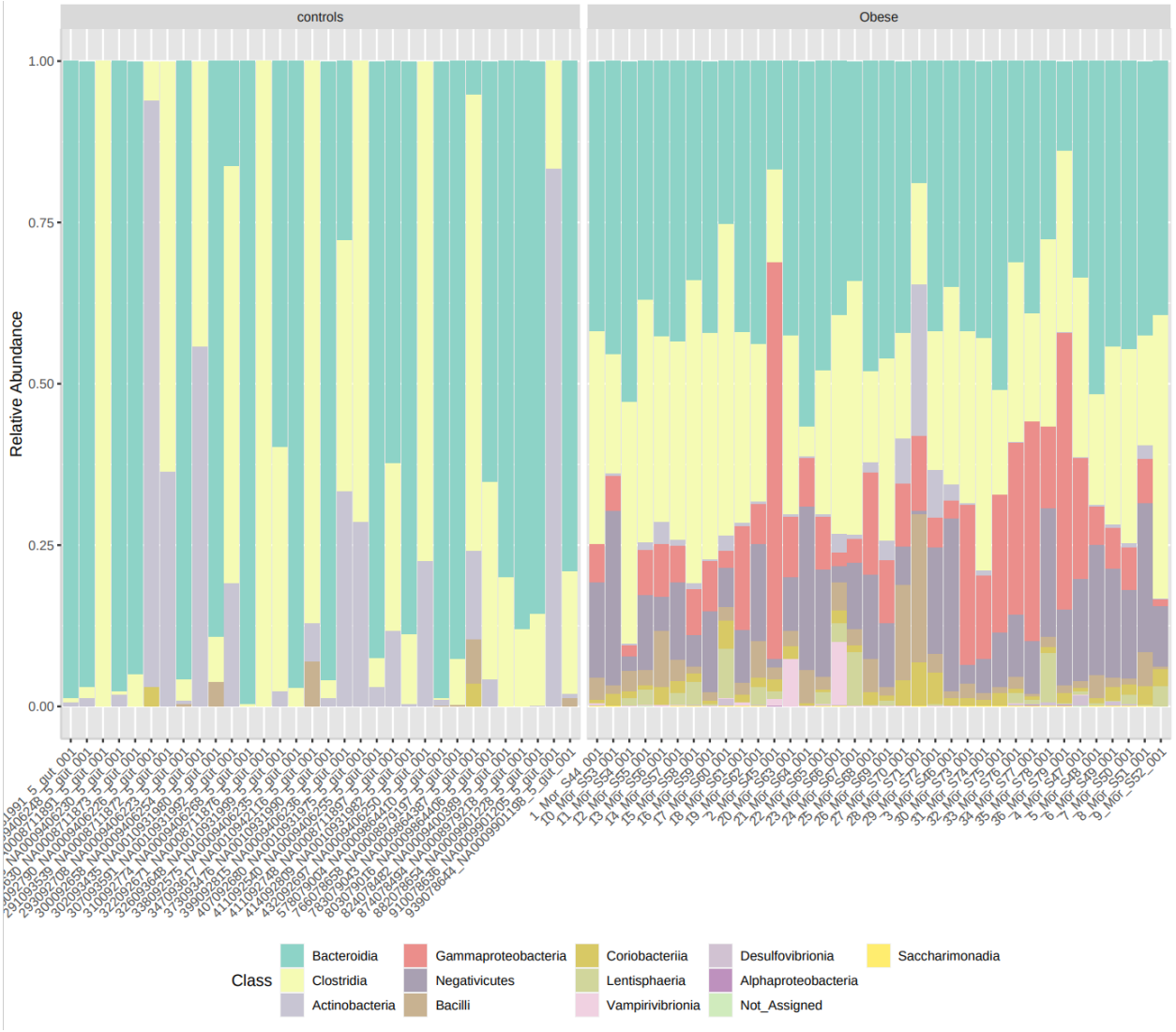


Figure 6.2: Relative abundance of different classes identified in gut microbiome of obese and control subjects

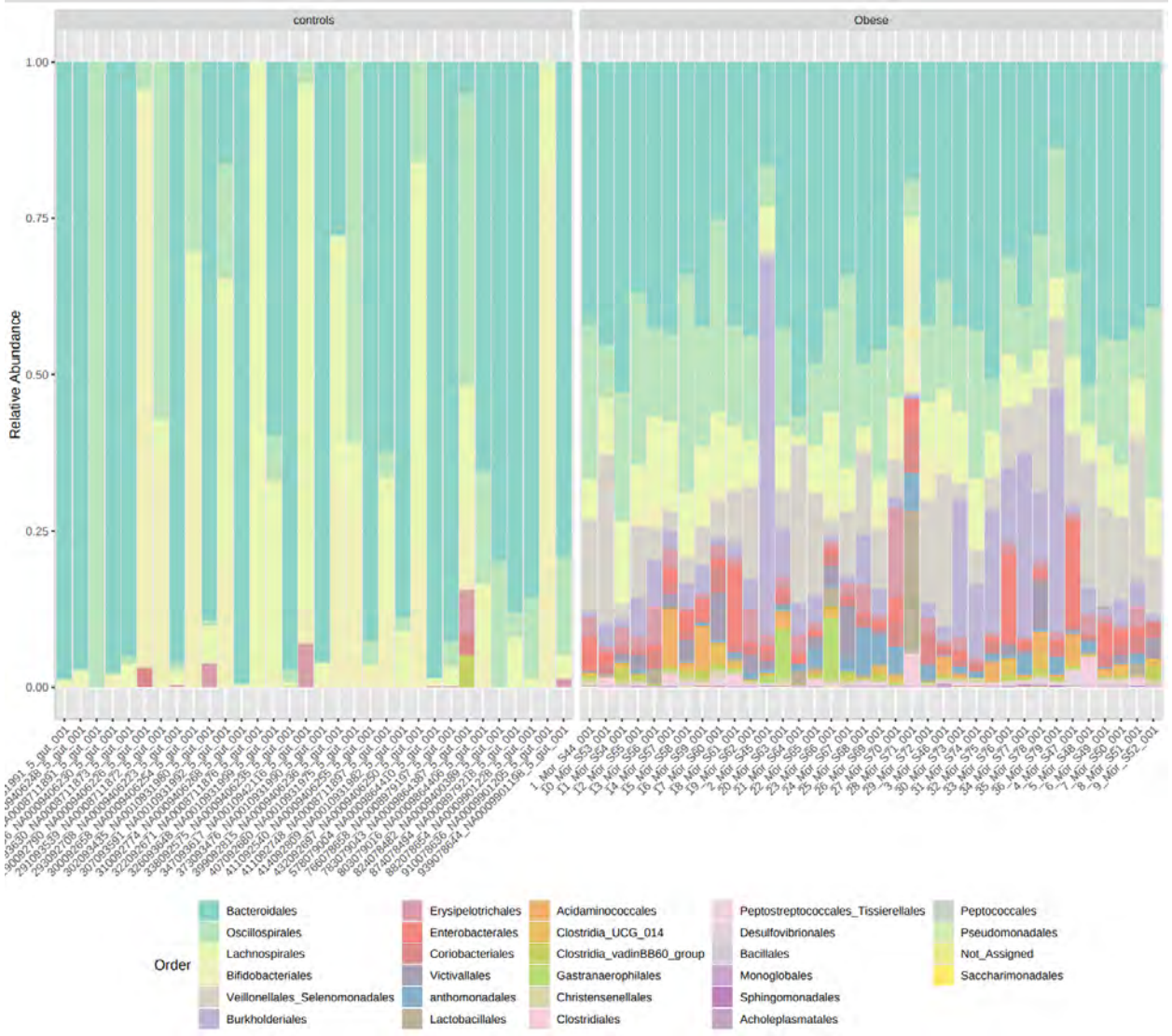


Figure 6.3: Relative abundance of different orders identified in gut microbiome of obese and control subjects

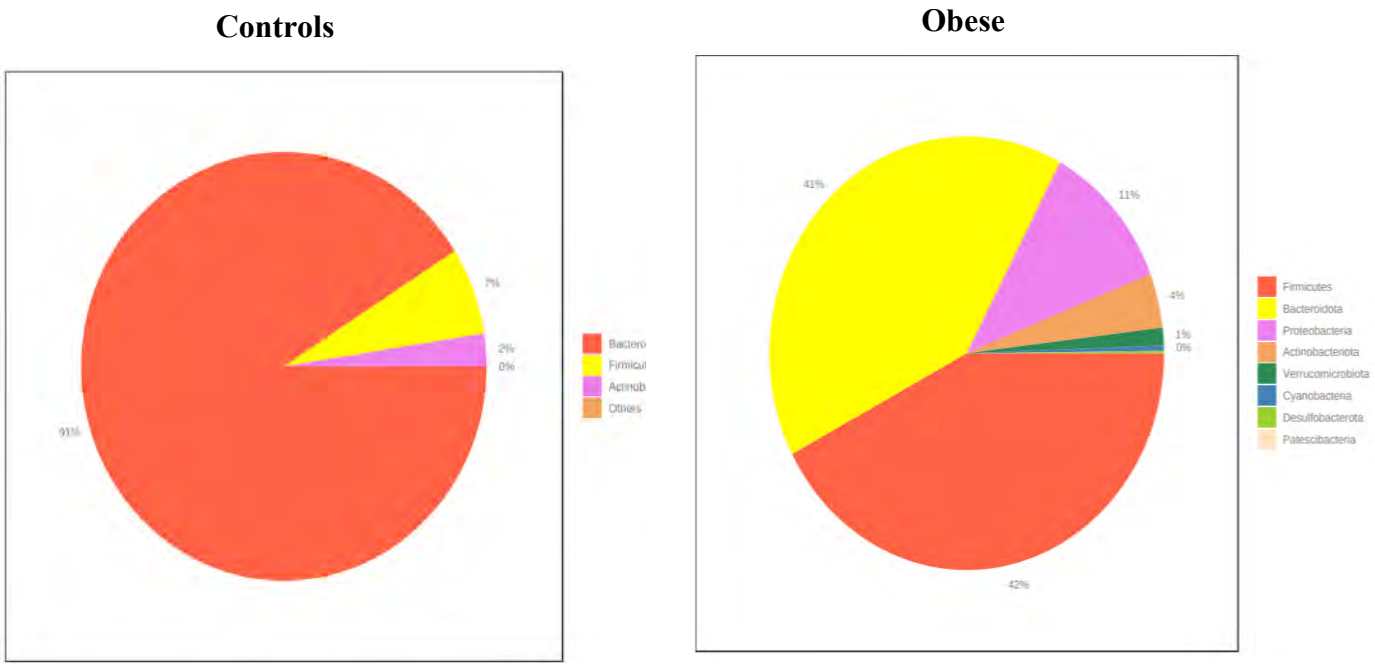


Figure 6.4: Relative percentage distribution of bacterial phyla identified in gut microbiome of obese and control subjects

Table 6.4. Relative abundance of gut microbiome at phylum, class and order level in obese (*n*=36) and controls subjects (*n*=32) after FDR adjustment using Kruskal-Wallis rank-sum tests.

Bacterial Taxonomy		**Relative Abundance**		***p*-value**	
		Obese	**Control**	***p*-value**	**FDR**
Bacteroidota	**Phylum**	**40.53%**	**90.95%**	**6.59e02***	**7.42e02***
Bacteroidia	Class	40.52%	90.95%	0.065917*	0.076903*
Bacteroidales	Order	40.49%	90.95%	0.065917*	0.0708*
Firmicutes	**Phylum**	**42.31%**	**6.59%**	**2.66e03***	**3.42e03***
Clostridia	Class	26.52%	6.42%	0.14882	0.16027
Oscillospirales	Order	16.06%	3.2%	0.000847*	0.000945*
Lachnospirales	Order	8.17%	3.19%	0.27686	0.27686
Clostridia_UCG_014	Order	0.59%	0.0%	3.00e11*	6.21e11*
Clostridia_vadinBB60_group	Order	0.43%	0.0%	1.37e10*	2.48e10*
Christensenellales	Order	0.46%	0.0%	7.97e14*	4.63e13*
Clostridiales	Order	0.37%	0.0%	3.08e12*	8.94e12*
Peptostreptococcales_Tissierellales	Order	0.36%	0.0%	2.79e13*	1.16e12*
Bacilli	Class	3.83%	0.17%	2.41e09*	4.21e09*
Erysipelotrichales	Order	2.42%	0.17%	1.09e08*	1.76e08*
Victivallales	Order	1.31%	0.0%	3.08e12*	8.94e12*
Lactobacillales	Order	1.28%	0.0%	2.79e13*	1.16e12*
Bacillales	Order	0.1%	0.0%	7.97e14*	4.63e13*
Negativicutes	Class	11.96%	0.0%	7.97e14*	5.58e13*
Acidaminococcales	Order	1.01%	0.0%	2.58e10*	4.40e10*
Veillonellales_Selenomonadales	Order	10.95%	0.0%	7.97e14*	4.63e13*

Actinobacteriota	**Phylum**	**4.07%**	**2.45%**	**3.86e01***	**3.86e01***
Actinobacteria	Class	4.07%	2.43%	0.27122	0.27122
Bifidobacteriales	Order	2.24%	2.43%	0.27122	0.27686
Coriobacteriales	Order	1.83%	0.0%	4.93e11*	9.53e11*
Proteobacteria	**Phylum**	**11.2%**	**0.0%**	**7.97e14***	**7.18e13***
Gammaproteobacteria	Class	11.16%	0.0%	7.97e14*	5.58e13*
Enterobacterales	Order	2.47%	0.0%	7.97e14*	4.63e13*
Pseudomonadales	Order	0.01%	0.0%	1.36e08*	2.07e08*
Xanthomonadales	Order	1.53%	0.0%	3.08e12*	8.94e12*
Alphaproteobacteria	Class	0.04%	0.0%	9.77e12*	2.73e11*
Betaproteobacteria_ Burkholderiales	Order	7.13%	0.0%	7.97e14*	4.63e13*
Verrucomicrobiota	**Phylum**	**1.31%**	**0.0%**	**3.08e12***	**1.39e11***
Lentisphaeria	Class	1.31%	0.0%	3.08e12*	1.44e11*
Cyanobacteria	**Phylum**	**0.4%**	**0.0%**	**4.74e07***	**8.53e07***
Vampirivibrionia	Class	0.4%	0.0%	4.74e07*	6.64e 07*
Desulfobacterota	**Phylum**	**0.17%**	**0.0%**	**8.43e08***	**1.90e07***
Desulfovibrionia	Class	0.17%	0.0%	8.43e08*	1.31e07*
Patescibacteria	**Phylum**	**0.01%**	**0.0%**	**9.77e12***	**2.93e11***
Saccharimonadia	Class	0.01%	0.0%	9.77e12*	2.73e11*

6.3.3.3-Community Based Analysis in Gut Microbiome of Obese and Control Subjects

6.3.3.3.1-Alpha diversity

Alpha diversity of gut microbiota within each sample was assessed by the Shannon index, Simpson index, Observed Species box plot and Chao_1 box plot. These are the comprehensive indicators of species richness and uniformity in community microflora. Significant difference was observed between obese individuals and controls in community richness and evenness which evident from high values calculated for alpha diversity (Figures 6.5).

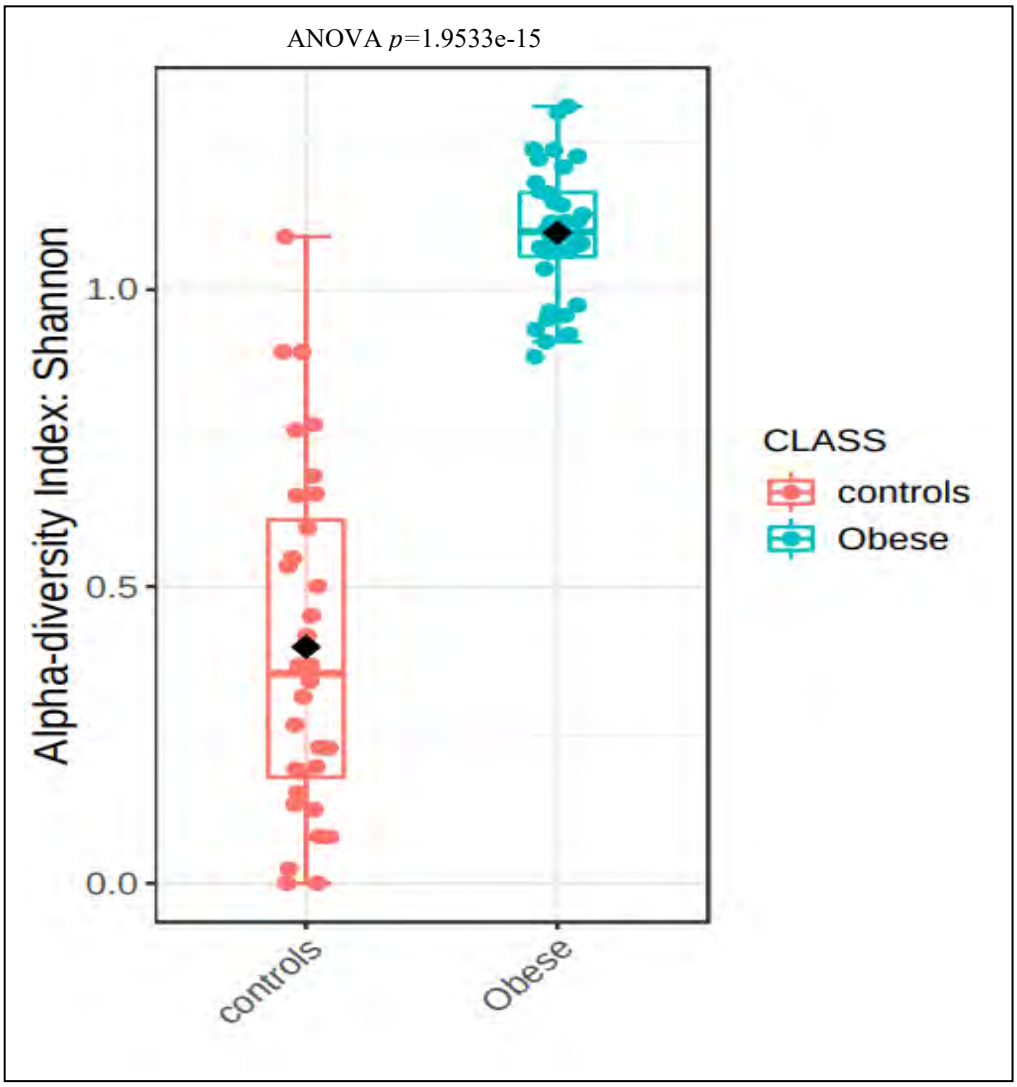


Figure 6.5: Alpha diversity of gut microbiome by comparison of boxplots of Phyla between obese (n=36) and control subjects (n=32) where Shannon index indicates the number of Phyla richness

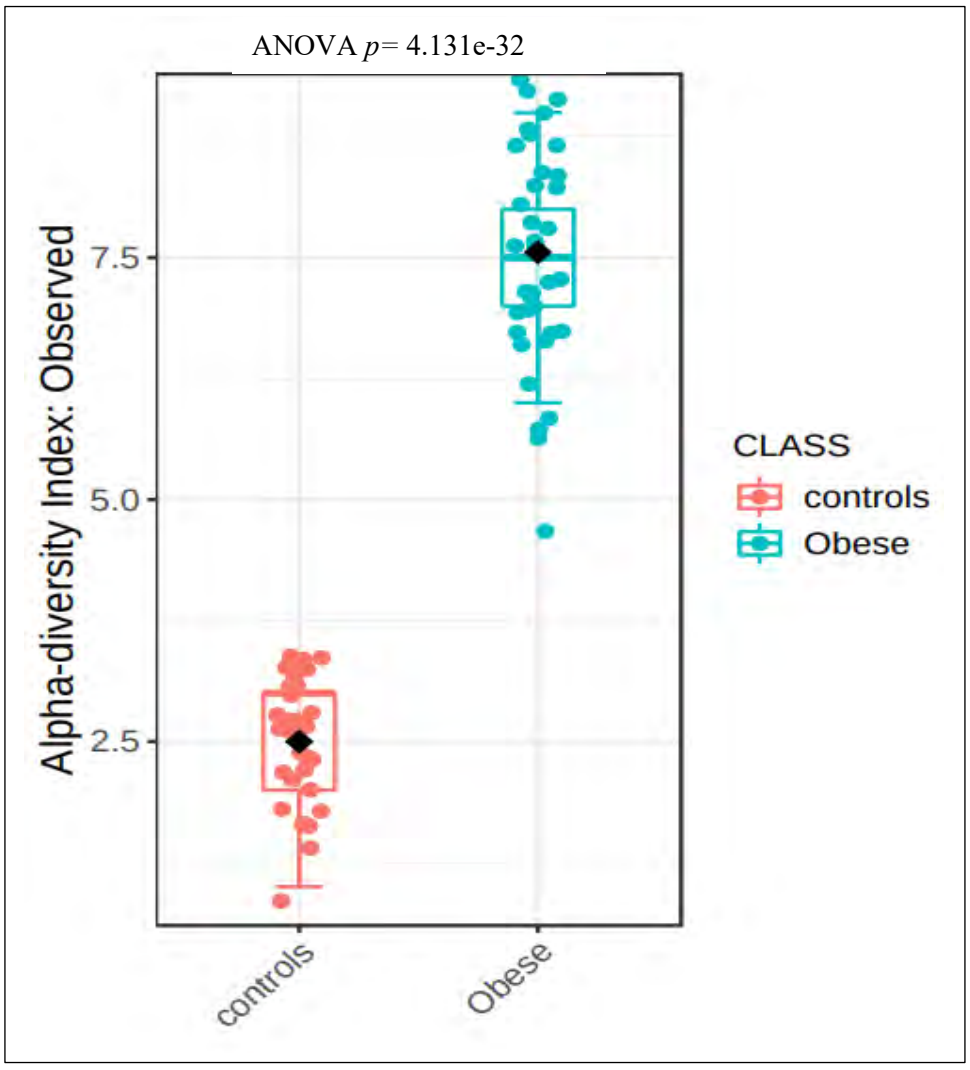


Figure 6.6: Alpha diversity of gut microbiome of obese (36) and control subjects (n=32), where observed species box plots indicate the number of actually observed Phyla richness.

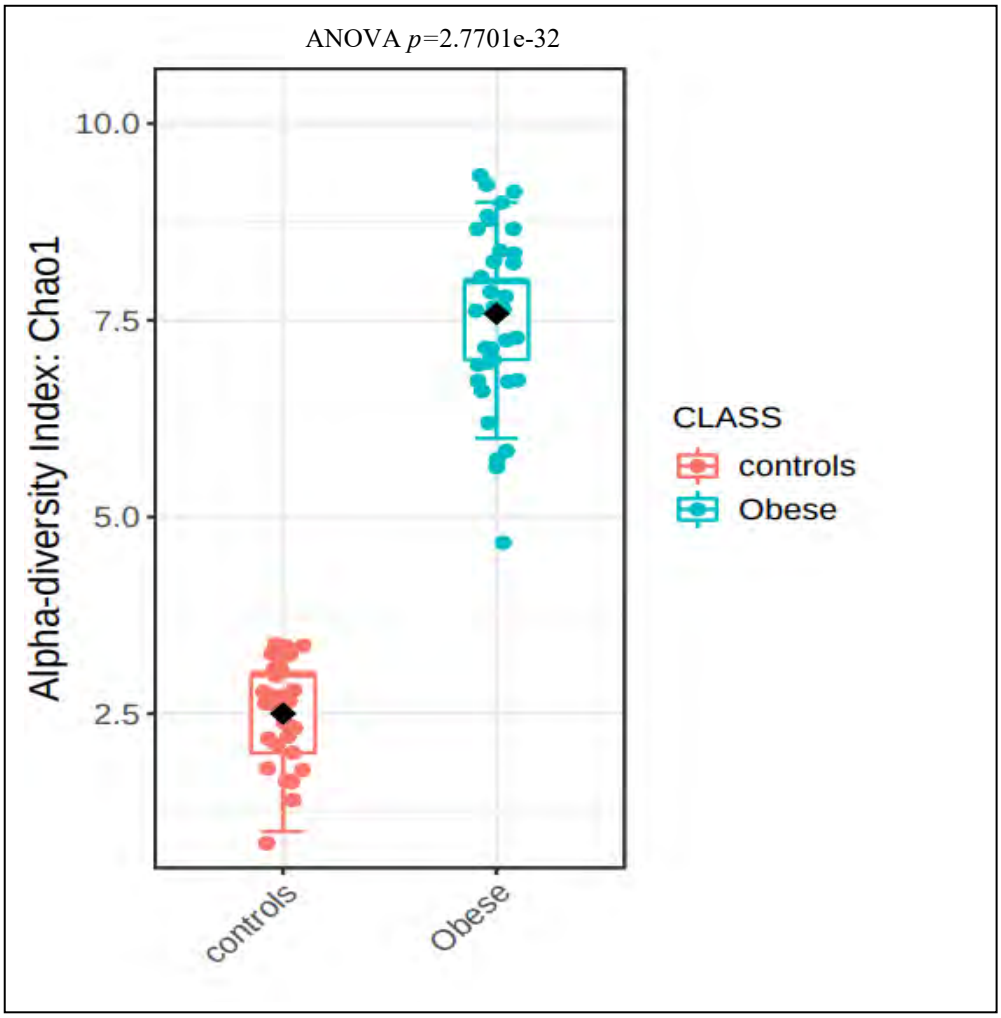


Figure 6.7: Alpha diversity gut microbiome of obese (36) and control subjects (n=32), where Chao 1 box plots indicates the number of Phyla richness

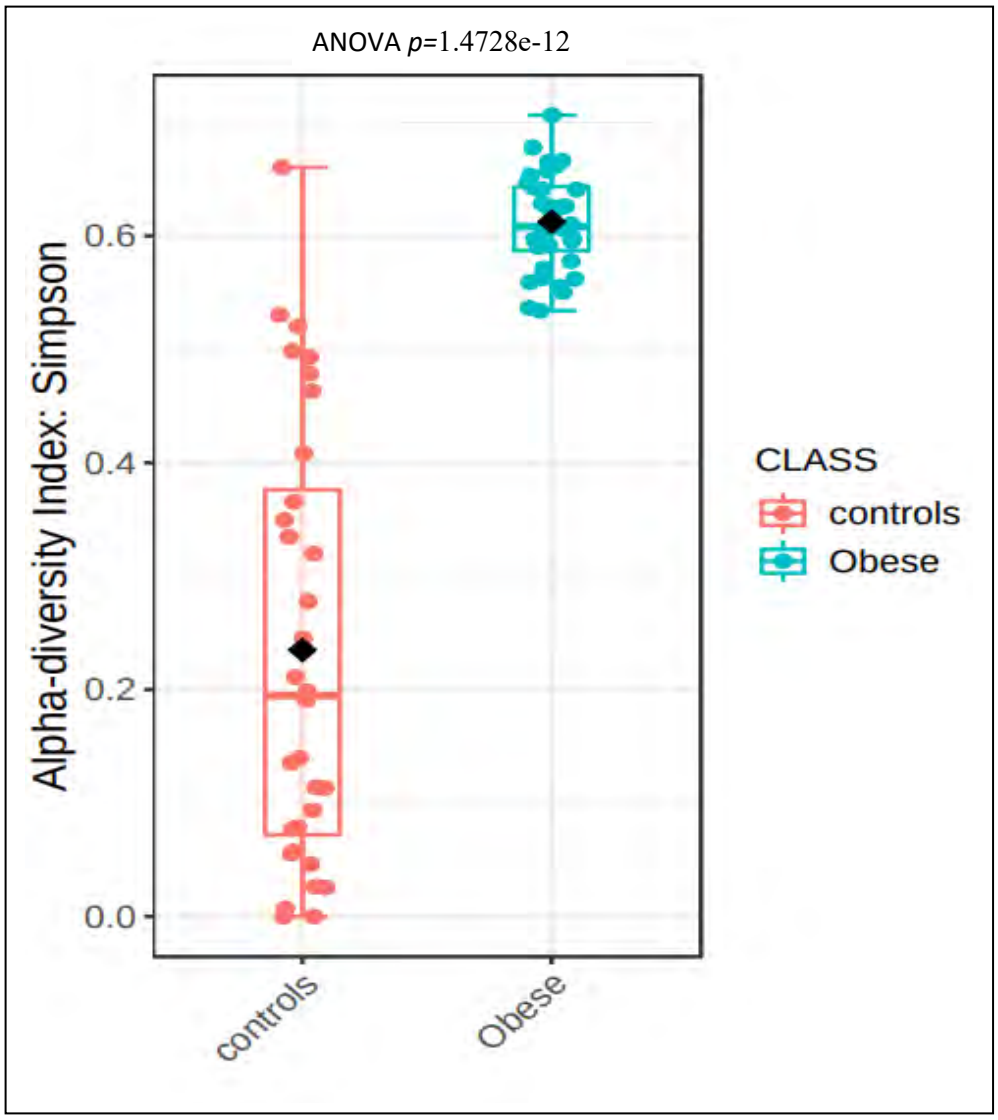


Figure 6.8: Alpha diversity of gut microbiome obese (36) and control subjects (n=32) where Simpson box plots indicates the number of Phyla richness

6.3.3.3.2- Beta diversity

In Principal Coordinates analysis (PCoA) and Non-metric multidimensional scaling (NMDS) (NMDS) scaling abundance-based Bray–Curtis metric has been used to calculate the dissimilarity among all samples as observed by beta diversity. PERMANOVA and PERMDISP were rigorously used to identify location vs. dispersion effects, respectively. In case of PCoA, the PERMANOVA showed significantly different abundance among obese and control individuals as it can be seen by statistical analysis values; F = 11.99, R^2 = 0.15374, *p*-value = 0.001. These differences were also evident by the PERMDISP analysis showing F= 17.63, *p*-value = 8.2082e-05 (Figure 6.8). The NMDS ordination of dissimilarity and the PERMANOVA showed significant differences (F= 46.41; R^2= 0.41, p-value= 0.001) among the obese and controls gut microbial composition with Stress = 0.06. While the PERMDISP did show any significant difference (F= 0.004; p-value= 0.94) with stress = 0.06.

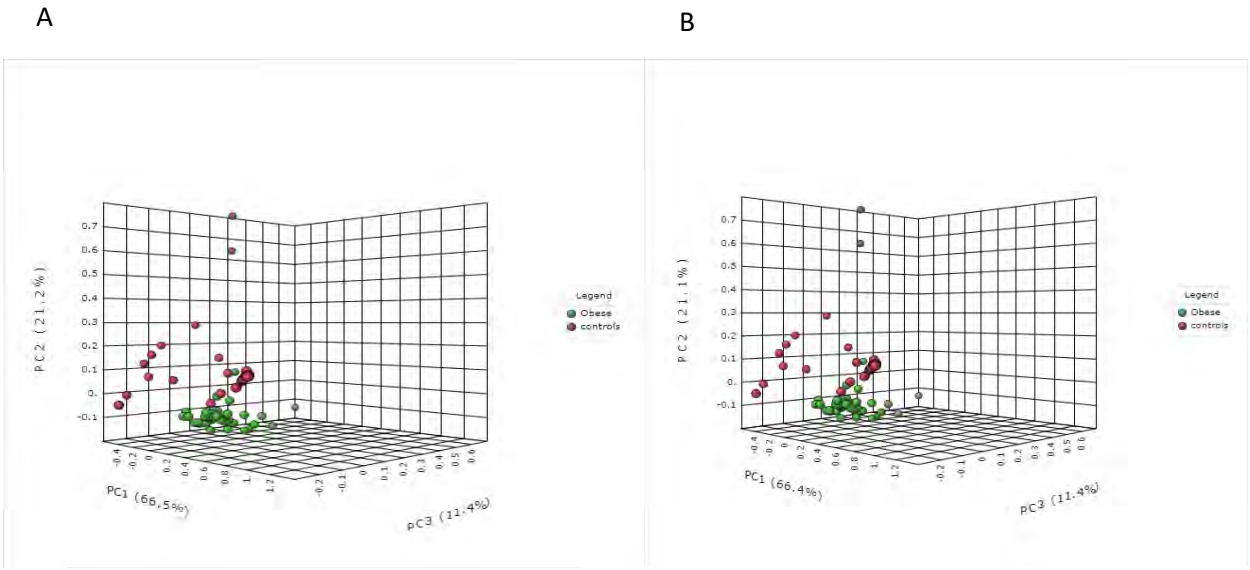


Figure 6.9: PCoA of beta-diversity comparison using Bray Curtis distances revealed significant separation of microbial communities p<0.001, using PERMANOVA, additional analysis using PERMDISP indicates dispersion contributes significantly to these differences.

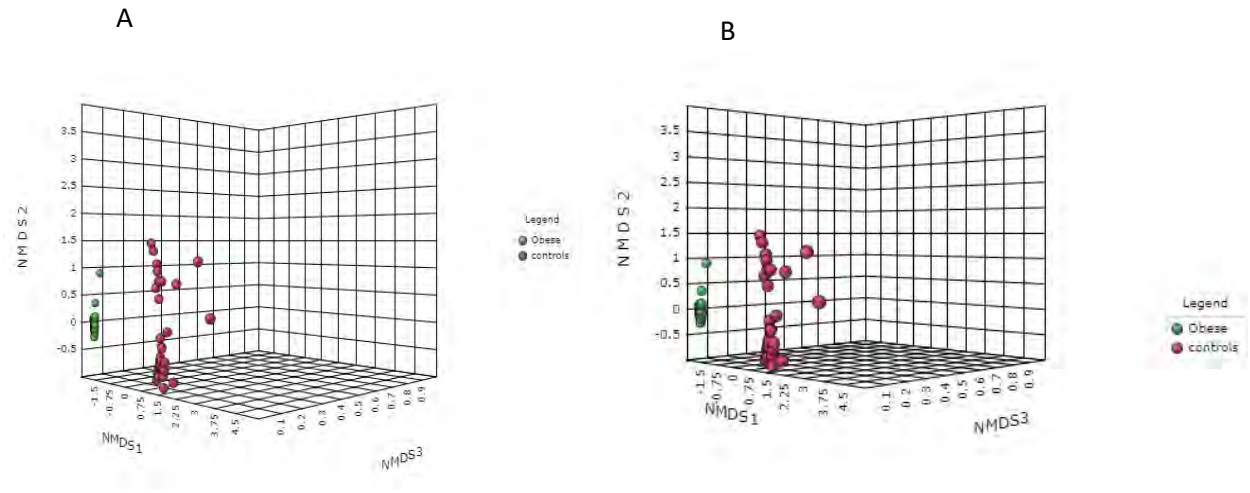


Figure 6.10: NMDS of beta-diversity comparison using Bray Curtis distances revealed significant separation of microbial communities p<0.001, using PERMANOVA, additional analysis using PERMDISP indicates dispersion does not contributes significantly to these differences.

6.3.3.3.3- Core microbiome

The core microbiome of obese gut was found to have various microbial taxa which were shared by all obese individual based on relative abundance of different Phylums. The top taxa comprising core microbiome of obese consisted of Proteobacteria, Firmicutes, Bacteroidota, Actinobacteriota, Verrucomicrobiota, Cyanobacteria, Desulfobacterota, Patescibacteria and Euryarchaeota. In case of control subjects. the top taxa comprised of Firmicutes, Bacteroidota, Actinobacteriota, Verrucomicrobiota, Proteobacteria, Patescibacteria, Euryarchaeota, Desulfobacterota and Cyanobacteria in the gut microbiome.

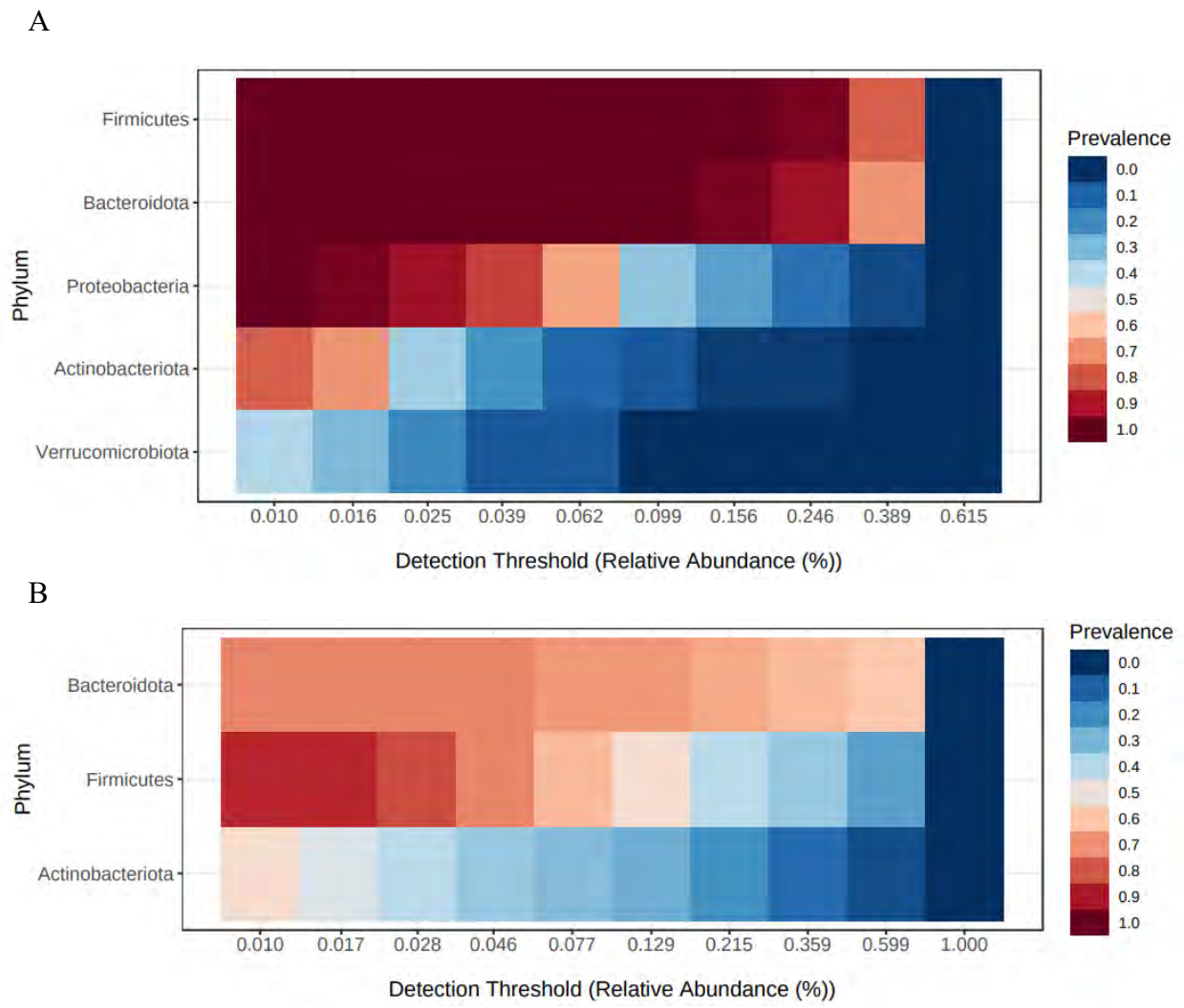


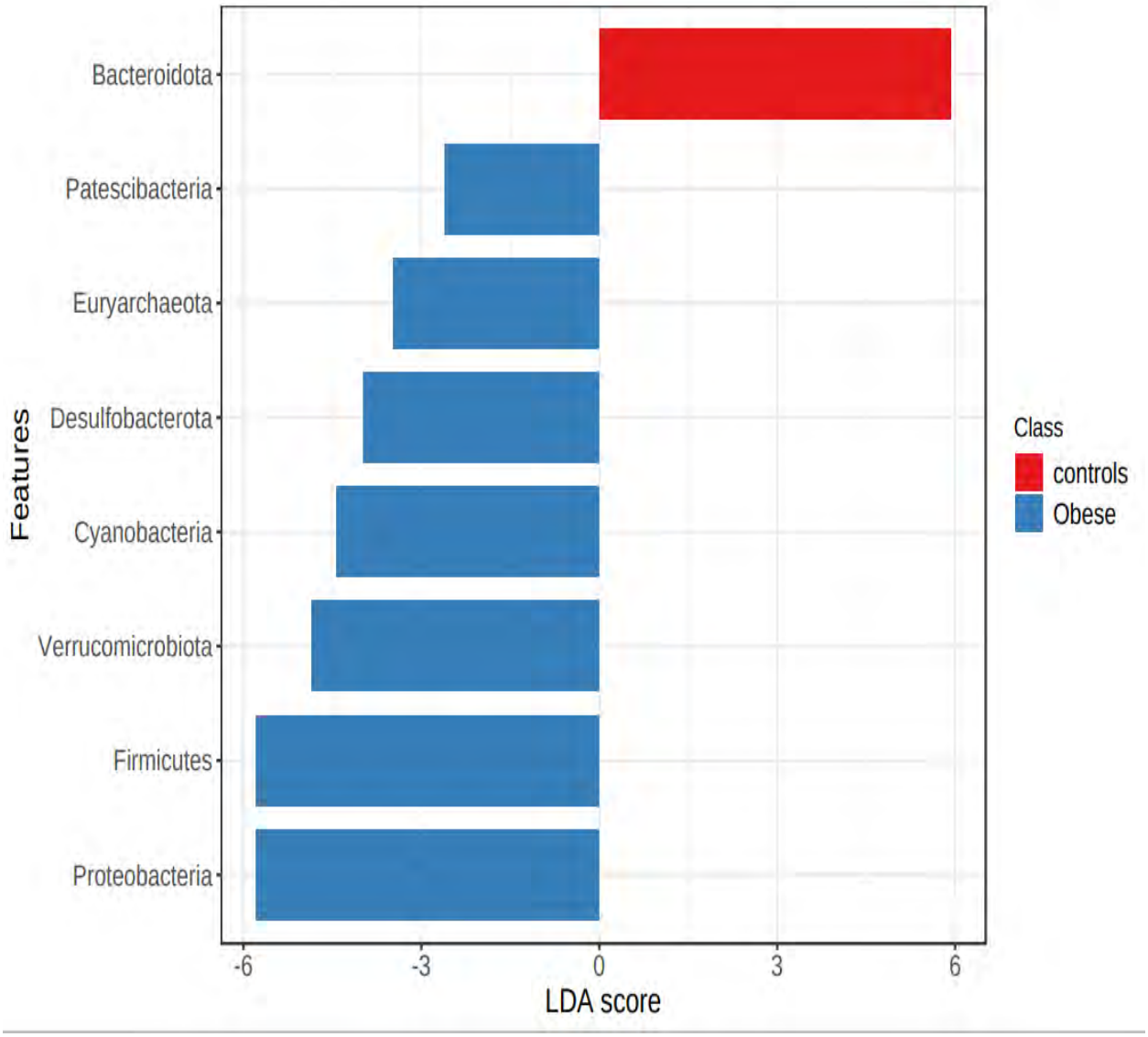
Figure 6.11: Core microbiome analysis of faecal samples from A) obese and B) control subjects at Phylum level. Heatmap clustering for core microbiome with prevalence ranging from 0 to 1.

6.3.3.3.4-Correlation analysis of gut microbial composition of obese with controls subjects

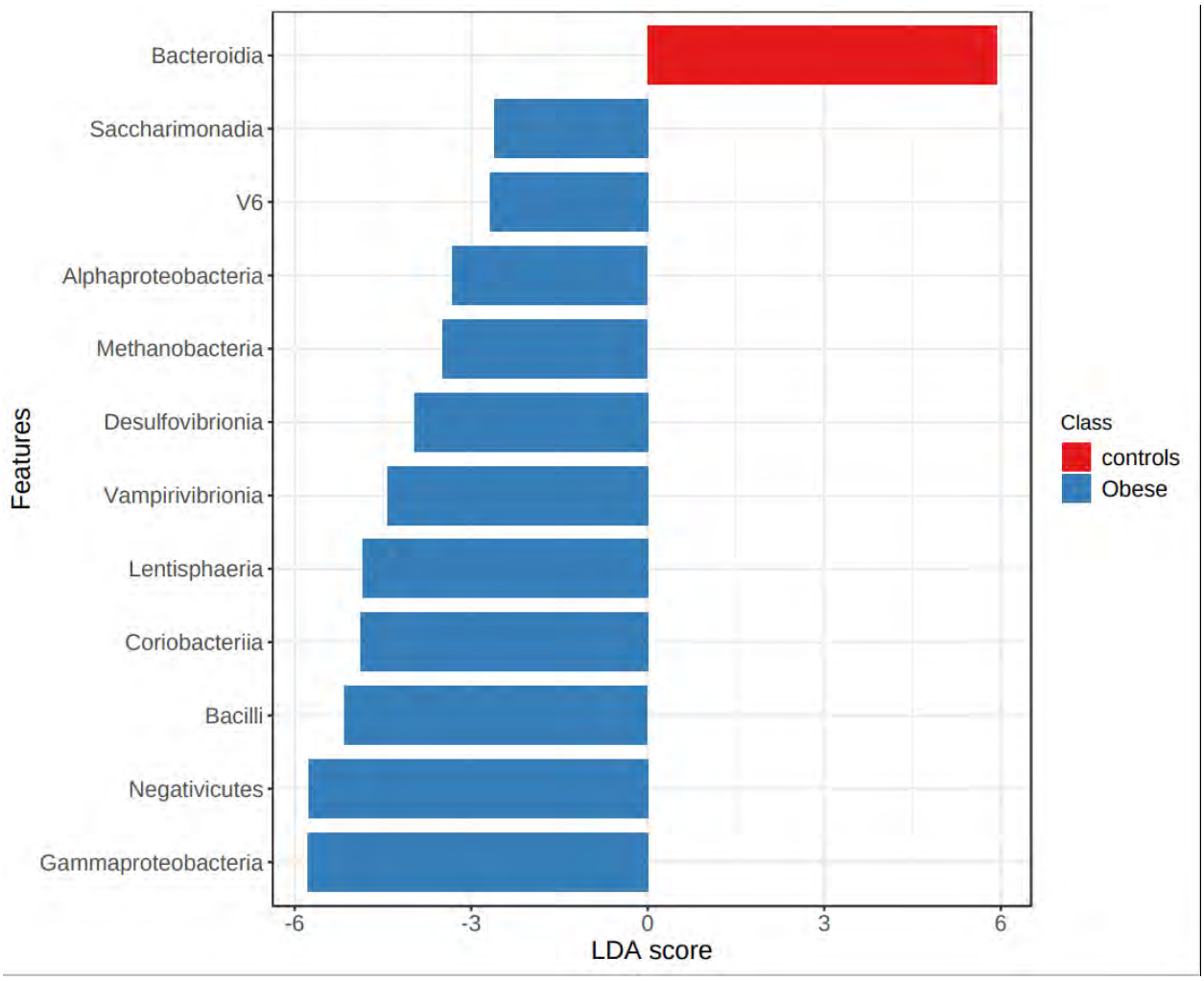
In order to compare the gut microbial composition among obese and controls based on specific phylotypes by using the linear discriminant analysis (LDA) of effect size (LEfSe) algorithm and edgeR, most abundant taxa in obese group compared to the control group observed were given in Figure 6.11 and 6.12. Eight Phyla-level phylotypes were discovered as high dimensional biomarkers which were discriminating gut microbiota of obese from control gut flora (Fig 6.11A). In the obese group, phylotype alterations were more dramatic compared to the control groups. Top fifteen class level phylotypes were discovered as biomarkers discriminating gut microbiota of obese from control individuals (Fig 6.11 B). Top fifteen order level phylotypes were seen as biomarkers for discriminating gut microbiota of obese from and controls.

Spearman's rank correlation analysis was carried out to investigate the relationship among the taxa in obese and control gut environment (Figure 3.21). Based on the Microbial dysbiosis (MD) index, the taxa (Phyla) based difference was significantly higher in obese than that in the control group (Obese/control MD= 0.40, $P < 0.05$, Spearman's rank -sum test). The Phyla Firmicutes have shown positive correlation with the Actinobacteriota, Verrucomicrobiota, Desulfobacteriota and the others inside the gut microflora. The Phylum Bacteroidota was negatively correlated with the Firmicutes and Actinobacteriota.

A


Bacteroidota
Patescibacteria
Euryarchaeota
Desulfobacterota
Cyanobacteria
Verrucomicrobiota
Firmicutes
Proteobacteria
Features
-6
-3
0
3
6
LDA score
Class
controls
Obese

B


Bacteroidia
Saccharimonadia
V6
Alphaproteobacteria
Methanobacteria
Desulfovibrionia
Vampirivibrionia
Lentisphaeria
Coriobacteriia
Bacilli
Negativicutes
Gammaproteobacteria
Features
-6
-3
0
3
6
LDA score
Class
controls
Obese

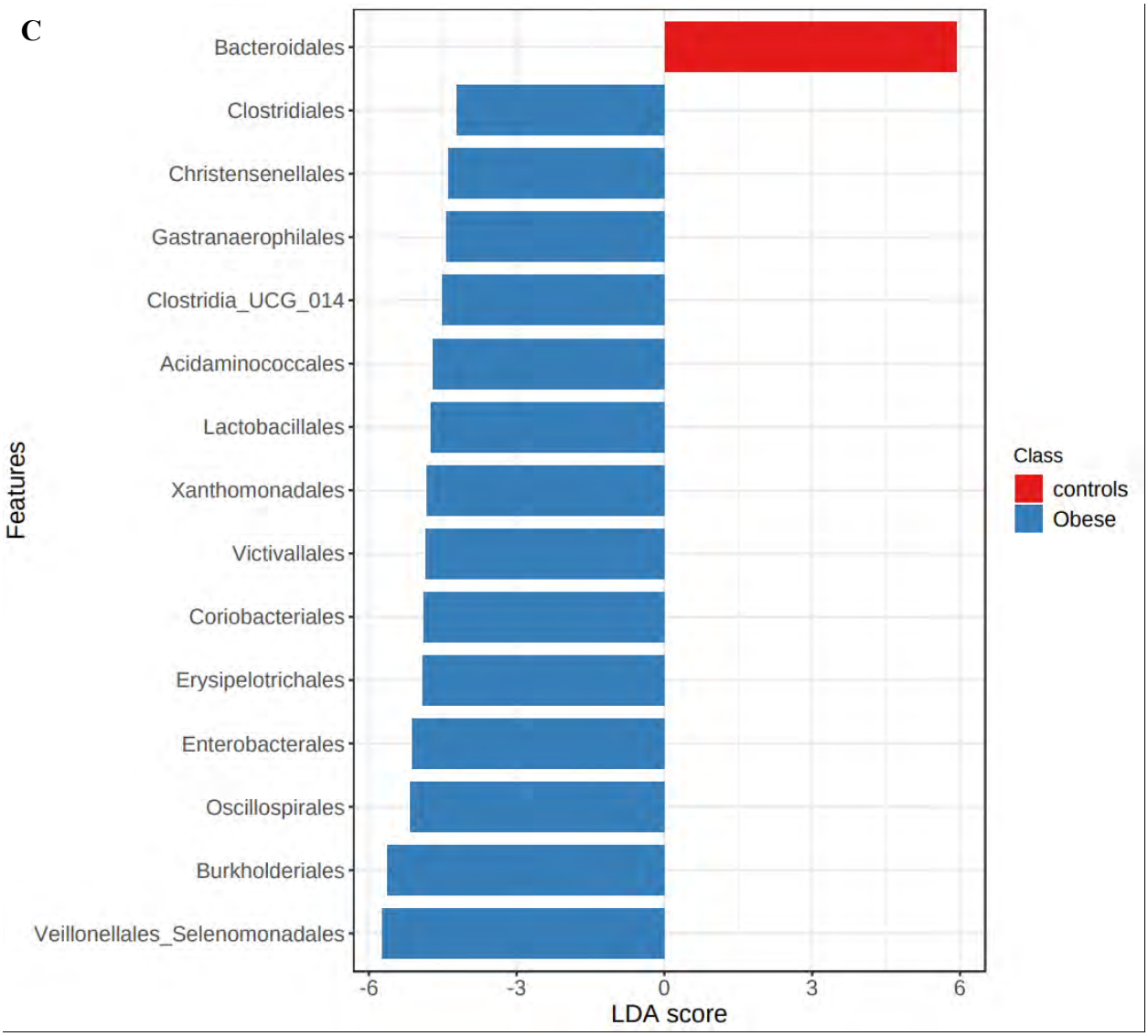


Figure 6.12: HistoGram show linear discriminant analysis (LDA) scores calculated for differences in A) Phyla-level B) class level and C) order level abundance showing Effect size (LEfSe) analysis among obese and controls. The LDA scores of obese were negative, while those of controls were positive. The absolute values of the effect size indicate the scale of the difference between 2 groups regardless of the positivity or negativity.

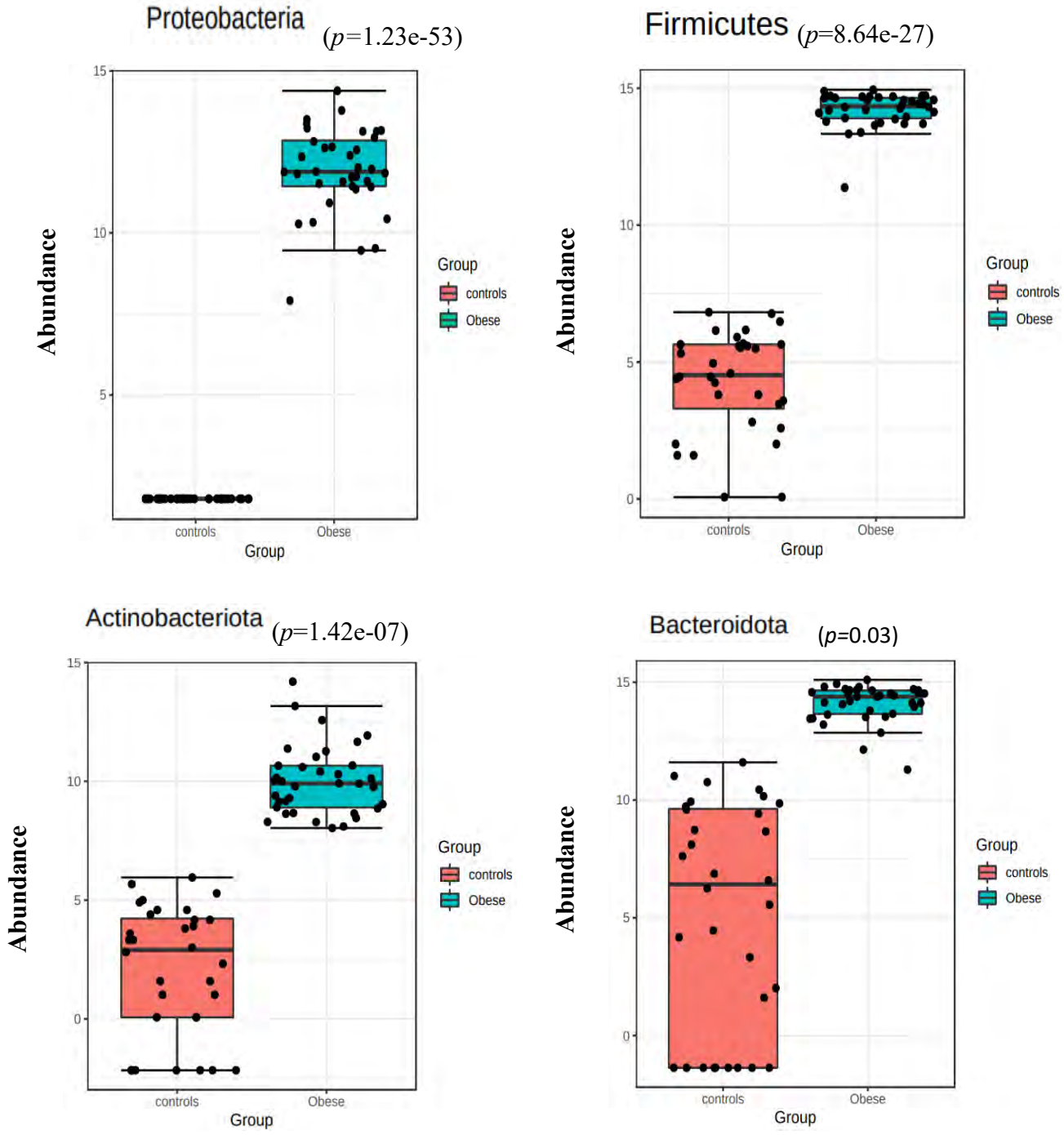


Figure 6.13: Box plot of multivariate differential abundance analysis (edgeR by default RLE :relative log expression) at Phyla level with significantly different relative abundances in obese and control subjects (Blueboxes: Obese ; red boxes: Control)

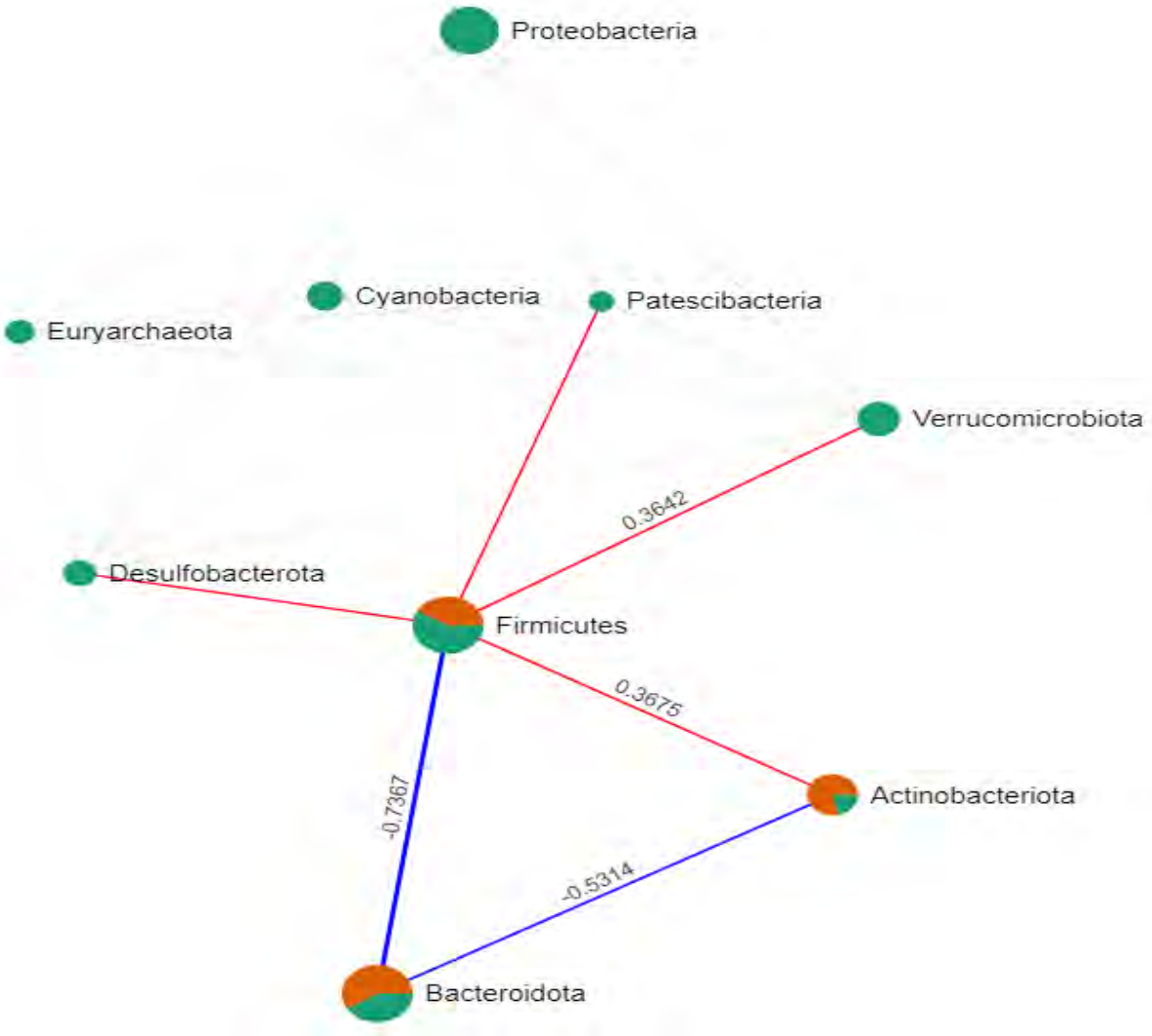


Figure 6.14: Spearman`s rank-sum test correlation network where *p* value < 0.05 was significant, a positive correlation is indicated by red line and negative correlation as blue line among abundant different gut Phylum

Table 6.5: Lefse analysis for differential abundance of taxas among obese and control subjects

		Obese	Controls	LDA-score	*p*-value	FDR
Differential Phylum	Proteobacteria	1228800	0	-5.79	7.59e-14	6.83e-13
	Verrucomicrobiota	140810	0	-4.85	2.94e-12	1.32e-11
	Patescibacteria	808.2	0	-2.61	9.32e-12	2.80e-11
	Desulfobacterota	18804	0	-3.97	8.09e-08	1.82e-07
	Cyanobacteria	53604	0	-4.43	4.56e-07	8.20e-07
	Euryarchaeota	6039.1	0	-3.48	2.34e-06	3.52e-06
	Firmicutes	4233100	3017100	-5.78	0.002	0.003
	Bacteroidota	3954800	5652600	5.93	0.065	0.073
	Actinobacteriota	363180	1330300	5.68	0.382	0.382
Differential Classes	Negativicutes	1165300	0	-5.77	7.59E-14	5.31E-13
	Gammaproteobacteria	1224800	0	-5.79	7.59E-14	5.31E-13
	Lentisphaeria	140810	0	-4.85	2.94E-12	1.37E-11
	Alphaproteobacteria	4182	0	-3.32	9.32E-12	2.61E-11
	Saccharimonadia	808.4	0	-2.61	9.32E-12	2.61E-11
	V6	980.23	0	-2.69	2.86E-11	6.68E-11
	Coriobacteria	174180	20246	-4.89	4.72E-11	9.45E-11
	Bacilli	343940	60901	-5.15	2.50E-09	4.37E-09
	Desulfovibrionia	18804	0	-3.97	8.09E-08	1.26E-07
	Vampirivibrionia	53629	0	-4.43	4.56E-07	6.38E-07
	Methanobacteria	6042.6	0	-3.48	2.34E-06	2.98E-06
	Bacteroidia	3954400	5652600	5.93	0.065	0.075
	Clostridia	2723000	2956200	5.07	0.143	0.154
	Actinobacteria	189090	1310000	5.75	0.268	0.268
	Veillonellales_Selenomonadales	1065800	0	-5.73	7.59e-14	4.40e-13
	Enterobacterales	256960	0	-5.11	7.59e-14	4.40e-13
	Bacillales	11264	0	-3.75	7.59e-14	4.40e-13
	Burkholderiales	836150	0	-5.62	7.59e-14	4.40e-13
	Christensenellales	48004	0	-4.38	7.59e-14	4.40e-13
	Peptostreptococcales_Tissierellales	31941	0	-4.2	2.66e-13	1.10ee-12
	Lactobacillales	108790	0	-4.74	2.66e-13	1.10e-12
	Victivallales	140850	0	-4.85	2.94e-12	8.53e-12
	Xanthomonadales	130820	0	-4.82	2.94e-12	8.53e-12
	Clostridiales	33285	0	-4.22	2.94e-12	8.53e-12

Differential Orders						
	Saccharimonadales	808.52	0	-2.61	9.32e-12	2.25e-11
	Sphingomonadales	4184.3	0	-3.32	9.32e-12	2.25e-11
	Clostridia_UCG_014	64405	0	-4.51	2.86e-11	5.93e-11
	V13	980.92	0	-2.69	2.86e-11	5.93e-11
	Coriobacteriales	174230	20246	-4.89	4.72e-11	9.13e-11
	Clostridia_vadinBB60_group	46977	16515	-4.18	1.31e-10	2.38e-10
	Acidaminococcales	99805	0	-4.7	2.47e-10	4.21e-10
	Erysipelotrichales	223880	60901	-4.91	1.05e-08	1.70e-08
	Pseudomonadales	1043.5	0	-2.72	1.30e-08	1.99e-08
	Desulfovibrionales	18813	0	-3.97	8.09e-08	1.12e-07
	Peptococcales	1368.4	0	-2.84	8.09e-08	1.12e-07
	Gastranaerophilales	53612	0	-4.43	4.56e-07	6.01e-07
	Acholeplasmatales	2685.2	0	-3.13	1.05e-06	1.26e-06
	Monoglobales	4617.2	0	-3.36	1.05e-06	1.26e-06
	Methanobacteriales	6041.8	0	-3.48	2.34e-06	2.72e-06
	Oscillospirales	1654300	1357600	-5.17	0.0008	0.0009
	Bacteroidales	3955000	5652600	5.93	0.065	0.069
	Bifidobacteriales	189070	1310000	5.75	0.268	0.274
	Lachnospirales	834290	1582100	5.57	0.274	0.274

6.3.3.4- Endotoxin detection in serum samples:

All the samples sent for the 16S rRNA gene sequencing were further checked for the presence of bacterial LPS in serum samples of obese using Inter Quartile Range (IQR), the LPS activity was detected among obese was 23.8 EU/mL.

6.4. DISCUSSION:

Over the last decade, gut microbiome's comprehensive knowledge advanced has due development of omics techniques and reduction in the cost. There is still undiscovered microbial data and it necessitate investigation into this field, as culture dependent approaches have established a link between human health and resident flora but still there are gaps in complete understanding of any adverse conditions exist. The recent improvement and availability of high throughput sequencing are opening avenues for better understanding of any diseases. The data on the gut microorganisms has revealed that there is involvement of these microbes in development of host immune system and also vice versa is affected by it (Wells *et al*., 2011;Pott & Hornef, 2012). The presence of gut microbes and their metabolites after digestion of vitamins, carbohydrate, lipid and amino acid play

an important and also some microbes produce these for the host (Tremaroli and Bäckhed 2012). The gut microbial communities have critical job performing various metabolic activities for the host human, which human cannot form (Balzola *et al*., 2010).

It is now revealed, the specific change of phyla and specie occurs in obesity which is in comparison to healthy individuals differs (Delzenne & Cani, 2011). These complex host bacteria relationships are very critical for human health to understand, it can be dissected by using both culture-dependent and culture-independent approach. This is an attempt to understand the composition of gut microflora in obese Pakistani population. The culture dependent techniques were coupled with cultural independent techniques to better and comprehensively understand the gut profile to determine the basis for obesity especially in Pakistani population.

The first phase of the current study was to identify the cultureable diversity among study participants. The average CFUs in aerobic condition were 4.42 $x10^6$ CFU/mL in obese, 5.88 $x10^6$ CFU/mL in pre-obese and 6.16$x10^6$ CFU/mL in controls. While in anaerobic conditions the average CFUs in obese was 1.3 $x10^5$CFU/mL, pre-obese 1.7 $x10^5$ CFU/mL and controls 15 $x10^5$ CFU/mL. In both conditions the obese group had lower abundance in comparison with the pre-obese and control group.

Previous studies have found dominance of *Enterobacteriaceae* in obesity. In case of present study, Gram negatives isolated were mostly member of *Enterobacteriaceae* family belonging to phylum *Proteobacteria* where were predominant *E. coli* followed by *P. aeruginosa* obese gut samples. Likewise, *E. coli* and *Enterobacter* cloacae dominated pre-obese faecal samples where as in controls *E. aerogenes* were comparatively high in number. In case of facultative anaerobes, the trend for the isolation was higher for *E.coli* compared to *K. pneumoniae* in obese. These findings are supported by the studies on Korean population, where comparatively higher frequency of phylum *Proteobacteria* was observed among obese individuals (Shin *et al*., 2015). A mouse model study where effect of endotoxin was assessed by administration of purified endotoxin from *E. coli* , in these mice weight gain and insulin resistant phenotypes developed (Ichinose *et al*., 2006).

The presence of opportunistic Gram negative bacteria in higher percentages gut flora makes the obese individuals more prone to infections, as these bacteria have ability to release LPS from their outer membrane (Löwik *et al*., 2019). In the current study, a higher Gram negative isolates

percentage among obese than in the control group samples indicate that it might be also involved in releasing proinflammatory metabolites. The high prevalence of these bacteria is supported by a study on germ free mice which were inoculated with *E. cloacae,* later these mice developed endotoxemia co-occurrence of obesity (Fei & Zhao, 2013). The similar findings were also observed in genetically obese (ob/ob) mice as well as in lean mice (C57bl6/J) fed with obesogenic diet (Cani *et al*., 2007;Cani *et al*., 2008). In study with healthy human, administration of lower to moderate doses (0.2–2 ng/kg) of *E.coli* derived LPS causes led to release of pro-inflammatory cytokines along with the metabolic disturbance (Patel *et al*., 2015;Mohammad & Thiemermann, 2021). These experimental model showed that there was Gram negatives abundance change from which it can be inferred that change in abundance of cultureable Gram negatives found in this study in obese subjects compared to healthy controls may be involved in endotoxima, which was detected in LAL assay. In current study the serum LPS level was lower with IQR 23.8 EU/mL (0.23ng/mL) in the obese samples. Such detection of low level LPS is supported by Cani *et al.,* in mouse model study where lower levels of plasma LPS at 5.1±7.3 pg/mL were found and causing metabolic endotoxemia.

A previous study have showed that *Enterobacter* was involved in increasing obesity by increasing serum LPS acting as vital ligand for regulation of inflammatory pathways in *TLR-4* (Fei & Zhao, 2013). In a recent study on obese mice reduction in Gram-negative Proteobacteria and Deferribacteres that causes improvement in intestinal epithelial integrity was observed. This bacterial diversity change lowers the serum levels of LPS and TNF-α as well as causes the down regulation of *TLR-4* expression (Meng *et al*., 2018; Li *et al*., 2020). Various other studies have confirmed LPS from *E. coli* causes increase of proinflammatory pathways and more recently macrophage and platelet activation through the *TLR-4* dependent pathway increasing inflammation (Maker & Washington, 1974; Jones *et al*., 1978; Biswas *et al*., 2020; Carpino *et al*., 2020). These studies support the findings of the current study where increased *E.coli* among cultureable diversity and distinctively increased Phyla *Proteobacteria* among unculturable diversity showed their possible contribution in increased LPS at lower levels in serum samples of the obese.

In the present study among Gram positives, *S. aureus* were high in number from obese faecal and pre-obese samples in comparison to controls. These findings are in line with work where specie

specific study found association of *S. aureus* with increased weight in pre-obese children in Carolina, USA (Kalliomäki *et al*., 2008). A study from Spain on pregnant women detected increase in weight was positively associated with increased prevalence of gut *Staphylococcus*, *Enterobacteriaceae* and *E. coli* in comparison with the non-pregnant females (Santacruz *et al*., 2010). The current findings as well as the above mentioned studies have shown that the gut *Enterobacteriaceae* might have role in obesity, where cross talk of gut flora with skeletal muscles and adipose tissue is critical for weight gain (Bleau *et al*., 2015).

It can be summed up after phase one after analysis of the cultureable bacteria, that in obese a decrease in abundance based on CFU/mL was seen. Although, the all groups shared some dominant Gram negatives (*E. coli, E. cloacae. K. pneumonia*, *P. aeruginosa* etc.) and Gram positive (*Staphlycoccus aureus*). But there was change in diversity in case the obese group, where *Proteus vulgris* and *Streptococcus* were additional observed species. Interestingly lower diversity was seen in the pre-obese group even compared to controls. There was cultureable bacterial dysbiosis especially of Gram negatives, where change in their abundance and diversity was also recorded. This increase in Gram negatives show potential to alter the host immune responses by interacting with the endotoxin production, which play an important role in obesity through change in inflammation profiling of an individual(Cani *et al*., 2012; Hersoug *et al*., 2018). Another inflammatory disorder (colitis) also showed similar trend where high prevalence of *Enterobacteriaceae* especially *K. pneumonia* was present which was involved in manipulating the host immunity causing inflammation (Garrett *et al*., 2010). Non fermenter *P. aeruginosa* was highly abundant in obese group but there was reduction of facultative anaerobes. This show that with further investigation an indicator bacterial strain can be identified for early detection of obesity development.

Studies found that there is only 30% recovery of gut bacteria by culture dependent method (Fraher *et al*., 2012). As in obese group, the gut bacterial diversity was more and varied, which indicated that there could be several other compositional and functional disruptions due to change in microbial populations and their gene frequencies which are still not found by culturing the gut samples(Debédat et al., 2019). The data on cultureable gut microflora is still valuable as there is scarcity of existing data on it in obesity. However, routine microbiological technique alone does

not provide the in depth of gut microbial profiling, therefore, high throughput 16S rRNA gene sequencing carried out to acquire the complete knowledge of uncultured bacteria flora.

As extensive work on animal models has been done, even several metagenomic studies for involvement of gut flora in gut microbial diversity change by compromising the gut health and activating immunity by involvement of different immune pathways involved in releasing pro-inflammatory cytokines increasing inflammation in the body (Hassanein *et al*., 1995;Guarner & Malagelada, 2003; Khalif *et al*., 2005; Uzbay, 2019;Cai *et al*., 2022). The role of gut flora in humans is well established due to metagenomic studies in case of colon cancers, Inflammatory bowel diseases and fatty liver disease, where abundance of specific bacterial Phyla involved with the disease outcomes under the influence of different factors including age, gender, BMI , diet, and habits (Matsuoka & Kanai, 2015; Ni *et al*., 2017;Campo *et al*., 2019). However the gut flora in obesity is the recent topic of researcher's interest, in which most of the advanced metagenomic and initial studies were focused on the mice, transgenic mice and germ free mice models only (Tilg, 2010; Evans *et al*., 2014; Bakker & Nieuwdorp, 2017; Moretti *et al*. 2021). Such animal model studies have linked obesity with the gut microflora dysbiosis. In case of obesity associated gut flora in human population-based studies are very limited. There is huge gap in our understanding of obesity and a need for population-based gut microbial diversity studies to decipher the shift of gut microflora due to change in body weight.

As from last few decades, change in bacterial diversity with age, physiology, immune status, metabolism and gut related complications is found to be associated with resident microflora. The gut microflora develops from infancy to adulthood, in which three dominant Phyla colonized the human gut namely Firmicutes (*Ruminococcaceae* and *Lachnospiraceae*), *Bacteroidota* (*Bacteroidaceae* and *Prevotellaceae*), and *Actinobacteriota* (*Coriobacteriaceae* and *Bifidobacteriaceae*). The maturation of gut microflora with the time is also influenced by various factors including genetics, environment, diet, lifestyle, and gut physiology (Yatsunenko et al., 2012; Alou *et al*., 2016). The gut microflora is recently categorized into three enterotypes based on bacterial clusters that make-up the enterotype. These enterotypes are characterized by three dominant bacteria clusters: *Bacteroides* (enterotype I), *Prevotella* (enterotype II) and *Ruminococcus* (enterotype III). Each enterotype harbours different bacterial genera and

characteristics of distinctive functions. The abundance of these enterotypes have shown to be influenced by diet due to their capability of generating energy from fermentation of available substrates. All the previous gut related studies focused on to understand the complex link of bacteria with human health (Arumugam *et al*., 2011), where dysbiosis of these enterotypes was found to linked with the disease outcomes.

There are couple of studies on gut flora from Pakistan, but only study on gut microflora of diabetic individuals with obesity is reported in 2019 (Ahmad *et al*., 2019). No previous study has evaluated differences in microbiota between the gut of obese and control individuals only. The obesity is a distinct body state where physiological condition is influenced by genetic and environmental factors which have potential to altered gut microbiota. There is still debate on obesity as a disease condition, where altered gut microflora could act as risk factor for obesity. The current study was focused on the gut microbiome profiling among local obese individuals without any comorbid condition targeting the V4 region by 16S rRNA sequencing. Earlier work detected change in two major Phyla comprising 90% of the bacterial diversity in the obese gut namely *Firmicutes* and *Bacteroidota* (John & Mullin, 2016). *Firmicutes* phylum includes Gram positive bacteria comprised of more than 200 different genera such as *Fecalibacterium*, *Lactobacillus*, *Roseburia*, *Catenibacterium*, *Clostridium*, *Eubacterium*, *Dorea*, *Ruminococcus*, and *Veillonella*. *Bacteroidota* phylum comprised of Gram negative bacteria including 20 genera such as *Prevotella*, *Bacteroides*, *Odoribacter*, and *Tannerella* (Tremaroli and Bäckhed 2012). These results were in line with this study as clear shift can be observed with increased relative abundance of *Firmicute* (42.31%) in obese group whereas in controls these 6.59%. This finding is substantiated by previous studies, in which development of obesity was associated with the increased *Firmicutes* in the faecal material and also changing the gut bacterial diversity , such change was considered to be good biomarker of obesity and weight loss regime (Ley, 2010). In a study on Japanese individuals, increased gut bacterial diversity was recorded in the obese individuals. These results are also in line with current findings as increased bacterial diversity in local obese subject was linked with decreased abundance of *Bacteroidota* (Kasai *et al*., 2015). However, in Chinese study on university student`s gut microbiome the ratio of *Firmicutes* to *Bacteroidota* changed with decreased *Firmicutes* in obese students. The Chinese study showed decrease in microbial diversity with increase of BMI, however, in this study increase in microbial diversty was detected (Lv *et al*. 2019). These results

showed that whether increase or decrease in diversity among obese there is always clear shift in obese gut flora. *Firmicutes* in comparison with the *Bacteroidota* are found to be more effective in harvesting energy from food, thus promoting weight gain efficiently by absorbing the extra calories (Brown *et al*. 2012).

The alpha diversity index Shannon, showed that the diversity was comparatively higher in obese gut flora of study subjects. The richness of microbial species was also observed to be greater as calculated by Observed and Chao-1 index in case of local obese individuals. Similarly, the uniformity of gut flora was also higher in obese individuals than in the gut of controls by Simpson's index. There was distinct pattern of beta diversity observed among the obese and healthy groups as computed by PCoA and NDM analysis. These alpha and beta diversity analyses indicate that the composition of gut bacteria was significant different between the obese and control group. These results are supported by various studies on obese animal models and humans (Bäckhed *et al*., 2004; Ley *et al*., 2005;Turnbaugh *et al*., 2009;P. Xu *et al*., 2012), where increased gut diversity was the characteristics feature in obesity in comparison to health status.

The LEfSe and edge R analysis found that all the Phyla with negative LDA scores which was significantly associated with obesity (Table 6.5). Among the Phyla, False-Discovery rate corrected p value <0.05 has shown significant association of *Proteobacteria* Phyla with obesity giving highest LDA score (-5.79) while it was absent in control samples. These findings are in line with the previous studies among Chinese individuals, where obese gut microflora showed dominance of Phyla *Proteobacteria* as a signature of dysbiosis in obesity in comparison with the control gut (Gao *et al*., 2018;Crovesy *et al*., 2020).

Proteobacteria is comprised of opportunistic pathogens, in which it member species are usually indicator of imbalanced and unstable gut microbial community in host (Shin *et al*., 2015). The existence of Phyla *Proteobacteria* in the studied obese individuals is an indication of dysbiosis in local individuals too. The phylotypes based on class level in the obese subjects found three taxa (*Bacteroidia*, *Clostridia* and *Actinobacteria*) among top fifteen classes, their abundance was decreased in the obese only with positive LDA score (5.93, 5.07 and 5.75 respectively) showing their association with the healthy gut. Rest of 13 classes (Table 6.5) have shown negative LDA score and were strongly associated with obesity. Among them *Negativicutes*,

Gammaproteobacteria and Bacilli showed highest LDA score (-5.77, -5.79 and -5.15 respectively) an indication of strong association with obesity ($p < 0.05$). The gut microbiome study on *Proteobacteria* and Bacilli in Chinese population found these classes as risk for obesity(R. Gao *et al*., 2018). *Negativicutes* were increased in the current obese gut microbiota, this finding is in contrast with the previous Pakistani study on obese T2DM individuals (Ahmad *et al*., 2019). This may be due to the comorbid diabetic condition in these obese individuals. In a study on gut microbiota in Spain, *Gammaproteobacteria* and *Proteobacteria* were the main class and Phyla in obesity related microbiome (Quiroga *et al*., 2020).

The analysis of orders in the microbes in the study groups, found that *Bacteroidales* and *Bifidobacteriales* were top 2 order among the15 orders with positive LDA scores of 5.93 and 5.75 respectively in control gut. While all other taxa were showing negative LDA score showing association with obese gut with FDR <0.05. In a study on obese murine model induced by high fat diet have also shown the decrease of *Bifidobacterium* in case of obesity (Yin *et al*., 2010). *Bifidobacterium* belong *Actinomycetes* which produces abundant probiotic helping *Bacteroidetes* in degrading polysaccharides and act as beneficial commensal (Sonnenburg *et al*., 2006). These studies are in line with the current study as *Bifidobacterium* was found to be negatively associated with the risk of obesity in the local study subjects.

The correlation analysis by Spearman's` rank sum test based on relatively abundant Phyla was used to determine if any the change in interactions among different Phyla in the obesity. There was change in interaction of Phyla in obesity when compared with controls. These results show a significant positive correlation of *Firmicutes* with *Verrucomicrobiota*, *Patescibacteria*, *Desulfobacterota* and *Actinobacteriota* with p-value ≤ 0.05. While in case of controls only *Bacteroidota*, *Firmicutes* and *Actinomycetes* have shown association. The results of current study showed that the core microbiome of the obese gut is in dysbiosis in comparison with control gut. As abundance of *Proteobacteria* was more in obesity, and most opportunistic pathogens are its member there is possibility that they change the immune status and energy harvesting abilities in the obese. A number of interaction were change to positive and negative indicate that there is intricate networking existing where different Phyla play the role, if any change in these in networks can be pathogenic.

In the current study significant differences of relative abundance of gut flora was detected among obese and controls at multiple levels of classifications. This is the first study in Pakistan population in which culture dependent coupled with culture independent approaches have been used to classify and to differentiate gut microbiota in obesity. There was great difference in the cultureable diversity which was fraction of the bacterial diversity identified through the microbiome analysis. In case of cultureable bacteria, Gram negatives were predominating the gut flora where as after 16S rRNA gene V4 based high throughput analysis, *Firmicutes* were dominant in obesity which are Gram positives interestingly. After the comprehensive analysis of bacterial diversity by both approaches, microbiome of Pakistani obese population has striking difference with other population yet have some similar bacterial diversity. Functional mechanisms of these bacteria are needed to be validated to get insights into bacterial communities of the Pakistani obese gut flora with a rising trend of obesity in this region.

CHAPTER 7: FINAL DISCUSSION

Obesity is one of the alarming health issue in the world and initially it was thought to be the problem of developed countries but now it has also seen at alarming rate in developing countries. Especially while looking at low to middle income countries, there is the upward trend of the disease. This study was conceived to understand the underlined genetic basis and their association with the gut micro flora along impact of the demographic, lifestyle and dietary habits. In overall, in the previous chapters, the demographic, dietary, life style, genetic of TLRs and gut microbial diversity were analysed separately in obese individuals. These various risk factors were assessed as are believed to effect the weight gain by imitating and sustaining low grade inflammation in the adipose tissue in obesity. As low grade inflammation is thought a role in obesity, the pattern recognition receptors with focus was on *TLR-2* and *TLR-4* was investigated as these recognise both microbial and dietary ligands. Initially, *TLR-2 and 4* genes were dissected if any changes in the gene is present among obese individuals which might be responsible for their altered expression, either lower expression or over expression of gene. Further the gut cultureable and un-cultureable biodiversity was determine to detect if dysbiosis in case of the obesity. After analysing *TLR-2 and 4* polymorphism and microbes (both bacterial and fungal diversity), their association with the other risk factors (dietary, demographics and life styles) was assessed.

In the first part of the study various variants (153688371T>C, 153702295T>C, 153703504T>C, and 153705074C>A) in *TLR-2* and (117707870 G>A, 117708080 A>G, 117708777 C>G and 117713024 A>G) *TLR-4* were significantly associated with the obesity. All these SNPs as homozygous genotype were observed in the majority of obese females. Moreover, these SNPs were more common in age group 35-50 years especially females. While males were more likely at risk of obesity in age group of 28-39 years. Most of SNPs detected in both TLRs were dominant in obese females, different studies on TLR polymorphism also observed age and gender effecting TLR response in patients with different diseases like hepatitis, diabetes and cancers (Chen *et al*., 2004; Li *et al*., 2012; Gao *et al*. 2018b). Similarly, in case of obesity FTO gene polymorphisms was found to be a risk in increasing BMI in female carriers, if BMI increased 2.36 kg/m^2 per year (Alvarez *et al*., 2016) Different other metabolic diseases like hypertension and atherosclerosis have shown higher risk of disease development due to polymorphisms at higher age that showed specific

differential impact on disease outcome (Jin *et al*., 2011; Park *et al*. 2011;Wang *et al*. 2020). The detected SNPs in present study obese individuals might be contributing as risk for obesity and difference in obesity between genders especially in obese females indicate that such polymorphisms along with anatomical and physiological factors effect development of female obesity. Also, it is possible to infer that the polymorphisms in *TLR-2* and *TLR-4* the demographic feature like age and gender have role in obesity among the local Pakistani population. These obese individuals were from low- to middle-income group living as urban dwellers with high education indicating that the presence of polymorphism was affected with life style which increased the likelihood of developing obesity. Also survey based studies conducted in Thailand and China showed influence of the demographic characteristics (gender, age, income, level of education, and place of residence) with risk of obesity (Aekplakorn *et al*., 2007; Lee & Um, 2021).

There was no association of *TLR-2* variations with the cultureable fungal and bacterial gut colonization in the obese individuals of this study. While *TLR-4* SNPs showed strong association with the both cultureable fungal and bacterial colonization as well as demographic and dietary habits of the study participants. The data was then stratified based on homozygous and heterozygous genotypes where upon regression analysis strong association was seen with mutant genotypes for most detected SNPS except for few. The association of variant 117707870 G>A of exon 2 in homozygous genotype was found to increase risk (OR=2.0, CI=0.05-78.25) in obese for *Pseudomonas aeruginosa* colonization in individuals with sedentary life style.

The second variant 117708080 A>G of exon 2, their heterozygous genotype was significantly promoting the *E. coli* H_2S colonization (p value$\leq$0.05). Also this variant's genotype was found to be significantly associated with each factor which were urban dwellers, low income, marital status, intake of poultry, no regular exercise routine and gut colonization with *E. cloacae.* Moreover, *Pseudomonas* colonization among obese was a risk factor for obesity with ingestion of beef, fruits/vegetables, poultry or sedentary life style.

The exon 7 variant (117715853 G>C), mutant genotype in heterozygous was significantly associated (p value= 0.03) with *P. aeruginosa* colonization in the obese individuals living in urban area sedentary life style. Gender, marital status and ingestion of fruits/vegetables increased risk

of obesity in the individuals with homozygous genotype significantly associated (*p* value = 0.03) with the *K. pneumonia* colonization too. *E. coli* H_2S variant and *C.kefyr* were significantly (*p* value < 0.00) associated with heterozygous genotype in those having sedentary life style routine. While urban dwellers with poultry use showed significant association with *Pseudomonas* colonization and was risk (OR=1.00, CI=0.04-24.54) for obesity. While use of beef and poultry were significantly (*p* value <0.01) increasing risk for obesity by *Enterobacter* colonization.

After determination of association between factors where each factor was assessed individually againt other, next multiple factors correlation was done. Among obese individuals with exon 2 variant 117707870 G>A, *Shigella*, *S. aureus*, *C. krusei*, and *C. utilis* were significantly correlated with one another, the positive correlation among them showed that the increase in colonization of one specie substantially promotes the growth of others in the gut of obese individuals. While *Pseudomonas* was positively correlated with the *C. kefyr* in the gut of obese people, demonstrating possibility of co carriage of both fungi with bacteria has a role in obesity. In case of variant 117708080 A>G, a negative correlation of this variant with *Klebsiella* and *Shigella* was found, their co-inhibition was keeping the healthy status. In case of variant (117715853 G>C), *Shigella, C.utilis* and *C.krusei* together were positively correlated with obesity.

In the current study, cultreable bacterial-bacterial and bacterial-fungal correlations were seen with *TLR-4*, which show that the gut microbial profile is important along with host genetics. Any dysbiosis with polymorphism might results in either obesity development or obesity directly or indirectly change the microbial diversity, which need further research work in experimental models. These findings support the hypothesis that the TLR (*TLR-2* and *TLR-4*) genetic alterations with the gut microbial diversity in obese individuals is significantly associated to dietary, demographic, and life-style risk factors of obese individuals.

Similarly, the unculturable diversity based on 16S rRNA gene based sequencing showed that the gut microbial composition in obesity had dysbiosis as microbial dysbiosis index was 0.40. Significantly high percentage of phyla *Firmicutes* especially of class *Clostridia*, *Negativicutes* and *Gammaproteobacteria* was increased in obese individuals while there was reduction in percentage of *Bacteroidota* in obese individuals. At phyla level dominance of *Firmicutes* is associated with

obesity as like many earlier studies (Indiani *et al*., 2018;Sutoyo *et al*., 2020; Crovesy *et al*., 2020). The metadata analysis in this study on the sequenced microbiome revealed that a significant positive correlation of *Firmicutes* and *Actinobacteriota* with study factors where a negative correlation was observed between phylum *Bacteroidota* and multiple studied factors like demographic, life style and dietary factors. The MD index was 2.26, when gender as factors was assessed against microbiome, indicated obese females' gender as risk factor for the dysbiosis of the gut microflora when obese males were set as reference group. The marital status was also positively associated as MD index was 0.36 while among the dietary patterns beef intake was negative for dysbiosis (MD index = -0.46). As previous work on obese and lean twin mice model, *Firmicutes* were found to be efficient in energy uptake while *Bacteroidota* in production of short-chain fatty acids (SCFAs) and *Actinobacteriota* had key role in biodegradation of resistant starch. The microbial diversity in these mice model indicate that each Phyla have distinctive role in the host gut.

The altered bacterial diversity detected after sequencing in this study indicate that the weight gains and development of obesity was affected by the bacterial gut diversity changes by making available metabolites like SCFA, LPS *etc*. As the intestinal microbiota participate in the energy harvesting by the uptake of monosaccharide and other carbohydrates (Turnbaugh *et al*., 2009; Zhang *et al*., 2013), which are suggested to effect the abundance of gut microbiota, which not contribute in health but also in development of metabolic disorders, characterized by low-grade inflammation.

A model based on lean mice (C57bl6/J) found high circulating plasma LPS endotoxin after consumption of high-carbohydrate / high-energy diets for one month (Amar *et al*., 2008). As in current study the staple food of study participants was wheat (Carbohydrates), it could be one of the factor for increased LPS detected in the plasma of obese individuals of present study. Although the obese individuals microbiome was dominated with Gram positive but core microbiome was found to be rich with *Proteobacteria* phylum which are gram negatives which were the major cultureable bacterial diversity of obese subjects too. An increase in Gram-negative bacteria causes LPS to be released, which increases gut permeability and causes endotoxemia. That in turn alter innate immune responses by making the gut microbiota the best source of ligands for TLRs (TLR-2 and TLR-4). A mouse model was used to investigate colonization of germ free mice with a

complex gut microbiota where modulation of TLR-2 was seem with enhanced TLR-2 expression in the immune cells(Hörmann *et al*., 2014). It is also evident from another study on TLR-4 and TLR-2 deficient mice for investigation of susceptibility too diet-induced obesity and a decreased in obesity was recorded(Tsukumo *et al*., 2007; Ji *et al*., 2019) showing vital role of microbial as well as dietary factors in development of obesity accompanied by inflammation. Hence, increase in specific bacterial Phyla and species with LPS production have a role in obesity associated with *TLRs* signalling.

In a nutshell, it can be summarized that obesity is a multifactorial disease in which diet, lifestyle, and demographics act as risk factors, along with the polymorphisms of TLRs and microorganisms acted as risk factors. Based on the current study's findings, it is possible to hypothesize that functional polymorphisms in the TLR gene might have significant role in initiating or sustaining a low-grade inflammation under the influence of multiple endogenous (LPS) as well as exogenous (dietary) factors. These factors either alone or in combination with other factors affect or are affected by each other, forming a complex interdependent network. A thorough look into host genetics to their lifestyle, diet, gut profile and much more is required in order to understand obesity.

Conclusion:

Obesity and its risk factors among the local Pakistani population were examined along with the genetic changes in *TLR-2* and *TLR-4.* Additionally, the cultureable and unculturable microbial colonization in obese and control subjects were examined for any dysbiosis as well as the associations between risk variables with colonization. The conclusion based on the finding of research work are as follows:

1. Higher rate of obesity was among females belonging to lower socioeconomic level with secondary level education. Additionally, their dietary choices and sedentary lifestyle contributed in obesity
2. Dietary habits (meals, fruits/vegetables, meat, and snacking) and fluid intake (tea, green tea) were high risk factors of obesity
3. The lifestyles including screen time, sleep habits, dinner timing and smoking were directly associated as risk for obesity
4. The four detected SNPs in *TLR-2* were present in different exonic region of the gene and in *TLR-4* the seven detected SNPs were present in intronic and exonic region of the gene
5. Overall 3 novel SNPs were identified in *TLR-2* and *TLR-4* genes; *TLR-2* (153705074 C >A) and *TLR-4* (117708777 C>G; 117715449 T>A) in obesity
6. *In silico* analysis showed that due variant 153688371 T >C a novel binding site for hsa-miR-4523 in *TLR-2* and variant 117708080 A>G for hsa-miR-32-3p in *TLR-4* mRNA was created
7. The change Gibbs free energy was observed among detected mutant and wild sequences of TLR-2 and TLR-4 mRNA indication overall change in translation of these transcripts
8. The impact of detected SNPs in *TLR-2* and *TLR-4* were predicted to alter their expression on plasma membrane of the immune cells as were in important domain like TIR for signal transduction and ligand binding LRR5
9. Such predicted changes in the TLR-2 and TLR-4 might affect downstream signalling and generation of pro-inflammatory cytokines and chemokine
10. The three most abundant species among all study groups were *C. kefyr, C. albicans* and *Teunomyces krusei*, of which *C. kefyr* was highest in obese individuals

11. All *Candida* species were observed in obese group were highly pathogenic compared to *Candida* in other two groups on basis of virulence profiling as phospholipase, coagulase, caseinolytic, strong biofilm formation and resistance to antimicrobials were significantly associated risk to obesity
12. Altered cultureable bacterial diversity with increased *P. aeruginosa, K. pneumonia, Acinetobacter, P. vulgaris* in case obese individuals
13. After microbiome analysis, an increased *Firmicutes* (42.31%/6.59%) with decreased *Bacteroidota* (40.53%/90.95%) among obese was observed
14. Most abundant classes in Phylum *Firmicutes* were *Clostridia*, *Negativicutes* and *Gammaproteobacteria* in obese where class in the healthy individuals were members of phylum *Bacteroidota*
15. LPS were present in serum of obese individuals which was indicating endotoxima and it was found to be associated to microbial cultureable and unculture Gram negative diversity
16. Such levels of serum LPS were indicating low grade inflammation
17. Individually variants 117708080 A>G, 117707870 G>A and 117715853 G>C in *TLR-4* were significantly associated with both cultureable dysbiosis, dietary choices (beef, poultry and fruits/vegetable) and lifestyle (no physical exercise) changes
18. Gut microbial dysbiosis (cultureable and uncultureable) was found to be associated with demographics, dietary habits and lifestyle choices along with TLR mutation in obesity
19. Correlation analysis between cultureable bacterial and fungal diversity found such interactions change with each other positively and negative in obesity
20. In the microbiome, the detected phyla interact with each other forming complex and intricate networks which positively or are negatively effected in the obesity

Future perspective

This research work has analysed multiple aspects of obesity that showed genomic alterations significantly associated with demographic, dietary and life style habits. Additionally, the microbial diversity and TLRs were significantly associated to dietary and demographic factors leading to low grade inflammation. Further advancement of knowledge on causes and risks of obesity, following further research work needed:

1. A comprehensive study is required for the estimation of the obesity at national level with its underlying causes such as demographic, behavioural, life style, dietary environmental and any other genes as risk factors
2. Expressional studies on detected SNPs in *TLR-2* and *TLR-4* for their effects on production and release of cytokines and chemokine in obesity for low grade inflammation.
3. Investigation on the other metabolites released by identified microbial species that could play a role in sustaining the TLR mediated obesity inflammation and biomarker.
4. Comprehensive study on mycobiome and virome association with the TLR mediated obesity for development of prognostic and diagnostic assay.
5. For formulation of personalized diet for weight healthy management and modulate gut diversity for weight reduction by replacing healthy microbial flora
6. Develop understanding for risk factors which would helpful for health professionals for better prevention, interventions and controlling obesity.

SIGNIFICANCE OF STUDY

This study is first of its kind in this region that provide a snapshot of multiple interacting risk factors in obesity. Three novel mutations in *TLR-2* and *TLR-4* genes, innate immune receptors of inflammation were found in the obese individuals, which were detected in crucial domains and were significantly associated with multiple risk factors including demographics, dietary and life style. There was clear dysbiosis in cultureable and unculturable gut microflora among these obese individuals. The presence of serum LPS was due to high Gram negative abundance in cultureable gut flora which was substantiated with the presence of Phylum *Proteobacteria* in the unculturable gut microbiome, was clear indicator of potential endotoxemia a trigger of innate immunity TLRs for inflammation. Hence, presence of serum LPS is a biomarker of dysbiosis in obesity. Also Gram negatives can be possible indicator bacteria.

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Appendix I: Consent form and Questionnaire for collecting data of the study individuals

Quaid-i-Azam University Islamabad
Department of Microbiology
Obesity Questionnaire

Certificate of Consent
I have read the information carefully. I have had the opportunity to ask questions relating it and all have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research.

Name of Participant_____

Signature of Participant _____

Date _____
Day/month/year

Witness in case, if the participant is illiterate
I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness_____

Signature of witness _____

Date _____
Day/month/year

Thumb print of participant

Statement by the researcher/person taking consent
I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. Their information will be kept confidential
2. Their blood sample will be collected by a trained medical professional.
3. All information gathered , will only be used for this research purpose.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been forced into giving consent, and the consent has been given freely and voluntarily.

Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

Day/month/year

Quaid-I-Azam University Islamabad
Department of Microbiology
Obesity Questionnaire

All information about you that will be collected during the research will be kept confidential.
Thank you for volunteering in this research. You were selected by a scientific sampling procedure, and your cooperation is very important to the source of the study. This is a questionnaire you are asked to fill out. Please answer the questions honestly and accurately.
The questions can be answered by marking the most suited option or by filling in a blank with a number or word.

Name: _____ **DOB**: ___/___/_____ (Date/Month/Year) **Age**:_____
Gender: Male / Female
Weight: _____ **Height**: _____ **BMI**:_____
***healthy *pre-obese *obese**
How long have you been obese?
since age: ***1-12 *13-25 *26-40 *above**
Marital status: ***Single *Married *Separated *Divorced Widowed**
Number of children:_____
Age of last child: _____

Read carefully and mark the box that best suits your situation.

1. **Medical history**
   - Do you suffer from any of the following:

Allergies	High blood pressure
Autoimmune disorders	Insulin resistance
Arthritis	Type 2 diabetes
Asthma	Gallbladder disease
Cardiovascular disease	Obstructive sleep Apnea
Fatty Liver disease	Poly cystic ovarian disease
Diabetes (Type not known)	Thyroid problem
Cancer	Acid reflux

2. **Genetic history**
   - Is any other family member obese?
     ***Father *mother *sister *brother *Aunt or uncle**
   - Disease family history:
     ***Diabetes *high blood pressure *allergies *others**
     In case of others please specify:
3. **Demographics**
   - Province:_____ city**:** _____
   - Urban/rural
   - Occupation:_____ Income Level:_____
   - Birthplace:_____ Ethnic group:_____
- Education: ***school *collage *university *uneducated *other**

4. **Dietary trends and habits**
   - How many meals or snacks do you eat, each day?
     Meal:_____ Snacks: Fruits/vegetables:_____
   - What time you take your last meal _____, before going to bed?

- What time you sleep_____.
- How many times a week do you eat meat (beef____, mutton_____, chicken____)
- What sort of food do you eat the most?
  ***healthy food** ***fast food** (deep fried items specially)
- What is your average fluid intake per day?
  Answer:_____ glass.
- Which type of food do you prefer:
  ***Sweet** ***savoury** ***both**
- How frequently do you take **tea/coffee** per day:
  ***None** ***once** ***twice** ***thrice** ***more**
- How much sugar do u take in **tea/coffee**:
  ***No sugar** ***1 teaspoon** ***2 teaspoon** ***more**
- What particularly in fluids you consume the most? In what quantity(glass)?

Water _____	Whole milk_____	Soda _____	Tea _____
Juice _____	Skim milk _____	Diet soda _____	Green tea_____
		Energy drink_____	Coffee_____

5. Lifestyle

(a) Smoking: Tobacco exposure: I never smoked (skip to other question)

Smoke regularly	Cigarettes/day: _____
Smoke occasionally	
Former smoker	
Chain smoker	

(b) Sleep pattern

- On average how many hours do you sleep each day?
  ***2-4 hours** ***4-8 hours** ***more**
- You have **regular/irregular** sleep pattern.

(c) Activities

- How many hours of television do you watch each day?
  ***don't watch** ***1-2** ***2-4** ***more**
- Do you do exercise? **(yes/no)**
  ***daily** ***occasionally**

(d) Stress

- Is your stress level high? **(yes/no)**

(e) Medication

Are you taking any sort of medication? **(yes/no)**
***Antidepressants** ***steroids**

Appendix II: A multinomial regression model for assessing the relationship between various sociodemographic characteristics and obesity

		Obese vs controls						**Pre-obese vs control**					
Socio-demographic characteristics	**Risk factors**	**β**	**Wald χ^2**	***p*-value**	**Odd ratio**	**95% Confidence Interval**		**β**	**Wald χ^2**	***p*-value**	**Odd ratio**	**95% Confidence Interval**	
						L	**U**					**L**	**U**
Gender	**Female**	.362	3.458	.063	1.436	.981	2.104	.512	3.154	.076	1.669	.948	2.936
	Male	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Marital status	**Married**	.928	19.919	.000*	2.529	1.683	3.802	1.429	16.197	.000	4.176	2.082	8.376
	Single	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Residence area	**Urban**	.737	11.708	.001*	2.090	1.370	3.189	1.605	19.009	.000	4.977	2.419	10.241
	Rural	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Socioeconomic status	**Lower strata**	1.065	9.267	.002*	2.900	1.461	5.757	.162	.102	.750	1.176	.435	3.178
	Middle strata	.811	6.511	.011*	2.250	1.207	4.193	.303	.464	.496	1.354	.566	3.236
	Upper strata	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Education level	**Secondary School level**	.351	1.377	.241	1.420	.791	2.551	-.488	1.262	.261	.614	.262	1.438
	Graduated	-.529	2.634	.105	.589	.311	1.116	-1.742	11.705	.001	.175	.065	.475
	Masters	-1.260	17.640	.000*	.284	.157	.511	-2.050	21.471	.000	.129	.054	.306
	Uneducated	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.

*Significant association was considered for *p*-value ≤0.05
L=lower, U=upper

Appendix III: A multinomial regression model for assessing the relationship between various dietary habits and obesity

		Obese vs Control						Pre-obese vs Control					
Risk factors	**numbers**	**Estimated β**	**Wald χ^2**	***p*-value**	**Odd ratio**	**95% Confidence Interval for Exp(B)**		**Estimated β**	**Wald χ^2**	***p*-value**	**Odd ratio**	**95% Confidence Interval for Exp(B)**	
						Lowe	**U**					**L**	**U**
Number of meals/day	**4**	3.83	7.64	0.006*	46.45	3.05	705.45	-18.476	0.000	0.998	9.464E-009	0.000	.[b]
	3	2.09	11.16	0.001*	8.12	2.37	27.78	-0.236	0.291	0.589	0.790	0.336	1.859
	2	0[b]	.	.	.	.	.	0[c]	.	.	.	.	.
Number of Snacks/day	**3**	-5.99	15.08	0.000*	0.002	0.00	0.05	-3.283	6.631	0.010*	0.038	0.003	0.456
	2	1.89	5.38	0.020*	6.61	1.34	32.67	0.006	0.000	0.994	1.006	0.196	5.175
	1	0.144	0.06	0.798	1.15	0.38	3.46	1.421	8.505	0.004*	4.141	1.594	10.761
	None	0[b]	.	.	.	.	.	0[c]	.	.	.	.	.
Portion of fruits and vegetables /day	**3-4**	3.27	17.55	0.000*	26.54	5.72	123.06	1.945	8.470	0.004*	6.993	1.887	25.912
	1-2	2.17	12.47	0.000*	8.76	2.62	29.22	2.196	13.575	0.000*	8.987	2.795	28.900
	None	0[b]	.	.	.	.	.	0[c]	.	.	.	.	.

Preferred flavour	**Sweet**	27.35	0.00	0.994	760881361010.13	0.00	.[c]	-4.418	45.920	0.000*	0.012	0.003	0.043
	Savoury	23.25	0.00	0.995	126004655 21.831	0.00	.[c]	-4.915	47.767	0.000*	0.007	0.002	0.030
	Both types	0[b]	.	.	.	.	.	0[c]	.	.	.	.	.
Preferred meal	**Fast food**	0.205	0.375	0.540	1.228	0.636	2.369	0.497	0.815	0.367	1.644	0.559	4.840
	homecooked	3.378	78.276	0.000*	29.314	13.870	61.956	4.915	47.767	0.000*	136.307	33.822	549.336
	Both types	0[b]	.	.	.	.	.	0[c]	.	.	.	.	.
Mutton consumption/week	**>3**	0.35	0.05	0.815	1.43	0.07	28.93	-13.978	.	.	8.502E-007	8.502E-007	8.502E-007
	1-2	-0.50	0.77	0.378	0.60	0.19	1.86	0.634	1.428	0.232	1.885	0.666	5.331
	None	0[b]						0[c]	.	.	.	.	.
Beef consumption/week	**>3**	0.62	0.25	0.611	1.87	0.16	21.00	0.365	0.711	0.399	1.440	0.617	3.360
	1-2	1.28	5.75	0.016*	3.60	1.26	10.25	0.326	0.119	0.730	1.385	0.218	8.812
	None	0[b]	.	.	.	.	.	0[c]	.	.	.	.	.
Chicken consumption/week	**>3**	0.26	0.	0.821	1.30	0.13	12.66	-3.540	18.277	0.000*	0.029	0.006	0.147

			05										
	1-2	0.68	0.36	0.545	1.98	0.21	18.37	-3.004	14.169	0.000*	0.0500	0.010	0.237
	None[b]	0[b]	.	.	.	.	.	0[c]	.	.	.	.	.
*Significant association was considered for *p*-value ≤0.05 L=lower, U=upper													

Appendix IV: A multinomial regression model for assessing the relationship between various drinking habits and obesity

Risk factors	numbers	Obese vs control						Pre-obese vs control					
		Estimated β	Wald χ^2	*p*-value	Odd ratio	95% Confidence Interval for Exp(B)		Estimated β	Wald χ^2	*p*-value	Odd ratio	95% Confidence Interval for Exp(B)	
						L	U					L	U
Fluid intake (litre)	**3**	-2.539	2.510	0.113	0.079	0.003	1.825	-0.860	0.887	0.346	0.423	0.071	2.533
	2	5.065	37.525	0.000*	158.405	31.329	800.927	2.433	32.911	0.000*	11.398	4.963	26.174
	1	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Tea intake cups/day	**<3**	19.717	0.000	0.995	365579151.997	0.000	.[c]	22.420	0.000	0.993	5457205447.667	0.000	.[c]
	3	1.899	5.691	0.017*	6.681	1.403	31.809	0.455	0.573	0.449	1.576	0.485	5.121
	2	2.060	7.699	0.006*	7.847	1.831	33.630	1.484	7.222	0.007*	4.410	1.494	13.016
	None	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Sugar teaspoon/ cup	**3**	22.491	0.000	0.995	585595541142	0.000	.[c]	17.894	0.000	0.997	59028505.509	0.000	.[c]
	2	-5.693	26.099	0.000*	0.003	0.000	0.030	-3.126	27.600	0.000*	0.044	0.014	0.141
	1	-4.074	14.473	0.000*	0.017	0.002	0.139	-2.486	19.679	0.000*	0.083	0.028	0.250
	None	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Water intake(litre/day)	**>3**	-.835	2.580	0.108	0.434	0.157	1.202	-.937	3.859	0.049*	0.392	0.154	0.998
	3	-0.764	2.158	0.142	0.466	0.168	1.291	-1.167	4.353	0.037*	0.311	0.104	0.932
	1-2	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.

Fresh Juice consumption/day	**2**	-9.506	.	.	7.439E-005	7.439E-005	7.439E-005	20.486	0.000	0.997	788508616.560	0.000	.[c]
	1	-1.450	3.358	0.067	0.235	0.050	1.106	-0.562	1.007	0.316	0.570	0.190	1.708
	None	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Whole milk/day	**2**	-1.420	0.934	0.334	0.242	0.014	4.305	-0.636	1.391	0.238	0.529	0.184	1.523
	1	-1.592	5.464	0.019*	0.204	0.054	0.773	-0.550	0.406	0.524	0.577	0.106	3.131
	None	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Soda intake/day	**2**	13.462	0.000	0.999	702547.054	0.000	.[c]	17.183	.	.	28993648.547	289936 48.547	289936 48.547
	1	-2.410	7.578	0.006*	0.090	0.016	0.500	-2.613	5.548	0.019*	0.073	0.008	0.645
	None	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Green tea cup/day	**2**	20.255	0.000	0.998	626009084.218	0.000	.[c]	18.807	0.000	0.997	147204964.936	0.000	.[c]
	1	2.076	8.938	0.003*	7.976	2.044	31.115	1.009	4.993	0.025*	2.743	1.132	6.647
	None	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.

*Significant association was considered for *p*-value ≤0.05

L=lower, U=upper

Appendix V: A multinomial regression model for assessing the relationship between lifestyle and obesity

Risk factors	numbers	Estimated β	Wald χ^2	*p*-value	Odd ratio	95% Confidence Interval for Exp(B)		Estimated β	Wald χ^2	*p*-value	Odd ratio	95% Confidence Interval for Exp(B)	
						L	U					L	U
Sleeping (hrs)	**>8**	8.477	20.813	0.000*	4804.214	125.874	183361.167	1.594	3.101	0.078	4.924	0.835	29.039
	4-8	5.802	22.697	0.000*	330.844	30.412	3599.181	1.314	2.628	0.105	3.721	0.760	18.220
	2-4	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Sleeping pattern	**Irregular**	-1.579	8.194	0.004*	0.206	0.070	0.608	-1.000	12.240	0.000*	0.368	0.210	0.644
	Regular	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Gap between last meal and sleep(hrs)	**≥3**	-2.696	9.653	0.002*	0.067	0.012	0.370	-1.043	4.272	0.039*	0.352	0.131	0.947
	3	-.193	0.159	0.690	0.824	0.319	2.133	0.242	0.677	0.411	1.273	0.716	2.265
	1-2	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
TV/mobile time (hrs/day)	**2-4**	1.509	5.503	0.019*	4.524	1.282	15.966	-0.423	0.928	0.335	0.655	0.277	1.549
	1-2	1.072	3.612	0.057*	2.920	0.967	8.818	0.026	0.008	0.930	1.026	0.577	1.825
	None	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Exercise	**Yes**	0.909	2.656	0.103	2.482	0.832	7.404	-1.173	17.135	0.000*	0.310	0.178	0.539
	No	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.
Smoking history	**Yes**	-2.661	10.814	0.001*	0.070	0.014	0.341	-1.130	9.278	0.002*	0.323	0.156	0.668
	No	0[b]	.	.	.	.	.	0[b]	.	.	.	.	.

*Significant association was considered for p-value ≤0.05
L=lower, U=upper

Appendix VI: Preparation of stock solution for DNA extraction

Sr. No.	Solution	Chemicals	Amount	Initially distill water (mL)	Final volume upto (mL)
1	1 M Tris HCL	Tris HCL	14.532(mg)	120	150
2	0.1 M of EDTA (PH-8.8)	EDTA	73 (g)	450	500
		NaOH	For pH adjustment only		
3	20 % of sodium dodecyl sulphate	SDS	20 (g)	50	100
4	70 % of ethanol	Absolute Ethanol	70 (mL)	30	100
5	Sodium acetate	CH3COONa	9.84 (g)	25	40
6	T.E Buffer	EDTA (0.2M)	10 (mL)	70	100
		Tris HCL(1M)	20 (mL)		

Appendix VII: Preparation of working solutions for DNA extraction

Sr. No.	Solutions	Chemicals	Amount	Distilled water (mL)	Final Volume (mL)
1	Solution A	Sucrose(0.32M)	27.63g	150	250
		Tris (10mM)	0.303g		
		MgCl2 (5mM)	0.254g		
		Triton X 100	2.5mL		
2	Solution B	Tris (10mM)	0.364g	200	300
		NaCl (400mM)	7.02g		
		EDTA	0.1752g		
3	Solution C	Tris HCL (10mM)	0.605g	____	500
		Phenol	500mL		
4	Soultion D	chloroform	20mL	____	500
		Iso-amyl Alcohol	480mL		

Appendix VIII: Preparation of 10X TBE for Gel Electrophoresis

Sr. No.	Chemicals	Amount (g)	Distilled water (mL)	Final Volume (mL)
1	Tris Base	27	220	250
2	EDTA	2.325		
3	Boric Acid	14.5		

Appendix IX: Preparation of Bromophenol Blue stock solution for DNA loading

Sr. No.	Chemical	Amount (g)	Distilled water (mL)	Final Volume (mL)
1	Bromophenol Blue	0.05	20	50
2	Sucrose	8.0		

Appendix X. List of primers for the *TLR-2* gene polymerase chain reaction

Exons/Primer name	Primers	Length (bp)	GC (%)	Product Size (bp)
Exon 1/Exon 1	F: CTTCTTCGACCCAGCCTTG	19	57.9	397
	R:GTGCAGAGAGACCACACGAG	20	60.0	
Exon 2/Exon 2	F:TCACTGCTCACACTGTTGGA	20	50.0	393
	R:AGAGCACCAGTCCAGTTTCAG	21	52.4	
Exon 3/Exon 3	F:ATTGTGCCCATTGCTCTTTC	20	45.0	833
	R:CCAGATTTGGCCATAAACAA	20	40.0	
Exon 4/Exon 4A	F:AGCCCTCACCAGACAACAGT	20	55.0	849
	R:TCCTGCCTAGAAGTAAATGCAA	22	40.9	
Exon 4/Exon 4B	F:AATCTGAACAGCCCATCCAG	20	50.0	836
	R:TACCATTGCGGTCACAAGAC	20	50.0	
Exon4/Exon 4C	F:TGTAGGTTGAAGCACTGGACA	21	47.6	712
	R:CTCGCAGTTCCAAACATTCC	20	50.0	
Exon 4/Exon 4D	F:TGCTGGACTTACCTTCCTTGA	21	47.6	798
	R:TCTGGCCACTGACAAGTTTC	20	50.0	
Exon 4/Exon 4E	F:GCCTGGCCCTCTCTACAAAC	20	60.0	699
	R:ATACCACAGGCCATGGAAAC	20	50.0	
Exon 4/ Exon 4F	F:GCATGTGCTGTGCTCTGTTC	20	55.0	600
	R:GACTGGTCTTAAATATGGGAACC	23	43.5	
Exon 4/ Exon 4G	F:CTGAGAGCTGCGATAAAGTCC	21	52.4	699
	R:TGGCCACAGAGGAGTCTCTTA	21	52.4	

Appendix XI. List of primers for the *TLR-4* gene polymerase chain reaction

S. No:	Exons	Primers	Length(bp)	GC (%)	Product Size(bp)
1.	**Exon 1**	F: TTGGAAAATCACCGTCATCC	20	45	588
		R: TAAACAAACCAGGGCACACA	20	45	
2.	**Exon 2**	F: CTGGGCCATGACTAAGGAAG	20	55	684
		R: AGGGGCCTCTTTTTGTGC	18	55	
3.	**Exon 3**	F: CCTCTCCACCATCTCTGGTC	20	60	630
		R: CCTTAACAACAGGGGCTGTG	20	55	
4.	**Exon 4 Ex4 P-1**	F: CTCAGTCTGTGGGGCTTCTT	20	55	765
		R: CAGGCCCTCTAGAGCAGATT	20	55	
5.	**Ex4 P-2**	F: TCTGGCTGGTTTAGAAGTCCA	21	47	497
		R: TCATGGTAATAACACCATTGAAGC	24	37	
6.	**Ex4 P -3**	F: CCAACAAAGGTGGGAATGCT	20	50	696
		R: GCAGGAAACTCTGGTGTTCA	20	50	
7.	**Ex4 P-4**	F: TGTCTGAACTCCCTCCAGGT	20	55	527
		R: GCCACACCGGGAATAAAGT	19	52	
8	**Ex4 P-5**	F: CTCAAGCCAGGATGAGGACT	20	55	455
		R: TGCATCCTGTACCCACTGTT	20	50	
9	**Ex4 P-6**	F: CTGGAGACGACTCAGAAAAGC	21	52	585
		R: AAGGGGCATCTTGCATCA	18	50	
10	**Ex4 P-7**	F: GGGCTCCTGATGCAAGATG	19	57	845
		R: TGTTTCTGAGGAGGCTGGAT	20	50	
11	**Ex4 P-8**	F: TGCCAGGAGAACTACGTGTG	20	55	677
		R: TAATGGCCTCCGGGTTTATT	20	45	
12	**Ex4 P-9**	F: AATAAACCCGGAGGCCATT	19	47	791
		R: TCAAACAGCCATAGACATCCA	21	42	
13	**Ex4 P-10**	F: TGGATGTCTATGGCTGTTTGA	21	42	794
		R: CCCTTTATCCCTCTTAAATCCAG	23	43	
14	**Ex4 P-11**	F: TCAGTTCCCTCTCCCAGATG	20	55	996
		R: CTGAGAAGACCTCCCAGTGC	20	60	
15	**Ex4 P-12**	F: GGCACTAGGAGAAGCACTGG	20	60	924
		R: AGTCTGTGATGCCCGTCAG	19	57	
16	**Ex4 P-13**	F: GGTTATGGGGGAAGTTCCAA	20	50	832
		R: CATTCCCAGAACTGCTTGC	19	52	
17	**Ex4 P-14**	F: CTCAAGCCAGGATGAGGACT	20	50	767
		R: GGAAGGGGTTAAGGGATGAG	20	55	
18	**Ex4 P-15**	F: TGGGCTGTTAGCGTAACCAT	20	50	781
		R: GGCCAATCCTAGTATCCCAAA	21	47	

19	**Ex4 P-16**	F: ATAGCCCAGGCAAGGTCAG	19	57	869
		R: GGTGGCAGACCTGAATCCT	19	57	
20	**Ex4 P-17**	F: CAGGGAATACCTTTGCCAGT	20	50	809
		R: AGTGGTGGAGCCATTTCACT	20	50	
21	**Ex4 P-18**	F: ATGGCTCCACCACTAACAGG	20	55	741
		R: GGAGTGCTGGCACTAAGGAC	20	60	
22	**Ex4 P-19**	F: AGGGCTGCTATCATCCTTCA	20	50	772
		R: TTTGAGGGCACTTCTTGGTC	20	50	

Appendix XII: Acryl-Bisacrylamide 30% solution for vertical gel to detect SNP

S.no.	Chemicals	Amount (g)
1	Acryl Amide	145
2	Bisacrylamide	5
Dissolve in 250 mL of water and then raise the final volume up to 500mL		

Appendix XIII: Ammonium per Sulphate 10% solution for polyacryl amide gel

S.no.	**Chemicals**	**Amount**
1.	Ammonium per sulphate	5g
2.	Distill water	45mL

Appendix XIV: Predicted MicroRNA on mRNA Selected Regions of *TLR-2* accessed by miRWalk database

s.no	Gene Name	RefSeqID	MicroRNA	StemLoop ID	Seed Length	Start	Position	End	Region	Pvalue
1.	*TLR2*	NM_003264	hsa-miR-30c-2*	hsa-mir-30c-2	9	72	1	64	5UTR	0.0008
2.	*TLR2*	NM_003264	hsa-miR-191*	hsa-mir-191	9	23	1	15	5UTR	0.0008
3.	*TLR2*	NM_003264	hsa-miR-30c-1*	hsa-mir-30c-1	8	72	1	65	5UTR	0.0033
4.	*TLR2*	NM_003264	hsa-miR-21*	hsa-mir-21	8	84	1	77	5UTR	0.0033
5.	*TLR2*	NM_003264	hsa-miR-30c-2*	hsa-mir-30c-2	8	71	2	64	5UTR	0.0033
6.	*TLR2*	NM_003264	hsa-miR-1225-3p	hsa-mir-1225	8	160	1	153	5UTR	0.0033
7.	*TLR2*	NM_003264	hsa-miR-720	hsa-mir-720	8	14	1	7	5UTR	0.0033
8.	*TLR2*	NM_003264	hsa-miR-520f	hsa-mir-520f	8	214	2	207	5UTR	0.0033
9.	*TLR2*	NM_003264	hsa-miR-191*	hsa-mir-191	8	22	2	15	5UTR	0.0033
10.	*TLR2*	NM_003264	hsa-miR-1825	hsa-mir-1825	8	217	1	210	5UTR	0.0033
11.	*TLR2*	NM_003264	hsa-miR-1233	hsa-mir-1233	7	160	1	154	5UTR	0.0133
12.	*TLR2*	NM_003264	hsa-miR-488	hsa-mir-488	7	190	1	184	5UTR	0.0133
13.	*TLR2*	NM_003264	hsa-miR-625*	hsa-mir-625	7	112	2	106	5UTR	0.0133
14.	*TLR2*	NM_003264	hsa-miR-146b-5p	hsa-mir-146b	7	68	2	62	5UTR	0.0133
15.	*TLR2*	NM_003264	hsa-miR-141	hsa-mir-141	7	76	2	70	5UTR	0.0133
16.	*TLR2*	NM_003264	hsa-miR-30c-1*	hsa-mir-30c-1	7	71	2	65	5UTR	0.0133
17.	*TLR2*	NM_003264	hsa-miR-21*	hsa-mir-21	7	83	2	77	5UTR	0.0133
18.	*TLR2*	NM_003264	hsa-miR-200a	hsa-mir-200a	7	76	2	70	5UTR	0.0133
19.	*TLR2*	NM_003264	hsa-miR-1276	hsa-mir-1276	7	178	2	172	5UTR	0.0133

20.	*TLR2*	NM_003264	hsa-miR-604	hsa-mir-604	7	24	2	18	5UTR	0.0133
21.	*TLR2*	NM_003264	hsa-miR-145	hsa-mir-145	7	218	1	212	5UTR	0.0133
22.	*TLR2*	NM_003264	hsa-miR-1225-3p	hsa-mir-1225	7	159	2	153	5UTR	0.0133
23.	*TLR2*	NM_003264	hsa-miR-639	hsa-mir-639	7	12	2	6	5UTR	0.0133
24.	*TLR2*	NM_003264	hsa-miR-30b*	hsa-mir-30b	7	121	1	115	5UTR	0.0133
25.	*TLR2*	NM_003264	hsa-miR-614	hsa-mir-614	7	43	1	37	5UTR	0.0133
26.	*TLR2*	NM_003264	hsa-miR-720	hsa-mir-720	7	13	2	7	5UTR	0.0133
27.	*TLR2*	NM_003264	hsa-miR-30b*	hsa-mir-30b	7	72	1	66	5UTR	0.0133
28.	*TLR2*	NM_003264	hsa-miR-615-3p	hsa-mir-615	7	62	1	56	5UTR	0.0133
29.	*TLR2*	NM_003264	hsa-miR-1233	hsa-mir-1233	7	160	1	154	5UTR	0.0133
30.	*TLR2*	NM_003264	hsa-miR-647	hsa-mir-647	7	25	2	19	5UTR	0.0133
31.	*TLR2*	NM_003264	hsa-miR-1825	hsa-mir-1825	7	216	2	210	5UTR	0.0133
32.	*TLR2*	NM_003264	hsa-miR-146a	hsa-mir-146a	7	68	2	62	5UTR	0.0133
33.	*TLR2*	NM_003264	hsa-miR-367*	hsa-mir-367	12	429	1	418	CDS	0.0001
34.	*TLR2*	NM_003264	hsa-miR-367*	hsa-mir-367	11	428	2	418	CDS	0.0006
35.	*TLR2*	NM_003264	hsa-miR-135a	hsa-mir-135a-2	10	2453	2	2444	CDS	0.0022
36.	*TLR2*	NM_003264	hsa-miR-20b	hsa-mir-20b	10	1211	2	1202	CDS	0.0022
37.	*TLR2*	NM_003264	hsa-miR-135a	hsa-mir-135a-1	10	2453	2	2444	CDS	0.0022
38.	*TLR2*	NM_003264	hsa-miR-548c-3p	hsa-mir-548c	10	871	1	862	CDS	0.0022
39.	*TLR2*	NM_003264	hsa-miR-106b	hsa-mir-106b	9	1212	1	1204	CDS	0.0089
40.	*TLR2*	NM_003264	hsa-miR-548c-3p	hsa-mir-548c	9	870	2	862	CDS	0.0089
41.	*TLR2*	NM_003264	hsa-miR-140-3p	hsa-mir-140	9	2069	1	2061	CDS	0.0089
42.	*TLR2*	NM_003264	hsa-miR-20a	hsa-mir-20a	9	1212	1	1204	CDS	0.0089

43.	*TLR2*	NM_003264	hsa-miR-370	hsa-mir-370	9	2130	2	2122	CDS	0.0089
44.	*TLR2*	NM_003264	hsa-miR-30c-2*	hsa-mir-30c-2	9	2381	2	2373	CDS	0.0089
45.	*TLR2*	NM_003264	hsa-miR-1201	hsa-mir-1201	9	297	1	289	CDS	0.0089
46.	*TLR2*	NM_003264	hsa-miR-516b*	hsa-mir-516b-2	9	2110	2	2102	CDS	0.0089
47.	*TLR2*	NM_003264	hsa-miR-374a*	hsa-mir-374a	9	1118	2	1110	CDS	0.0089
48.	*TLR2*	NM_003264	hsa-miR-512-3p	hsa-mir-512-1	9	1988	2	1980	CDS	0.0089
49.	*TLR2*	NM_003264	hsa-miR-516b*	hsa-mir-516b-1	9	2110	2	2102	CDS	0.0089
50.	*TLR2*	NM_003264	hsa-miR-636	hsa-mir-636	9	1870	2	1862	CDS	0.0089
51.	*TLR2*	NM_003264	hsa-miR-512-3p	hsa-mir-512-2	9	1988	2	1980	CDS	0.0089
52.	*TLR2*	NM_003264	hsa-miR-516a-3p	hsa-mir-516a-1	9	2110	2	2102	CDS	0.0089
53.	*TLR2*	NM_003264	hsa-miR-135b	hsa-mir-135b	9	2453	2	2445	CDS	0.0089
54.	*TLR2*	NM_003264	hsa-miR-1233	hsa-mir-1233	9	370	1	362	CDS	0.0089
55.	*TLR2*	NM_003264	hsa-miR-516a-3p	hsa-mir-516a-2	9	2110	2	2102	CDS	0.0089
56.	*TLR2*	NM_003264	hsa-miR-505*	hsa-mir-505	9	2095	2	2087	CDS	0.0089
57.	*TLR2*	NM_003264	hsa-miR-1233	hsa-mir-1233	9	370	1	362	CDS	0.0089
58.	*TLR2*	NM_003264	hsa-miR-329	hsa-mir-329-2	8	446	2	439	CDS	0.0353
59.	*TLR2*	NM_003264	hsa-miR-17	hsa-mir-17	8	1211	2	1204	CDS	0.0353
60.	*TLR2*	NM_003264	hsa-miR-526b*	hsa-mir-526b	8	1211	2	1204	CDS	0.0353
61.	*TLR2*	NM_003264	hsa-miR-1254	hsa-mir-1254	8	459	1	452	CDS	0.0353
62.	*TLR2*	NM_003264	hsa-miR-148a*	hsa-mir-148a	8	1666	1	1659	CDS	0.0353
63.	*TLR2*	NM_003264	hsa-miR-485-5p	hsa-mir-485	8	271	1	264	CDS	0.0353
64.	*TLR2*	NM_003264	hsa-miR-873	hsa-mir-873	8	2020	1	2013	CDS	0.0353
65.	*TLR2*	NM_003264	hsa-miR-580	hsa-mir-580	8	2422	2	2415	CDS	0.0353

66.	*TLR2*	NM_003264	hsa-miR-1233	hsa-mir-1233	8	3691	2	3624	CDS	0.0353
67.	*TLR2*	NM_003264	hsa-miR-520b	hsa-mir-520b	8	1211	1	1204	CDS	0.0353
68.	*TLR2*	NM_003264	hsa-miR-106b	hsa-mir-106b	8	1211	2	1204	CDS	0.0353
69.	*TLR2*	NM_003264	hsa-miR-618	hsa-mir-618	8	1071	1	1064	CDS	0.0353
70.	*TLR2*	NM_003264	hsa-miR-1261	hsa-mir-1261	8	1552	2	1545	CDS	0.0353
71.	*TLR2*	NM_003264	hsa-miR-553	hsa-mir-553	8	2054	2	2047	CDS	0.0353
72.	*TLR2*	NM_003264	hsa-miR-543	hsa-mir-543	8	899	1	892	CDS	0.0353
73.	*TLR2*	NM_003264	hsa-miR-140-3p	hsa-mir-140	8	2068	2	2061	CDS	0.0353
74.	*TLR2*	NM_003264	hsa-miR-1200	hsa-mir-1200	8	1860	1	1853	CDS	0.0353
75.	*TLR2*	NM_003264	hsa-miR-20a	hsa-mir-20a	8	1211	2	1204	CDS	0.0353
76.	*TLR2*	NM_003264	hsa-miR-520c-3p	hsa-mir-520c	8	1211	1	1204	CDS	0.0353
77.	*TLR2*	NM_003264	hsa-miR-558	hsa-mir-558	8	339	1	332	CDS	0.0353
78.	*TLR2*	NM_003264	hsa-miR-370	hsa-mir-370	8	1942	2	1935	CDS	0.0353
79.	*TLR2*	NM_003264	hsa-miR-1200	hsa-mir-1200	8	342	2	335	CDS	0.0353
80.	*TLR2*	NM_003264	hsa-miR-520d-3p	hsa-mir-520d	8	1211	1	1204	CDS	0.0353
81.	*TLR2*	NM_003264	hsa-miR-630	hsa-mir-630	8	1348	1	1341	CDS	0.0353
82.	*TLR2*	NM_003264	hsa-miR-1272	hsa-mir-1272	8	264	1	257	CDS	0.0353
83.	*TLR2*	NM_003264	hsa-miR-559	hsa-mir-559	8	1285	2	1278	CDS	0.0353
84.	*TLR2*	NM_003264	hsa-miR-372	hsa-mir-372	8	1211	1	1204	CDS	0.0353
85.	*TLR2*	NM_003264	hsa-miR-495	hsa-mir-495	8	1020	1	1013	CDS	0.0353
86.	*TLR2*	NM_003264	hsa-miR-143	hsa-mir-143	8	664	1	657	CDS	0.0353
87.	*TLR2*	NM_003264	hsa-miR-630	hsa-mir-630	8	1312	1	1305	CDS	0.0353
88.	*TLR2*	NM_003264	hsa-miR-181a-2*	hsa-mir-181a-2	8	1521	2	1514	CDS	0.0353

89.	*TLR2*	NM_003264	hsa-miR-561	hsa-mir-561	8	2341	1	2334	CDS	0.0353
90.	*TLR2*	NM_003264	hsa-miR-145	hsa-mir-145	8	2373	1	2366	CDS	0.0353
91.	*TLR2*	NM_003264	hsa-miR-1201	hsa-mir-1201	8	296	2	289	CDS	0.0353
92.	*TLR2*	NM_003264	hsa-miR-26a	hsa-mir-26a-1	8	1352	1	1345	CDS	0.0353
93.	*TLR2*	NM_003264	hsa-miR-130b*	hsa-mir-130b	8	1232	1	1225	CDS	0.0353
94.	*TLR2*	NM_003264	hsa-miR-1283	hsa-mir-1283-2	8	875	1	868	CDS	0.0353
95.	*TLR2*	NM_003264	hsa-miR-203	hsa-mir-203	8	1788	1	1781	CDS	0.0353
96.	*TLR2*	NM_003264	hsa-miR-1283	hsa-mir-1283-1	8	875	1	868	CDS	0.0353
97.	*TLR2*	NM_003264	hsa-miR-607	hsa-mir-607	8	1545	1	1538	CDS	0.0353
98.	*TLR2*	NM_003264	hsa-miR-1208	hsa-mir-1208	8	431	1	424	CDS	0.0353
99.	*TLR2*	NM_003264	hsa-miR-26a-1*	hsa-mir-26a-1	8	1490	2	1483	CDS	0.0353
100.	*TLR2*	NM_003264	hsa-miR-26a	hsa-mir-26a-2	8	1352	1	1345	CDS	0.0353
101.	*TLR2*	NM_003264	hsa-miR-298	hsa-mir-298	8	2469	2	2462	CDS	0.0353
102.	*TLR2*	NM_003264	hsa-miR-520e	hsa-mir-520e	8	1211	1	1204	CDS	0.0353
103.	*TLR2*	NM_003264	hsa-miR-1287	hsa-mir-1287	8	1555	1	1548	CDS	0.0353
104.	*TLR2*	NM_003264	hsa-miR-26b	hsa-mir-26b	8	1352	1	1345	CDS	0.0353
105.	*TLR2*	NM_003264	hsa-miR-26a-2*	hsa-mir-26a-2	8	1490	2	1483	CDS	0.0353
106.	*TLR2*	NM_003264	hsa-miR-640	hsa-mir-640	8	2276	1	2269	CDS	0.0353
107.	*TLR2*	NM_003264	hsa-miR-892a	hsa-mir-892a	8	1570	1	1563	CDS	0.0353
108.	*TLR2*	NM_003264	hsa-miR-1297	hsa-mir-1297	8	1352	1	1345	CDS	0.0353
109.	*TLR2*	NM_003264	hsa-miR-29a*	hsa-mir-29a	8	1740	1	1733	CDS	0.0353
110.	*TLR2*	NM_003264	hsa-miR-361-5p	hsa-mir-361	8	1118	1	1111	CDS	0.0353
111.	*TLR2*	NM_003264	hsa-miR-450b-5p	hsa-mir-450b	8	676	2	669	CDS	0.0353

112.	*TLR2*	NM_003264	hsa-miR-130a*	hsa-mir-130a	8	998	1	991	CDS	0.0353
113.	*TLR2*	NM_003264	hsa-miR-576-5p	hsa-mir-576	8	1309	1	1302	CDS	0.0353
114.	*TLR2*	NM_003264	hsa-miR-520a-3p	hsa-mir-520a	8	1211	1	1204	CDS	0.0353
115.	*TLR2*	NM_003264	hsa-miR-136	hsa-mir-136	8	1094	1	1087	CDS	0.0353
116.	*TLR2*	NM_003264	hsa-miR-1233	hsa-mir-1233	8	369	2	362	CDS	0.0353
117.	*TLR2*	NM_003264	hsa-miR-1299	hsa-mir-1299	8	1678	1	1671	CDS	0.0353
118.	*TLR2*	NM_003264	hsa-miR-93	hsa-mir-93	8	1211	2	1204	CDS	0.0353
119.	*TLR2*	NM_003264	hsa-miR-513a-5p	hsa-mir-513a-1	8	1839	1	1832	CDS	0.0353
120.	*TLR2*	NM_003264	hsa-miR-362-3p	hsa-mir-362	8	446	2	439	CDS	0.0353
121.	*TLR2*	NM_003264	hsa-miR-329	hsa-mir-329-1	8	446	2	439	CDS	0.0353
122.	*TLR2*	NM_003264	hsa-miR-15a*	hsa-mir-15a	8	2064	1	2057	CDS	0.0353
123.	*TLR2*	NM_003264	hsa-miR-106a	hsa-mir-106a	8	1211	2	1204	CDS	0.0353
124.	*TLR2*	NM_003264	hsa-miR-513a-5p	hsa-mir-513a-2	8	1839	1	1832	CDS	0.0353
125.	*TLR2*	NM_003264	hsa-miR-1826	hsa-mir-1826	9	2900	2	2892	3UTR	0.0032
126.	*TLR2*	NM_003264	hsa-miR-548d-3p	hsa-mir-548d-2	9	2774	1	2766	3UTR	0.0032
127.	*TLR2*	NM_003264	hsa-miR-582-5p	hsa-mir-582	9	3145	1	3137	3UTR	0.0032
128.	*TLR2*	NM_003264	hsa-miR-374b*	hsa-mir-374b	9	3232	1	3224	3UTR	0.0032
129.	*TLR2*	NM_003264	hsa-miR-561	hsa-mir-561	9	3390	2	3382	3UTR	0.0032
130.	*TLR2*	NM_003264	hsa-miR-328	hsa-mir-328	9	3138	2	3130	3UTR	0.0032
131.	*TLR2*	NM_003264	hsa-miR-575	hsa-mir-575	9	3161	2	3153	3UTR	0.0032
132.	*TLR2*	NM_003264	hsa-miR-548d-3p	hsa-mir-548d-1	9	2774	1	2766	3UTR	0.0032
133.	*TLR2*	NM_003264	hsa-miR-708*	hsa-mir-708	9	2608	1	2600	3UTR	0.0032
134.	*TLR2*	NM_003264	hsa-miR-708*	hsa-mir-708	8	2607	2	2600	3UTR	0.0128

135.	*TLR2*	NM_003264	hsa-miR-580	hsa-mir-580	8	3203	1	3196	3UTR	0.0128
136.	*TLR2*	NM_003264	hsa-miR-544	hsa-mir-544	8	3118	2	3111	3UTR	0.0128
137.	*TLR2*	NM_003264	hsa-miR-548d-3p	hsa-mir-548d-1	8	2773	2	2766	3UTR	0.0128
138.	*TLR2*	NM_003264	hsa-miR-548n	hsa-mir-548n	8	3086	1	3079	3UTR	0.0128
139.	*TLR2*	NM_003264	hsa-miR-548d-3p	hsa-mir-548d-2	8	2780	2	2773	3UTR	0.0128
140.	*TLR2*	NM_003264	hsa-miR-582-5p	hsa-mir-582	8	3144	2	3137	3UTR	0.0128
141.	*TLR2*	NM_003264	hsa-miR-548d-3p	hsa-mir-548d-2	8	2773	2	2766	3UTR	0.0128
142.	*TLR2*	NM_003264	hsa-miR-588	hsa-mir-588	8	3249	2	3242	3UTR	0.0128
143.	*TLR2*	NM_003264	hsa-miR-374b*	hsa-mir-374b	8	3231	2	3224	3UTR	0.0128
144.	*TLR2*	NM_003264	hsa-miR-654-3p	hsa-mir-654	8	2651	1	2644	3UTR	0.0128
145.	*TLR2*	NM_003264	hsa-miR-567	hsa-mir-567	8	3082	1	3075	3UTR	0.0128
146.	*TLR2*	NM_003264	hsa-miR-425	hsa-mir-425	8	3216	1	3209	3UTR	0.0128
147.	*TLR2*	NM_003264	hsa-miR-616	hsa-mir-616	8	3208	1	3201	3UTR	0.0128
148.	*TLR2*	NM_003264	hsa-miR-579	hsa-mir-579	8	3189	1	3182	3UTR	0.0128
149.	*TLR2*	NM_003264	hsa-miR-526b	hsa-mir-526b	8	3307	1	3300	3UTR	0.0128
150.	*TLR2*	NM_003264	hsa-miR-548l	hsa-mir-548l	8	3085	1	3078	3UTR	0.0128
151.	*TLR2*	NM_003264	hsa-miR-548d-3p	hsa-mir-548d-1	8	2780	2	2773	3UTR	0.0128

Appendix XV: Predicted MicroRNA on mRNA Selected Regions of *TLR-4* accessed by miRWalk database

MicroRNA	RefSeqID	Gene Name	Start	End	Pvalue	E-value
hsa-let-7f-5p	NM_138554	*TLR4*	951	963	0.9615385	-17.7
hsa-miR-1207-5p	NM_138554	*TLR4*	1565	1591	0.9615385	-28.2
hsa-miR-4437	NM_138554	*TLR4*	694	712	0.9615385	-21.6
hsa-miR-4802-5p	NM_138554	*TLR4*	819	839	0.9615385	-19.2
hsa-miR-6831-3p	NM_138554	*TLR4*	2422	2471	0.9615385	-22
hsa-miR-8089	NM_138554	*TLR4*	671	706	0.9615385	-29.4
hsa-miR-9718	NM_138554	*TLR4*	2398	2417	0.9615385	-26
hsa-miR-10392-5p	NM_138554	*TLR4*	412	433	0.9615385	-26.5
hsa-miR-4802-5p	NM_003266	*TLR4*	939	959	0.9615385	-19.2
hsa-miR-4784	NM_138554	*TLR4*	2280	2297	0.9692308	-22.8
hsa-miR-490-5p	NM_003266	*TLR4*	2566	2599	0.9692308	-25.3
hsa-miR-27b-3p	NM_138554	*TLR4*	454	475	0.974359	-23.3
hsa-miR-634	NM_138554	*TLR4*	301	321	0.974359	-21.2
hsa-miR-4724-3p	NM_138554	*TLR4*	817	834	0.974359	-19.6
hsa-miR-150-3p	NM_003266	*TLR4*	416	441	0.974359	-25.1
hsa-miR-4657	NM_003266	*TLR4*	723	744	0.974359	-22
hsa-miR-6817-3p	NM_003266	*TLR4*	2571	2586	0.974359	-19.3
hsa-miR-6083	NM_003266	*TLR4*	2414	2433	0.9807692	-23
hsa-let-7a-2-3p	NM_138554	*TLR4*	427	476	1	-25.6
hsa-let-7a-2-3p	NM_138554	*TLR4*	139	164	1	-25.1
hsa-let-7e-5p	NM_138554	*TLR4*	370	392	1	-22.4

hsa-miR-15a-3p	NM_138554	*TLR4*	2474	2516	1	-24.6
hsa-miR-15a-3p	NM_138554	*TLR4*	1860	1878	1	-23.3
hsa-miR-18a-5p	NM_138554	*TLR4*	873	910	1	-23.7
hsa-miR-21-3p	NM_138554	*TLR4*	1961	1978	1	-19.4
hsa-miR-22-5p	NM_138554	*TLR4*	915	934	1	-18.9
hsa-miR-22-3p	NM_138554	*TLR4*	1582	1599	1	-19.9
hsa-miR-27a-3p	NM_138554	*TLR4*	427	475	1	-19.7
hsa-miR-28-5p	NM_138554	*TLR4*	1207	1232	1	-20.1
hsa-miR-29a-3p	NM_138554	*TLR4*	1285	1305	1	-17.6
hsa-miR-33a-3p	NM_138554	*TLR4*	461	479	1	-23.9
hsa-miR-92a-2-5p	NM_138554	*TLR4*	392	414	1	-21.2
hsa-miR-182-3p	NM_138554	*TLR4*	814	838	1	-20.3
hsa-miR-204-3p	NM_138554	*TLR4*	2423	2441	1	-23.5
hsa-miR-205-5p	NM_138554	*TLR4*	454	476	1	-24.4
hsa-miR-216a-3p	NM_138554	*TLR4*	2294	2308	1	-22.3
hsa-miR-217-3p	NM_138554	*TLR4*	165	201	1	-21.6
hsa-miR-219a-1-3p	NM_138554	*TLR4*	2474	2504	1	-23.8
hsa-miR-224-3p	NM_138554	*TLR4*	2468	2485	1	-22.4
hsa-let-7g-5p	NM_138554	*TLR4*	907	963	1	-21.8
hsa-let-7g-3p	NM_138554	*TLR4*	1914	1932	1	-27.7
hsa-miR-124-5p	NM_138554	*TLR4*	462	478	1	-19.8
hsa-miR-138-2-3p	NM_138554	*TLR4*	1010	1026	1	-20.2
hsa-miR-125a-3p	NM_138554	*TLR4*	231	254	1	-25.5
hsa-miR-134-3p	NM_138554	*TLR4*	2265	2290	1	-23

hsa-miR-138-1-3p	NM_138554	*TLR4*	150	169	1	-23.6
hsa-miR-150-3p	NM_138554	*TLR4*	893	914	1	-25.1
hsa-miR-200c-3p	NM_138554	*TLR4*	2327	2346	1	-20.2
hsa-miR-128-2-5p	NM_138554	*TLR4*	1935	1988	1	-27.9
hsa-miR-29c-3p	NM_138554	*TLR4*	1285	1305	1	-17.6
hsa-miR-296-3p	NM_138554	*TLR4*	2164	2187	1	-30.7
hsa-miR-365b-5p	NM_138554	*TLR4*	692	713	1	-23.8
hsa-miR-376c-5p	NM_138554	*TLR4*	2160	2176	1	-18.4
hsa-miR-370-5p	NM_138554	*TLR4*	463	484	1	-21.7
hsa-miR-371a-3p	NM_138554	*TLR4*	343	363	1	-20.4
hsa-miR-378a-5p	NM_138554	*TLR4*	2444	2465	1	-27
hsa-miR-326	NM_138554	*TLR4*	2460	2480	1	-23.9
hsa-miR-331-3p	NM_138554	*TLR4*	2432	2481	1	-25.9
hsa-miR-196b-5p	NM_138554	*TLR4*	1744	1764	1	-19.9
hsa-miR-425-3p	NM_138554	*TLR4*	2459	2496	1	-23.3
hsa-miR-431-3p	NM_138554	*TLR4*	2507	2524	1	-23.9
hsa-miR-490-5p	NM_138554	*TLR4*	2446	2479	1	-25.3
hsa-miR-511-5p	NM_138554	*TLR4*	1016	1046	1	-19.1
hsa-miR-181d-3p	NM_138554	*TLR4*	989	1019	1	-22.7
hsa-miR-518c-5p	NM_138554	*TLR4*	1519	1540	1	-24.5
hsa-miR-518c-5p	NM_138554	*TLR4*	1890	1912	1	-21.8
hsa-miR-524-3p	NM_138554	*TLR4*	895	914	1	-22.5
hsa-miR-517a-3p	NM_138554	*TLR4*	895	915	1	-22.7
hsa-miR-521	NM_138554	*TLR4*	872	914	1	-24.1

hsa-miR-517b-3p	NM_138554	*TLR4*	895	915	1	-22.7
hsa-miR-520g-3p	NM_138554	*TLR4*	895	920	1	-22.4
hsa-miR-516b-5p	NM_138554	*TLR4*	1690	1714	1	-19.4
hsa-miR-518d-3p	NM_138554	*TLR4*	895	920	1	-24.2
hsa-miR-520h	NM_138554	*TLR4*	895	920	1	-21.7
hsa-miR-502-3p	NM_138554	*TLR4*	713	739	1	-22.7
hsa-miR-503-3p	NM_138554	*TLR4*	2451	2476	1	-26.7
hsa-miR-506-5p	NM_138554	*TLR4*	1553	1576	1	-20.1
hsa-miR-506-3p	NM_138554	*TLR4*	892	914	1	-22.6
hsa-miR-507	NM_138554	*TLR4*	1141	1165	1	-20.7
hsa-miR-508-3p	NM_138554	*TLR4*	892	918	1	-27.5
hsa-miR-551b-5p	NM_138554	*TLR4*	814	848	1	-20.3
hsa-miR-588	NM_138554	*TLR4*	2209	2223	1	-21.4
hsa-miR-593-5p	NM_138554	*TLR4*	1914	1937	1	-26.7
hsa-miR-595	NM_138554	*TLR4*	2440	2492	1	-24.4
hsa-miR-601	NM_138554	*TLR4*	212	252	1	-24.4
hsa-miR-612	NM_138554	*TLR4*	2492	2520	1	-31.6
hsa-miR-614	NM_138554	*TLR4*	2427	2482	1	-23.3
hsa-miR-618	NM_138554	*TLR4*	2380	2423	1	-22.5
hsa-miR-624-5p	NM_138554	*TLR4*	2543	2564	1	-19.8
hsa-miR-625-5p	NM_138554	*TLR4*	490	506	1	-18.5
hsa-miR-626	NM_138554	*TLR4*	2395	2415	1	-19
hsa-miR-632	NM_138554	*TLR4*	2565	2583	1	-20.7
hsa-miR-644a	NM_138554	*TLR4*	895	914	1	-19.3

hsa-miR-646	NM_138554	*TLR4*	1839	1873	1	-24.3
hsa-miR-648	NM_138554	*TLR4*	143	159	1	-20.1
hsa-miR-652-5p	NM_138554	*TLR4*	112	150	1	-25.1
hsa-miR-659-3p	NM_138554	*TLR4*	425	446	1	-27.3
hsa-miR-671-3p	NM_138554	*TLR4*	420	434	1	-25.6
hsa-miR-550a-3-5p	NM_138554	*TLR4*	1695	1717	1	-23.1
hsa-miR-550a-3-5p	NM_138554	*TLR4*	1624	1645	1	-21.7
hsa-miR-1296-5p	NM_138554	*TLR4*	351	373	1	-23.1
hsa-miR-1296-3p	NM_138554	*TLR4*	2362	2408	1	-25.1
hsa-miR-769-5p	NM_138554	*TLR4*	2329	2349	1	-23.8
hsa-miR-920	NM_138554	*TLR4*	281	304	1	-25.6
hsa-miR-943	NM_138554	*TLR4*	1034	1077	1	-23.4
hsa-miR-1178-3p	NM_138554	*TLR4*	2452	2483	1	-22.1
hsa-miR-1184	NM_138554	*TLR4*	456	474	1	-22.1
hsa-miR-1225-3p	NM_138554	*TLR4*	2452	2505	1	-27
hsa-miR-1227-5p	NM_138554	*TLR4*	826	851	1	-22.6
hsa-miR-1229-5p	NM_138554	*TLR4*	1779	1804	1	-23.3
hsa-miR-1238-5p	NM_138554	*TLR4*	645	684	1	-24.5
hsa-miR-1203	NM_138554	*TLR4*	819	853	1	-25.2
hsa-miR-663b	NM_138554	*TLR4*	80	113	1	-28.3
hsa-miR-663b	NM_138554	*TLR4*	2404	2430	1	-27.5
hsa-miR-1286	NM_138554	*TLR4*	2339	2386	1	-23.7
hsa-miR-1303	NM_138554	*TLR4*	2391	2408	1	-23.9
hsa-miR-1247-3p	NM_138554	*TLR4*	1480	1499	1	-25.1

hsa-miR-1263	NM_138554	*TLR4*	1984	2005	1	-22.2
hsa-miR-1275	NM_138554	*TLR4*	112	131	1	-21.6
hsa-miR-1288-5p	NM_138554	*TLR4*	2004	2025	1	-20.5
hsa-miR-1292-3p	NM_138554	*TLR4*	425	474	1	-23
hsa-miR-664a-5p	NM_138554	*TLR4*	978	1022	1	-27.3
hsa-miR-1307-5p	NM_138554	*TLR4*	819	856	1	-20.3
hsa-miR-1307-3p	NM_138554	*TLR4*	1915	1937	1	-22
hsa-miR-1322	NM_138554	*TLR4*	2466	2489	1	-21.7
hsa-miR-1539	NM_138554	*TLR4*	2550	2575	1	-23.6
hsa-miR-1911-3p	NM_138554	*TLR4*	2460	2478	1	-21.8
hsa-miR-1912-5p	NM_138554	*TLR4*	899	917	1	-20.4
hsa-miR-1915-5p	NM_138554	*TLR4*	2403	2444	1	-26.3
hsa-miR-2681-5p	NM_138554	*TLR4*	2451	2468	1	-21.7
hsa-miR-2681-3p	NM_138554	*TLR4*	2213	2245	1	-20.5
hsa-miR-718	NM_138554	*TLR4*	2464	2483	1	-23.9
hsa-miR-3122	NM_138554	*TLR4*	2268	2289	1	-25.2
hsa-miR-3124-5p	NM_138554	*TLR4*	1807	1829	1	-22.1
hsa-miR-3144-5p	NM_138554	*TLR4*	1973	1988	1	-17
hsa-miR-3150a-3p	NM_138554	*TLR4*	1186	1213	1	-22.6
hsa-miR-3074-5p	NM_138554	*TLR4*	2417	2447	1	-23.6
hsa-miR-3074-3p	NM_138554	*TLR4*	1922	1946	1	-21.8
hsa-miR-3154	NM_138554	*TLR4*	195	234	1	-24.7
hsa-miR-3160-3p	NM_138554	*TLR4*	2165	2187	1	-22.1
hsa-miR-3166	NM_138554	*TLR4*	2541	2564	1	-23.1

hsa-miR-3166	NM_138554	*TLR4*	2210	2230	1	-22
hsa-miR-3173-5p	NM_138554	*TLR4*	2454	2482	1	-25.2
hsa-miR-3176	NM_138554	*TLR4*	2276	2310	1	-21.1
hsa-miR-3180-3p	NM_138554	*TLR4*	2169	2192	1	-29.8
hsa-miR-3185	NM_138554	*TLR4*	2401	2437	1	-27.3
hsa-miR-3189-5p	NM_138554	*TLR4*	2400	2424	1	-35.2
hsa-miR-3191-3p	NM_138554	*TLR4*	815	837	1	-26.4
hsa-miR-3194-3p	NM_138554	*TLR4*	2401	2425	1	-27.5
hsa-miR-3200-5p	NM_138554	*TLR4*	2400	2440	1	-21.3
hsa-miR-4294	NM_138554	*TLR4*	151	173	1	-20
hsa-miR-4300	NM_138554	*TLR4*	1756	1790	1	-24.4
hsa-miR-4304	NM_138554	*TLR4*	1914	1927	1	-19.4
hsa-miR-4327	NM_138554	*TLR4*	293	316	1	-23.8
hsa-miR-4265	NM_138554	*TLR4*	410	430	1	-24.2
hsa-miR-2355-5p	NM_138554	*TLR4*	832	856	1	-22.6
hsa-miR-4281	NM_138554	*TLR4*	116	155	1	-27.6
hsa-miR-4286	NM_138554	*TLR4*	2424	2463	1	-22.7
hsa-miR-3617-3p	NM_138554	*TLR4*	1916	1935	1	-20.4
hsa-miR-3622b-5p	NM_138554	*TLR4*	1559	1579	1	-24.3
hsa-miR-3622b-5p	NM_138554	*TLR4*	2037	2057	1	-22.5
hsa-miR-3652	NM_138554	*TLR4*	1620	1635	1	-23.2
hsa-miR-3654	NM_138554	*TLR4*	1451	1488	1	-28.5
hsa-miR-3680-5p	NM_138554	*TLR4*	2438	2463	1	-20.4
hsa-miR-3681-3p	NM_138554	*TLR4*	2118	2134	1	-18.5

hsa-miR-3180	NM_138554	*TLR4*	2171	2192	1	-22.3
hsa-miR-3921	NM_138554	*TLR4*	1914	1936	1	-21.6
hsa-miR-3940-5p	NM_138554	*TLR4*	364	388	1	-25.7
hsa-miR-378f	NM_138554	*TLR4*	1467	1487	1	-20.4
hsa-miR-4435	NM_138554	*TLR4*	1983	2024	1	-25.5
hsa-miR-4450	NM_138554	*TLR4*	2284	2306	1	-23.7
hsa-miR-4451	NM_138554	*TLR4*	1981	1996	1	-19.7
hsa-miR-4462	NM_138554	*TLR4*	369	386	1	-27.1
hsa-miR-4463	NM_138554	*TLR4*	2169	2186	1	-21.7
hsa-miR-4467	NM_138554	*TLR4*	2180	2232	1	-22.8
hsa-miR-4469	NM_138554	*TLR4*	410	433	1	-25.8
hsa-miR-4470	NM_138554	*TLR4*	2245	2263	1	-19.5
hsa-miR-4508	NM_138554	*TLR4*	2165	2189	1	-24.7
hsa-miR-4508	NM_138554	*TLR4*	1475	1491	1	-24.2
hsa-miR-4521	NM_138554	*TLR4*	2549	2571	1	-26.3
hsa-miR-4533	NM_138554	*TLR4*	1502	1538	1	-24.4
hsa-miR-1587	NM_138554	*TLR4*	406	426	1	-25.1
hsa-miR-4538	NM_138554	*TLR4*	2282	2304	1	-24
hsa-miR-3972	NM_138554	*TLR4*	2469	2497	1	-26.3
hsa-miR-4633-3p	NM_138554	*TLR4*	805	829	1	-24
hsa-miR-4647	NM_138554	*TLR4*	2471	2492	1	-26.8
hsa-miR-4653-5p	NM_138554	*TLR4*	2388	2411	1	-22.5
hsa-miR-4675	NM_138554	*TLR4*	2511	2539	1	-24.2
hsa-miR-4683	NM_138554	*TLR4*	2474	2492	1	-22.9

hsa-miR-4686	NM_138554	*TLR4*	2532	2556	1	-24.2
hsa-miR-4689	NM_138554	*TLR4*	672	696	1	-25.7
hsa-miR-4690-3p	NM_138554	*TLR4*	2407	2427	1	-25.8
hsa-miR-4691-3p	NM_138554	*TLR4*	805	826	1	-25.7
hsa-miR-4701-3p	NM_138554	*TLR4*	1391	1410	1	-21.2
hsa-miR-4707-3p	NM_138554	*TLR4*	2462	2484	1	-25
hsa-miR-4709-3p	NM_138554	*TLR4*	2142	2183	1	-23.4
hsa-miR-4712-5p	NM_138554	*TLR4*	901	914	1	-20.3
hsa-miR-4713-5p	NM_138554	*TLR4*	2441	2467	1	-26.7
hsa-miR-4714-3p	NM_138554	*TLR4*	588	612	1	-20.6
hsa-miR-4715-3p	NM_138554	*TLR4*	2055	2087	1	-22.4
hsa-miR-4722-3p	NM_138554	*TLR4*	307	333	1	-25.3
hsa-miR-4724-5p	NM_138554	*TLR4*	1860	1883	1	-25.1
hsa-miR-4727-3p	NM_138554	*TLR4*	1091	1111	1	-23.4
hsa-miR-4731-5p	NM_138554	*TLR4*	1965	1991	1	-24.4
hsa-miR-4738-5p	NM_138554	*TLR4*	2347	2366	1	-20.1
hsa-miR-4748	NM_138554	*TLR4*	1535	1580	1	-23.3
hsa-miR-4750-5p	NM_138554	*TLR4*	231	271	1	-23.3
hsa-miR-4758-3p	NM_138554	*TLR4*	2408	2424	1	-27.5
hsa-miR-4761-5p	NM_138554	*TLR4*	2470	2491	1	-21.9
hsa-miR-4763-5p	NM_138554	*TLR4*	2437	2484	1	-27.5
hsa-miR-4781-5p	NM_138554	*TLR4*	145	158	1	-18.1
hsa-miR-4783-3p	NM_138554	*TLR4*	2278	2292	1	-23.8
hsa-miR-4784	NM_138554	*TLR4*	2352	2376	1	-21.5

hsa-miR-4785	NM_138554	*TLR4*	459	478	1	-24.6
hsa-miR-4786-5p	NM_138554	*TLR4*	1990	2025	1	-23.8
hsa-miR-4799-3p	NM_138554	*TLR4*	2351	2366	1	-19.4
hsa-miR-4804-3p	NM_138554	*TLR4*	2129	2145	1	-19.5
hsa-miR-5000-5p	NM_138554	*TLR4*	2421	2461	1	-25.3
hsa-miR-5001-3p	NM_138554	*TLR4*	1847	1868	1	-27.6
hsa-miR-5008-3p	NM_138554	*TLR4*	2457	2487	1	-25.7
hsa-miR-5010-3p	NM_138554	*TLR4*	2441	2470	1	-21.6
hsa-miR-5011-3p	NM_138554	*TLR4*	2351	2362	1	-16.3
hsa-miR-5187-5p	NM_138554	*TLR4*	676	705	1	-23.9
hsa-miR-5571-3p	NM_138554	*TLR4*	2436	2481	1	-28.3
hsa-miR-664b-3p	NM_138554	*TLR4*	461	482	1	-19.8
hsa-miR-5580-5p	NM_138554	*TLR4*	2129	2150	1	-22.1
hsa-miR-5580-3p	NM_138554	*TLR4*	2052	2073	1	-21.6
hsa-miR-5582-3p	NM_138554	*TLR4*	727	744	1	-18.9
hsa-miR-5587-5p	NM_138554	*TLR4*	2208	2224	1	-23.5
hsa-miR-5587-3p	NM_138554	*TLR4*	2457	2481	1	-26.4
hsa-miR-5589-3p	NM_138554	*TLR4*	1913	1932	1	-19.8
hsa-miR-5591-5p	NM_138554	*TLR4*	409	427	1	-20.6
hsa-miR-5690	NM_138554	*TLR4*	125	201	1	-24.6
hsa-miR-5693	NM_138554	*TLR4*	2290	2308	1	-24.3
hsa-miR-5739	NM_138554	*TLR4*	2169	2189	1	-21.4
hsa-miR-6068	NM_138554	*TLR4*	639	660	1	-23.1
hsa-miR-6080	NM_138554	*TLR4*	1917	1934	1	-24.3

hsa-miR-6083	NM_138554	*TLR4*	2294	2313	1	-23
hsa-miR-6086	NM_138554	*TLR4*	223	239	1	-23.2
hsa-miR-6125	NM_138554	*TLR4*	2421	2441	1	-27.1
hsa-miR-6165	NM_138554	*TLR4*	2036	2058	1	-23.1
hsa-miR-6500-5p	NM_138554	*TLR4*	2449	2476	1	-27.3
hsa-miR-6501-5p	NM_138554	*TLR4*	411	433	1	-29
hsa-miR-6509-3p	NM_138554	*TLR4*	458	476	1	-21.6
hsa-miR-6514-3p	NM_138554	*TLR4*	2452	2483	1	-32.8
hsa-miR-6515-5p	NM_138554	*TLR4*	1692	1712	1	-23.6
hsa-miR-6715b-5p	NM_138554	*TLR4*	2338	2366	1	-21.4
hsa-miR-892c-5p	NM_138554	*TLR4*	2334	2353	1	-20.3
hsa-miR-6727-3p	NM_138554	*TLR4*	126	169	1	-25.9
hsa-miR-6727-3p	NM_138554	*TLR4*	2512	2575	1	-22
hsa-miR-6733-3p	NM_138554	*TLR4*	316	352	1	-21.6
hsa-miR-6734-5p	NM_138554	*TLR4*	2171	2194	1	-27.9
hsa-miR-6744-5p	NM_138554	*TLR4*	2277	2299	1	-20.3
hsa-miR-6748-3p	NM_138554	*TLR4*	2457	2483	1	-23.3
hsa-miR-6756-5p	NM_138554	*TLR4*	2169	2192	1	-30.9
hsa-miR-6759-5p	NM_138554	*TLR4*	2179	2200	1	-21.1
hsa-miR-6760-3p	NM_138554	*TLR4*	2422	2473	1	-26.9
hsa-miR-6776-5p	NM_138554	*TLR4*	2282	2301	1	-22.4
hsa-miR-6784-5p	NM_138554	*TLR4*	405	429	1	-28.3
hsa-miR-6790-5p	NM_138554	*TLR4*	1421	1445	1	-25.1
hsa-miR-6792-5p	NM_138554	*TLR4*	2291	2310	1	-29.9

hsa-miR-6792-5p	NM_138554	*TLR4*	408	433	1	-24.8
hsa-miR-6792-3p	NM_138554	*TLR4*	428	476	1	-28.5
hsa-miR-6794-5p	NM_138554	*TLR4*	1620	1638	1	-25.8
hsa-miR-6801-3p	NM_138554	*TLR4*	2542	2574	1	-22.4
hsa-miR-6815-5p	NM_138554	*TLR4*	2197	2222	1	-24.8
hsa-miR-6821-3p	NM_138554	*TLR4*	2422	2469	1	-21.4
hsa-miR-6825-5p	NM_138554	*TLR4*	195	230	1	-27.5
hsa-miR-6830-5p	NM_138554	*TLR4*	1478	1499	1	-25.2
hsa-miR-6831-5p	NM_138554	*TLR4*	2169	2191	1	-28.9
hsa-miR-6780b-5p	NM_138554	*TLR4*	76	125	1	-32.2
hsa-miR-6839-3p	NM_138554	*TLR4*	2360	2403	1	-20.1
hsa-miR-6843-3p	NM_138554	*TLR4*	1947	1969	1	-19.3
hsa-miR-6847-5p	NM_138554	*TLR4*	1620	1638	1	-23
hsa-miR-6847-3p	NM_138554	*TLR4*	349	372	1	-20.9
hsa-miR-6855-5p	NM_138554	*TLR4*	697	714	1	-24.2
hsa-miR-6857-5p	NM_138554	*TLR4*	1473	1493	1	-26
hsa-miR-6860	NM_138554	*TLR4*	2171	2191	1	-24.7
hsa-miR-6862-5p	NM_138554	*TLR4*	1977	1998	1	-24.2
hsa-miR-6862-3p	NM_138554	*TLR4*	2114	2138	1	-25.1
hsa-miR-6863	NM_138554	*TLR4*	2181	2198	1	-20.6
hsa-miR-6864-5p	NM_138554	*TLR4*	1462	1488	1	-24.1
hsa-miR-6869-5p	NM_138554	*TLR4*	2210	2231	1	-26
hsa-miR-6871-3p	NM_138554	*TLR4*	425	471	1	-24
hsa-miR-6874-5p	NM_138554	*TLR4*	1346	1368	1	-22.2

hsa-miR-6884-5p	NM_138554	*TLR4*	2410	2441	1	-23.5
hsa-miR-6885-3p	NM_138554	*TLR4*	2567	2580	1	-20.5
hsa-miR-6889-3p	NM_138554	*TLR4*	2542	2560	1	-19.5
hsa-miR-7106-5p	NM_138554	*TLR4*	1517	1538	1	-24.7
hsa-miR-7114-5p	NM_138554	*TLR4*	397	418	1	-26
hsa-miR-7156-5p	NM_138554	*TLR4*	2091	2129	1	-22.2
hsa-miR-7156-3p	NM_138554	*TLR4*	293	314	1	-26.1
hsa-miR-7158-5p	NM_138554	*TLR4*	1840	1870	1	-25.6
hsa-miR-7161-3p	NM_138554	*TLR4*	460	485	1	-23.7
hsa-miR-7843-5p	NM_138554	*TLR4*	2164	2193	1	-25.2
hsa-miR-7851-3p	NM_138554	*TLR4*	279	306	1	-26
hsa-miR-8052	NM_138554	*TLR4*	2181	2214	1	-27.8
hsa-miR-8059	NM_138554	*TLR4*	281	304	1	-20.1
hsa-miR-8064	NM_138554	*TLR4*	1949	1996	1	-25.8
hsa-miR-8070	NM_138554	*TLR4*	1592	1613	1	-21.3
hsa-miR-8070	NM_138554	*TLR4*	1722	1738	1	-20.1
hsa-miR-8071	NM_138554	*TLR4*	2170	2190	1	-23.2
hsa-miR-8073	NM_138554	*TLR4*	2090	2121	1	-24.6
hsa-miR-8077	NM_138554	*TLR4*	2391	2412	1	-28.3
hsa-miR-8082	NM_138554	*TLR4*	2277	2297	1	-20.9
hsa-miR-9900	NM_138554	*TLR4*	894	912	1	-20.5
hsa-miR-9901	NM_138554	*TLR4*	459	478	1	-22.9
hsa-miR-9903	NM_138554	*TLR4*	1009	1041	1	-21.9
hsa-miR-10396a-3p	NM_138554	*TLR4*	2451	2481	1	-28.3

hsa-miR-10400-5p	NM_138554	*TLR4*	2286	2312	1	-31.4
hsa-miR-10396b-3p	NM_138554	*TLR4*	2452	2481	1	-26.1
hsa-miR-10522-5p	NM_138554	*TLR4*	2472	2493	1	-21.6
hsa-miR-10526-3p	NM_138554	*TLR4*	2171	2192	1	-25.6
hsa-miR-3085-3p	NM_138554	*TLR4*	2212	2232	1	-23.7
hsa-miR-9851-5p	NM_138554	*TLR4*	2212	2232	1	-27.1
hsa-miR-12116	NM_138554	*TLR4*	2457	2484	1	-23.3
hsa-miR-12118	NM_138554	*TLR4*	257	296	1	-20.6
hsa-miR-12120	NM_138554	*TLR4*	143	173	1	-26.5
hsa-let-7a-5p	NM_003266	*TLR4*	1059	1083	1	-20.2
hsa-let-7a-2-3p	NM_003266	*TLR4*	547	596	1	-25.6
hsa-let-7b-3p	NM_003266	*TLR4*	2659	2675	1	-18.1
hsa-let-7c-3p	NM_003266	*TLR4*	1018	1035	1	-17.6
hsa-let-7e-5p	NM_003266	*TLR4*	490	512	1	-22.4
hsa-miR-15a-3p	NM_003266	*TLR4*	1980	1998	1	-23.3
hsa-miR-21-3p	NM_003266	*TLR4*	2081	2098	1	-19.4
hsa-miR-22-5p	NM_003266	*TLR4*	1035	1054	1	-18.9
hsa-miR-22-3p	NM_003266	*TLR4*	568	590	1	-19.7
hsa-miR-22-3p	NM_003266	*TLR4*	1702	1719	1	-19.9
hsa-miR-28-5p	NM_003266	*TLR4*	1327	1352	1	-20.1
hsa-miR-33a-3p	NM_003266	*TLR4*	581	599	1	-23.9
hsa-miR-92a-2-5p	NM_003266	*TLR4*	512	534	1	-21.2
hsa-miR-196a-5p	NM_003266	*TLR4*	1725	1747	1	-19.1
hsa-miR-30c-2-3p	NM_003266	*TLR4*	387	426	1	-20.6

hsa-miR-30d-3p	NM_003266	*TLR4*	2598	2617	1	-18.7
hsa-miR-182-5p	NM_003266	*TLR4*	2332	2348	1	-19
hsa-miR-182-3p	NM_003266	*TLR4*	934	958	1	-20.3
hsa-miR-204-3p	NM_003266	*TLR4*	2543	2561	1	-23.5
hsa-miR-205-3p	NM_003266	*TLR4*	615	636	1	-19.9
hsa-miR-224-3p	NM_003266	*TLR4*	2588	2605	1	-22.4
hsa-let-7g-5p	NM_003266	*TLR4*	1027	1083	1	-21.8
hsa-let-7g-3p	NM_003266	*TLR4*	2034	2052	1	-27.7
hsa-miR-27b-3p	NM_003266	*TLR4*	532	551	1	-20.1
hsa-miR-124-5p	NM_003266	*TLR4*	582	598	1	-19.8
hsa-miR-138-2-3p	NM_003266	*TLR4*	1130	1146	1	-20.2
hsa-miR-150-3p	NM_003266	*TLR4*	1013	1034	1	-25.1
hsa-miR-128-2-5p	NM_003266	*TLR4*	2055	2108	1	-27.9
hsa-miR-299-5p	NM_003266	*TLR4*	2633	2651	1	-19.1
hsa-miR-363-3p	NM_003266	*TLR4*	2665	2688	1	-20.7
hsa-miR-365b-5p	NM_003266	*TLR4*	812	833	1	-23.8
hsa-miR-376c-5p	NM_003266	*TLR4*	2280	2296	1	-18.4
hsa-miR-370-5p	NM_003266	*TLR4*	583	604	1	-21.7
hsa-miR-370-5p	NM_003266	*TLR4*	2312	2342	1	-19.9
hsa-miR-371a-3p	NM_003266	*TLR4*	463	483	1	-20.4
hsa-miR-373-5p	NM_003266	*TLR4*	2255	2300	1	-21.9
hsa-miR-382-5p	NM_003266	*TLR4*	1750	1787	1	-19.2
hsa-miR-135b-3p	NM_003266	*TLR4*	526	550	1	-21.3
hsa-miR-331-5p	NM_003266	*TLR4*	520	562	1	-25

hsa-miR-331-3p	NM_003266	*TLR4*	2552	2601	1	-25.9
hsa-miR-324-5p	NM_003266	*TLR4*	2565	2604	1	-25.6
hsa-miR-324-3p	NM_003266	*TLR4*	2571	2615	1	-28.6
hsa-miR-338-5p	NM_003266	*TLR4*	2227	2244	1	-20.2
hsa-miR-196b-5p	NM_003266	*TLR4*	1864	1884	1	-19.9
hsa-miR-425-3p	NM_003266	*TLR4*	2579	2616	1	-23.3
hsa-miR-18b-3p	NM_003266	*TLR4*	2656	2676	1	-21.5
hsa-miR-376b-5p	NM_003266	*TLR4*	2562	2602	1	-18.8
hsa-miR-432-3p	NM_003266	*TLR4*	516	536	1	-22.3
hsa-miR-181d-3p	NM_003266	*TLR4*	2320	2340	1	-23.1
hsa-miR-525-3p	NM_003266	*TLR4*	1014	1034	1	-25.1
hsa-miR-518c-5p	NM_003266	*TLR4*	1639	1660	1	-24.5
hsa-miR-518c-5p	NM_003266	*TLR4*	2010	2032	1	-21.8
hsa-miR-521	NM_003266	*TLR4*	992	1034	1	-24.1
hsa-miR-516b-5p	NM_003266	*TLR4*	1810	1834	1	-19.4
hsa-miR-518d-3p	NM_003266	*TLR4*	1015	1040	1	-24.2
hsa-miR-502-3p	NM_003266	*TLR4*	833	859	1	-22.7
hsa-miR-503-3p	NM_003266	*TLR4*	2571	2596	1	-26.7
hsa-miR-505-5p	NM_003266	*TLR4*	2163	2183	1	-20
hsa-miR-505-5p	NM_003266	*TLR4*	1686	1702	1	-19.2
hsa-miR-508-3p	NM_003266	*TLR4*	1012	1038	1	-27.5
hsa-miR-557	NM_003266	*TLR4*	2034	2052	1	-26.7
hsa-miR-574-5p	NM_003266	*TLR4*	1547	1568	1	-19.8
hsa-miR-589-5p	NM_003266	*TLR4*	2524	2559	1	-23.9

hsa-miR-550a-5p	NM_003266	*TLR4*	374	396	1	-24.3
hsa-miR-593-5p	NM_003266	*TLR4*	2247	2294	1	-24.7
hsa-miR-593-3p	NM_003266	*TLR4*	520	538	1	-22.3
hsa-miR-595	NM_003266	*TLR4*	2560	2612	1	-24.4
hsa-miR-601	NM_003266	*TLR4*	332	372	1	-24.4
hsa-miR-612	NM_003266	*TLR4*	2612	2640	1	-31.6
hsa-miR-614	NM_003266	*TLR4*	2547	2602	1	-23.3
hsa-miR-615-5p	NM_003266	*TLR4*	716	740	1	-25
hsa-miR-622	NM_003266	*TLR4*	2449	2471	1	-21.4
hsa-miR-624-5p	NM_003266	*TLR4*	2663	2684	1	-19.8
hsa-miR-625-5p	NM_003266	*TLR4*	366	388	1	-20.5
hsa-miR-625-5p	NM_003266	*TLR4*	610	626	1	-18.5
hsa-miR-644a	NM_003266	*TLR4*	1015	1034	1	-19.3
hsa-miR-646	NM_003266	*TLR4*	1959	1993	1	-24.3
hsa-miR-648	NM_003266	*TLR4*	2586	2608	1	-21.6
hsa-miR-650	NM_003266	*TLR4*	1706	1729	1	-26.4
hsa-miR-663a	NM_003266	*TLR4*	2577	2638	1	-29.5
hsa-miR-659-3p	NM_003266	*TLR4*	545	566	1	-27.3
hsa-miR-671-3p	NM_003266	*TLR4*	540	554	1	-25.6
hsa-miR-550a-3-5p	NM_003266	*TLR4*	1815	1837	1	-23.1
hsa-miR-550a-3-5p	NM_003266	*TLR4*	1744	1765	1	-21.7
hsa-miR-1296-3p	NM_003266	*TLR4*	2482	2528	1	-25.1
hsa-miR-761	NM_003266	*TLR4*	2588	2639	1	-23
hsa-miR-675-5p	NM_003266	*TLR4*	544	566	1	-27

hsa-miR-298	NM_003266	*TLR4*	2578	2614	1	-26.3
hsa-miR-298	NM_003266	*TLR4*	2511	2532	1	-24.5
hsa-miR-874-5p	NM_003266	*TLR4*	1126	1159	1	-24.2
hsa-miR-890	NM_003266	*TLR4*	1877	1899	1	-17.3
hsa-miR-873-5p	NM_003266	*TLR4*	768	795	1	-20
hsa-miR-760	NM_003266	*TLR4*	2401	2425	1	-27.3
hsa-miR-301b-5p	NM_003266	*TLR4*	2015	2037	1	-20.2
hsa-miR-920	NM_003266	*TLR4*	401	424	1	-25.6
hsa-miR-943	NM_003266	*TLR4*	1154	1197	1	-23.4
hsa-miR-1178-3p	NM_003266	*TLR4*	2635	2676	1	-20.1
hsa-miR-1180-5p	NM_003266	*TLR4*	2325	2342	1	-22
hsa-miR-1225-3p	NM_003266	*TLR4*	2572	2625	1	-27
hsa-miR-1227-5p	NM_003266	*TLR4*	946	971	1	-22.6
hsa-miR-1228-5p	NM_003266	*TLR4*	812	832	1	-26.5
hsa-miR-1229-5p	NM_003266	*TLR4*	1899	1924	1	-23.3
hsa-miR-1237-3p	NM_003266	*TLR4*	2247	2288	1	-21.7
hsa-miR-1200	NM_003266	*TLR4*	943	975	1	-24.8
hsa-miR-1203	NM_003266	*TLR4*	939	973	1	-25.2
hsa-miR-1205	NM_003266	*TLR4*	2620	2641	1	-22.2
hsa-miR-1207-5p	NM_003266	*TLR4*	1685	1711	1	-28.2
hsa-miR-1290	NM_003266	*TLR4*	936	958	1	-19.2
hsa-miR-1293	NM_003266	*TLR4*	2406	2429	1	-22.3
hsa-miR-1295a	NM_003266	*TLR4*	2411	2433	1	-21.1
hsa-miR-1247-3p	NM_003266	*TLR4*	1600	1619	1	-25.1

hsa-miR-1248	NM_003266	*TLR4*	2671	2697	1	-20.8
hsa-miR-1263	NM_003266	*TLR4*	2104	2125	1	-22.2
hsa-miR-1272	NM_003266	*TLR4*	2331	2360	1	-26.4
hsa-miR-1288-5p	NM_003266	*TLR4*	2124	2145	1	-20.5
hsa-miR-1288-3p	NM_003266	*TLR4*	2024	2047	1	-25.6
hsa-miR-1307-5p	NM_003266	*TLR4*	939	976	1	-20.3
hsa-miR-1307-3p	NM_003266	*TLR4*	2035	2057	1	-22
hsa-miR-1322	NM_003266	*TLR4*	2586	2609	1	-21.7
hsa-miR-1539	NM_003266	*TLR4*	2670	2695	1	-23.6
hsa-miR-1912-5p	NM_003266	*TLR4*	1019	1037	1	-20.4
hsa-miR-1914-5p	NM_003266	*TLR4*	2657	2680	1	-26.3
hsa-miR-1915-5p	NM_003266	*TLR4*	2523	2564	1	-26.3
hsa-miR-1972	NM_003266	*TLR4*	2037	2061	1	-24
hsa-miR-2115-5p	NM_003266	*TLR4*	953	975	1	-19.4
hsa-miR-2277-5p	NM_003266	*TLR4*	2029	2053	1	-28.8
hsa-miR-2681-3p	NM_003266	*TLR4*	2333	2365	1	-20.5
hsa-miR-2682-5p	NM_003266	*TLR4*	2459	2483	1	-23.6
hsa-miR-3124-5p	NM_003266	*TLR4*	1927	1949	1	-22.1
hsa-miR-3144-5p	NM_003266	*TLR4*	2093	2108	1	-17
hsa-miR-3150a-3p	NM_003266	*TLR4*	1306	1333	1	-22.6
hsa-miR-3074-5p	NM_003266	*TLR4*	2537	2567	1	-23.6
hsa-miR-3074-3p	NM_003266	*TLR4*	2042	2066	1	-21.8
hsa-miR-3154	NM_003266	*TLR4*	315	354	1	-24.7
hsa-miR-3158-3p	NM_003266	*TLR4*	2497	2524	1	-29

hsa-miR-3160-3p	NM_003266	*TLR4*	2285	2307	1	-22.1
hsa-miR-1260b	NM_003266	*TLR4*	581	594	1	-20.7
hsa-miR-3173-5p	NM_003266	*TLR4*	2574	2602	1	-25.2
hsa-miR-3176	NM_003266	*TLR4*	2396	2430	1	-21.1
hsa-miR-3180-3p	NM_003266	*TLR4*	2289	2312	1	-29.8
hsa-miR-3185	NM_003266	*TLR4*	2521	2557	1	-27.3
hsa-miR-3189-5p	NM_003266	*TLR4*	2520	2544	1	-35.2
hsa-miR-3191-3p	NM_003266	*TLR4*	935	957	1	-26.4
hsa-miR-3194-3p	NM_003266	*TLR4*	2521	2545	1	-27.5
hsa-miR-4300	NM_003266	*TLR4*	1876	1910	1	-24.4
hsa-miR-4304	NM_003266	*TLR4*	2034	2047	1	-19.4
hsa-miR-4265	NM_003266	*TLR4*	530	550	1	-24.2
hsa-miR-2355-5p	NM_003266	*TLR4*	952	976	1	-22.6
hsa-miR-3617-3p	NM_003266	*TLR4*	2036	2055	1	-20.4
hsa-miR-3621	NM_003266	*TLR4*	2499	2532	1	-23.3
hsa-miR-3622b-5p	NM_003266	*TLR4*	1679	1699	1	-24.3
hsa-miR-3622b-5p	NM_003266	*TLR4*	2157	2177	1	-22.5
hsa-miR-3652	NM_003266	*TLR4*	1740	1755	1	-23.2
hsa-miR-3654	NM_003266	*TLR4*	1571	1608	1	-28.5
hsa-miR-3675-5p	NM_003266	*TLR4*	592	628	1	-23.6
hsa-miR-3680-5p	NM_003266	*TLR4*	2558	2583	1	-20.4
hsa-miR-3680-3p	NM_003266	*TLR4*	1012	1036	1	-22.5
hsa-miR-3681-3p	NM_003266	*TLR4*	2238	2254	1	-18.5
hsa-miR-3688-3p	NM_003266	*TLR4*	1623	1642	1	-19.2

hsa-miR-3689a-3p	NM_003266	*TLR4*	1426	1449	1	-23.4
hsa-miR-3921	NM_003266	*TLR4*	2034	2056	1	-21.6
hsa-miR-550b-3p	NM_003266	*TLR4*	2234	2258	1	-18.8
hsa-miR-378f	NM_003266	*TLR4*	1587	1607	1	-20.4
hsa-miR-4421	NM_003266	*TLR4*	405	436	1	-22.8
hsa-miR-4435	NM_003266	*TLR4*	2103	2144	1	-25.5
hsa-miR-4437	NM_003266	*TLR4*	814	832	1	-21.6
hsa-miR-4462	NM_003266	*TLR4*	489	506	1	-27.1
hsa-miR-4463	NM_003266	*TLR4*	2289	2306	1	-21.7
hsa-miR-4465	NM_003266	*TLR4*	1128	1153	1	-22.3
hsa-miR-4469	NM_003266	*TLR4*	530	553	1	-25.8
hsa-miR-4470	NM_003266	*TLR4*	2365	2383	1	-19.5
hsa-miR-4471	NM_003266	*TLR4*	2043	2059	1	-18.8
hsa-miR-4472	NM_003266	*TLR4*	337	359	1	-19.5
hsa-miR-4484	NM_003266	*TLR4*	2284	2312	1	-23.2
hsa-miR-4484	NM_003266	*TLR4*	474	493	1	-19.8
hsa-miR-4485-5p	NM_003266	*TLR4*	2525	2541	1	-17.1
hsa-miR-4493	NM_003266	*TLR4*	2529	2557	1	-22.1
hsa-miR-4498	NM_003266	*TLR4*	722	743	1	-25.3
hsa-miR-4508	NM_003266	*TLR4*	2285	2309	1	-24.7
hsa-miR-4508	NM_003266	*TLR4*	1595	1611	1	-24.2
hsa-miR-4516	NM_003266	*TLR4*	333	359	1	-24.6
hsa-miR-4521	NM_003266	*TLR4*	2669	2691	1	-26.3
hsa-miR-4533	NM_003266	*TLR4*	1622	1658	1	-24.4

hsa-miR-548an	NM_003266	*TLR4*	2200	2222	1	-16.5
hsa-miR-4538	NM_003266	*TLR4*	2402	2424	1	-24
hsa-miR-3960	NM_003266	*TLR4*	2302	2351	1	-28.8
hsa-miR-3972	NM_003266	*TLR4*	2589	2617	1	-26.3
hsa-miR-4632-3p	NM_003266	*TLR4*	2580	2605	1	-28
hsa-miR-4635	NM_003266	*TLR4*	772	791	1	-22.8
hsa-miR-4647	NM_003266	*TLR4*	2591	2612	1	-26.8
hsa-miR-4650-5p	NM_003266	*TLR4*	1331	1347	1	-18.5
hsa-miR-4653-3p	NM_003266	*TLR4*	1723	1744	1	-22.8
hsa-miR-4671-5p	NM_003266	*TLR4*	2597	2615	1	-18.1
hsa-miR-4671-5p	NM_003266	*TLR4*	746	779	1	-17.9
hsa-miR-4677-5p	NM_003266	*TLR4*	1966	1988	1	-19.4
hsa-miR-4683	NM_003266	*TLR4*	1444	1480	1	-26.3
hsa-miR-4689	NM_003266	*TLR4*	792	816	1	-25.7
hsa-miR-4690-3p	NM_003266	*TLR4*	2527	2547	1	-25.8
hsa-miR-4690-3p	NM_003266	*TLR4*	2163	2183	1	-25.1
hsa-miR-4691-3p	NM_003266	*TLR4*	925	946	1	-25.7
hsa-miR-4701-3p	NM_003266	*TLR4*	2406	2424	1	-21.2
hsa-miR-4709-3p	NM_003266	*TLR4*	2262	2303	1	-23.4
hsa-miR-4712-5p	NM_003266	*TLR4*	1021	1034	1	-20.3
hsa-miR-4712-5p	NM_003266	*TLR4*	2036	2054	1	-18.9
hsa-miR-4714-3p	NM_003266	*TLR4*	708	732	1	-20.6
hsa-miR-4722-3p	NM_003266	*TLR4*	427	453	1	-25.3
hsa-miR-4724-5p	NM_003266	*TLR4*	1980	2003	1	-25.1

hsa-miR-4724-3p	NM_003266	*TLR4*	937	954	1	-19.6
hsa-miR-4726-3p	NM_003266	*TLR4*	1974	1996	1	-23.8
hsa-miR-4727-3p	NM_003266	*TLR4*	1211	1231	1	-23.4
hsa-miR-4728-3p	NM_003266	*TLR4*	2572	2605	1	-29.2
hsa-miR-4731-5p	NM_003266	*TLR4*	2085	2111	1	-24.4
hsa-miR-4731-5p	NM_003266	*TLR4*	2389	2410	1	-23.4
hsa-miR-4732-3p	NM_003266	*TLR4*	2579	2601	1	-25.5
hsa-miR-4748	NM_003266	*TLR4*	1655	1700	1	-23.3
hsa-miR-4752	NM_003266	*TLR4*	764	787	1	-22.1
hsa-miR-4758-3p	NM_003266	*TLR4*	2528	2544	1	-27.5
hsa-miR-4763-5p	NM_003266	*TLR4*	2557	2604	1	-27.5
hsa-miR-4774-5p	NM_003266	*TLR4*	2219	2241	1	-22.1
hsa-miR-4783-3p	NM_003266	*TLR4*	2398	2412	1	-23.8
hsa-miR-4784	NM_003266	*TLR4*	2400	2417	1	-22.8
hsa-miR-4785	NM_003266	*TLR4*	579	598	1	-24.6
hsa-miR-4786-5p	NM_003266	*TLR4*	2110	2145	1	-23.8
hsa-miR-4794	NM_003266	*TLR4*	1602	1630	1	-20.1
hsa-miR-4797-3p	NM_003266	*TLR4*	2034	2056	1	-21.1
hsa-miR-4799-3p	NM_003266	*TLR4*	2471	2486	1	-19.4
hsa-miR-4802-3p	NM_003266	*TLR4*	2344	2375	1	-20.5
hsa-miR-4804-3p	NM_003266	*TLR4*	2249	2265	1	-19.5
hsa-miR-5000-5p	NM_003266	*TLR4*	2541	2581	1	-25.3
hsa-miR-5008-3p	NM_003266	*TLR4*	541	578	1	-27.1
hsa-miR-5008-3p	NM_003266	*TLR4*	2577	2607	1	-25.7

hsa-miR-5088-5p	NM_003266	*TLR4*	525	548	1	-27.4
hsa-miR-5089-5p	NM_003266	*TLR4*	940	957	1	-18.3
hsa-miR-5187-5p	NM_003266	*TLR4*	796	825	1	-23.9
hsa-miR-5188	NM_003266	*TLR4*	941	957	1	-17.4
hsa-miR-5192	NM_003266	*TLR4*	1684	1704	1	-23.1
hsa-miR-5196-5p	NM_003266	*TLR4*	334	355	1	-27.3
hsa-miR-5580-5p	NM_003266	*TLR4*	2249	2270	1	-22.1
hsa-miR-548au-3p	NM_003266	*TLR4*	2332	2352	1	-20.4
hsa-miR-5682	NM_003266	*TLR4*	2495	2526	1	-22.8
hsa-miR-5693	NM_003266	*TLR4*	2410	2428	1	-24.3
hsa-miR-5699-3p	NM_003266	*TLR4*	2655	2681	1	-19.6
hsa-miR-6068	NM_003266	*TLR4*	2545	2564	1	-23.6
hsa-miR-6068	NM_003266	*TLR4*	759	780	1	-23.1
hsa-miR-6070	NM_003266	*TLR4*	2235	2258	1	-20.7
hsa-miR-6080	NM_003266	*TLR4*	2037	2054	1	-24.3
hsa-miR-6086	NM_003266	*TLR4*	343	359	1	-23.2
hsa-miR-6125	NM_003266	*TLR4*	2541	2561	1	-27.1
hsa-miR-6131	NM_003266	*TLR4*	2325	2351	1	-22.5
hsa-miR-6134	NM_003266	*TLR4*	1067	1083	1	-18.5
hsa-miR-6165	NM_003266	*TLR4*	2156	2178	1	-23.1
hsa-miR-6500-5p	NM_003266	*TLR4*	2538	2596	1	-28.1
hsa-miR-6501-5p	NM_003266	*TLR4*	1976	1998	1	-31.9
hsa-miR-6503-3p	NM_003266	*TLR4*	2580	2601	1	-22.3
hsa-miR-6514-3p	NM_003266	*TLR4*	2572	2603	1	-32.8

hsa-miR-6515-5p	NM_003266	*TLR4*	1721	1753	1	-21.6
hsa-miR-6720-3p	NM_003266	*TLR4*	402	438	1	-26.4
hsa-miR-892c-5p	NM_003266	*TLR4*	1622	1646	1	-19.8
hsa-miR-6727-3p	NM_003266	*TLR4*	2632	2695	1	-22
hsa-miR-6733-3p	NM_003266	*TLR4*	436	472	1	-21.6
hsa-miR-6734-5p	NM_003266	*TLR4*	2291	2314	1	-27.9
hsa-miR-6751-3p	NM_003266	*TLR4*	2510	2530	1	-20.4
hsa-miR-6752-3p	NM_003266	*TLR4*	2578	2616	1	-22.7
hsa-miR-6754-3p	NM_003266	*TLR4*	2501	2543	1	-26.5
hsa-miR-6760-3p	NM_003266	*TLR4*	2542	2593	1	-26.9
hsa-miR-6761-3p	NM_003266	*TLR4*	2574	2600	1	-29.4
hsa-miR-6763-5p	NM_003266	*TLR4*	1724	1753	1	-26
hsa-miR-6765-5p	NM_003266	*TLR4*	1684	1705	1	-30.7
hsa-miR-6769a-5p	NM_003266	*TLR4*	2284	2311	1	-26.6
hsa-miR-6776-5p	NM_003266	*TLR4*	2402	2421	1	-22.4
hsa-miR-6784-5p	NM_003266	*TLR4*	2409	2430	1	-32.4
hsa-miR-6785-5p	NM_003266	*TLR4*	2349	2374	1	-23
hsa-miR-6788-5p	NM_003266	*TLR4*	2308	2335	1	-21.4
hsa-miR-6788-5p	NM_003266	*TLR4*	2542	2563	1	-21.4
hsa-miR-6790-5p	NM_003266	*TLR4*	1541	1565	1	-25.1
hsa-miR-6792-5p	NM_003266	*TLR4*	2411	2430	1	-29.9
hsa-miR-6792-5p	NM_003266	*TLR4*	528	553	1	-24.8
hsa-miR-6794-5p	NM_003266	*TLR4*	1740	1758	1	-25.8
hsa-miR-6795-3p	NM_003266	*TLR4*	2247	2290	1	-21.6

hsa-miR-6801-3p	NM_003266	*TLR4*	2662	2694	1	-22.4
hsa-miR-6813-5p	NM_003266	*TLR4*	359	389	1	-30.6
hsa-miR-6821-3p	NM_003266	*TLR4*	2542	2589	1	-21.4
hsa-miR-6825-5p	NM_003266	*TLR4*	315	350	1	-27.5
hsa-miR-6827-5p	NM_003266	*TLR4*	764	784	1	-25.2
hsa-miR-6827-3p	NM_003266	*TLR4*	2578	2603	1	-24.5
hsa-miR-6829-5p	NM_003266	*TLR4*	2547	2568	1	-25.5
hsa-miR-6831-5p	NM_003266	*TLR4*	2289	2311	1	-28.9
hsa-miR-6832-5p	NM_003266	*TLR4*	486	508	1	-23.5
hsa-miR-6836-3p	NM_003266	*TLR4*	2577	2605	1	-24.6
hsa-miR-6846-5p	NM_003266	*TLR4*	524	549	1	-27.2
hsa-miR-6847-5p	NM_003266	*TLR4*	1740	1758	1	-23
hsa-miR-6847-3p	NM_003266	*TLR4*	469	492	1	-20.9
hsa-miR-6854-5p	NM_003266	*TLR4*	1386	1414	1	-25.3
hsa-miR-6854-5p	NM_003266	*TLR4*	2129	2171	1	-24.1
hsa-miR-6854-3p	NM_003266	*TLR4*	2521	2548	1	-22.2
hsa-miR-6855-5p	NM_003266	*TLR4*	817	834	1	-24.2
hsa-miR-6857-5p	NM_003266	*TLR4*	1593	1613	1	-26
hsa-miR-6860	NM_003266	*TLR4*	2619	2640	1	-25.5
hsa-miR-6862-5p	NM_003266	*TLR4*	2097	2118	1	-24.2
hsa-miR-6863	NM_003266	*TLR4*	2301	2318	1	-20.6
hsa-miR-6868-3p	NM_003266	*TLR4*	2500	2543	1	-28.2
hsa-miR-6870-5p	NM_003266	*TLR4*	339	360	1	-23.6
hsa-miR-6881-5p	NM_003266	*TLR4*	1682	1704	1	-24.7

hsa-miR-6884-5p	NM_003266	*TLR4*	2530	2561	1	-23.5
hsa-miR-6885-3p	NM_003266	*TLR4*	2687	2700	1	-20.5
hsa-miR-6886-3p	NM_003266	*TLR4*	2574	2602	1	-23
hsa-miR-6890-5p	NM_003266	*TLR4*	815	836	1	-22.7
hsa-miR-7106-5p	NM_003266	*TLR4*	334	360	1	-24.5
hsa-miR-7108-5p	NM_003266	*TLR4*	2157	2185	1	-30
hsa-miR-7112-5p	NM_003266	*TLR4*	2595	2640	1	-27.1
hsa-miR-7114-5p	NM_003266	*TLR4*	517	538	1	-26
hsa-miR-7151-5p	NM_003266	*TLR4*	2526	2542	1	-23.3
hsa-miR-7156-3p	NM_003266	*TLR4*	413	434	1	-26.1
hsa-miR-7157-3p	NM_003266	*TLR4*	2406	2429	1	-20.9
hsa-miR-7158-5p	NM_003266	*TLR4*	1960	1990	1	-25.6
hsa-miR-7161-3p	NM_003266	*TLR4*	580	605	1	-23.7
hsa-miR-7703	NM_003266	*TLR4*	1012	1036	1	-22.9
hsa-miR-7851-3p	NM_003266	*TLR4*	399	426	1	-26
hsa-miR-8052	NM_003266	*TLR4*	2301	2334	1	-27.8
hsa-miR-8059	NM_003266	*TLR4*	401	424	1	-20.1
hsa-miR-8059	NM_003266	*TLR4*	2306	2333	1	-19.9
hsa-miR-8064	NM_003266	*TLR4*	2069	2116	1	-25.8
hsa-miR-8070	NM_003266	*TLR4*	1712	1733	1	-21.3
hsa-miR-8070	NM_003266	*TLR4*	1842	1858	1	-20.1
hsa-miR-8073	NM_003266	*TLR4*	2210	2241	1	-24.6
hsa-miR-8075	NM_003266	*TLR4*	2452	2482	1	-26
hsa-miR-8082	NM_003266	*TLR4*	2397	2417	1	-20.9

hsa-miR-9718	NM_003266	*TLR4*	524	544	1	-22.7
hsa-miR-9900	NM_003266	*TLR4*	1014	1032	1	-20.5
hsa-miR-9903	NM_003266	*TLR4*	1129	1161	1	-21.9
hsa-miR-10392-5p	NM_003266	*TLR4*	532	553	1	-26.5
hsa-miR-10394-3p	NM_003266	*TLR4*	2328	2343	1	-26.2
hsa-miR-10401-5p	NM_003266	*TLR4*	2518	2564	1	-23.6
hsa-miR-10396b-3p	NM_003266	*TLR4*	2572	2601	1	-26.1
hsa-miR-10522-5p	NM_003266	*TLR4*	2592	2613	1	-21.6
hsa-miR-10526-3p	NM_003266	*TLR4*	2291	2312	1	-25.6
hsa-miR-11181-5p	NM_003266	*TLR4*	2577	2601	1	-21.6
hsa-miR-3085-3p	NM_003266	*TLR4*	2332	2352	1	-23.7
hsa-miR-6529-3p	NM_003266	*TLR4*	2637	2680	1	-19.7
hsa-miR-9851-5p	NM_003266	*TLR4*	2332	2352	1	-27.1
hsa-miR-12116	NM_003266	*TLR4*	2577	2604	1	-23.3
hsa-miR-12117	NM_003266	*TLR4*	2087	2108	1	-18.9
hsa-miR-12128	NM_003266	*TLR4*	804	829	1	-26.9

Appendix XVI: List of miRNA binding with *TLR-2* sequence in miRDB database

Target Detail	Target Rank	Target Score	miRNA Name
Details	1	91	hsa-miR-561-3p
Details	2	90	hsa-miR-4720-5p
Details	3	90	hsa-miR-5588-5p
Details	4	90	hsa-miR-4799-3p
Details	5	89	hsa-miR-548ap-3p
Details	6	89	hsa-miR-4307
Details	7	89	hsa-miR-548aa
Details	8	89	hsa-miR-548t-3p
Details	9	87	hsa-miR-4793-5p
Details	10	87	hsa-miR-6165
Details	11	84	hsa-miR-6768-5p
Details	12	83	hsa-miR-548l
Details	13	83	hsa-miR-3200-3p
Details	14	82	hsa-miR-3126-3p
Details	15	81	hsa-miR-7110-3p
Details	16	78	hsa-miR-580-3p
Details	17	78	hsa-miR-654-3p
Details	18	77	hsa-miR-4475
Details	19	76	hsa-miR-6504-3p
Details	20	76	hsa-miR-10399-5p
Details	21	75	hsa-miR-4318
Details	22	74	hsa-miR-519b-5p
Details	23	74	hsa-miR-518f-5p
Details	24	74	hsa-miR-519a-5p
Details	25	74	hsa-miR-522-5p
Details	26	74	hsa-miR-520c-5p
Details	27	74	hsa-miR-519c-5p
Details	28	74	hsa-miR-518e-5p
Details	29	74	hsa-miR-523-5p
Details	30	74	hsa-miR-526a-5p
Details	31	74	hsa-miR-518d-5p

Details	32	73	hsa-miR-3942-3p
Details	33	73	hsa-miR-496
Details	34	72	hsa-miR-5692b
Details	35	72	hsa-miR-892c-5p
Details	36	72	hsa-miR-5692c
Details	37	72	hsa-miR-4639-3p
Details	38	71	hsa-miR-12124
Details	39	67	hsa-miR-7108-5p
Details	40	66	hsa-miR-548v
Details	41	63	hsa-miR-3128
Details	42	62	hsa-miR-514b-5p
Details	43	62	hsa-miR-513c-5p
Details	44	60	hsa-miR-5699-5p
Details	45	58	hsa-miR-575
Details	46	58	hsa-miR-4676-5p
Details	47	57	hsa-miR-6868-5p
Details	48	57	hsa-miR-4305
Details	49	57	hsa-miR-5092
Details	50	57	hsa-miR-6865-3p
Details	51	56	hsa-miR-3663-5p
Details	52	56	hsa-miR-4699-3p
Details	53	55	hsa-miR-410-3p
Details	54	54	hsa-miR-4430
Details	55	54	hsa-miR-567
Details	56	54	hsa-miR-3652
Details	57	51	hsa-miR-582-5p

Appendix XVII: List of miRNA binding with *TLR-4* sequence in miRDB database

Target Detail	Target Rank	Target Score	miRNA Name
Details	1	93	hsa-miR-448
Details	2	91	hsa-miR-3924
Details	3	89	hsa-miR-627-3p
Details	4	88	hsa-miR-338-5p
Details	5	88	hsa-miR-4272
Details	6	87	hsa-miR-10397-5p
Details	7	87	hsa-miR-5583-3p
Details	8	85	hsa-miR-4760-3p
Details	9	85	hsa-miR-4652-3p
Details	10	84	hsa-miR-1243
Details	11	81	hsa-miR-587
Details	12	81	hsa-miR-4678
Details	13	80	hsa-miR-6857-3p
Details	14	79	hsa-miR-140-5p
Details	15	79	hsa-miR-331-5p
Details	16	78	hsa-miR-656-3p
Details	17	78	hsa-miR-5697
Details	18	78	hsa-miR-153-5p
Details	19	77	hsa-miR-217-5p
Details	20	77	hsa-miR-6807-3p
Details	21	76	hsa-miR-4742-3p
Details	22	76	hsa-miR-6755-3p
Details	23	74	hsa-miR-642a-5p
Details	24	73	hsa-miR-4478
Details	25	73	hsa-miR-3929
Details	26	72	hsa-miR-3664-3p
Details	27	72	hsa-miR-219a-1-3p

Details	28	72	hsa-miR-571
Details	29	72	hsa-miR-892c-5p
Details	30	72	hsa-miR-6124
Details	31	72	hsa-miR-23b-5p
Details	32	71	hsa-miR-4724-5p
Details	33	70	hsa-miR-4775
Details	34	70	hsa-miR-630
Details	35	69	hsa-miR-651-3p
Details	36	68	hsa-miR-4261
Details	37	68	hsa-miR-1306-5p
Details	38	67	hsa-miR-4439
Details	39	67	hsa-miR-499b-5p
Details	40	67	hsa-miR-3136-5p
Details	41	66	hsa-miR-3148
Details	42	65	hsa-miR-5011-5p
Details	43	65	hsa-miR-3646
Details	44	65	hsa-miR-4692
Details	45	64	hsa-miR-23a-5p
Details	46	64	hsa-miR-10395-3p
Details	47	62	hsa-miR-4666a-3p
Details	48	62	hsa-miR-6889-3p
Details	49	62	hsa-miR-548ar-3p
Details	50	62	hsa-miR-4514
Details	51	62	hsa-miR-1250-3p
Details	52	62	hsa-miR-6529-3p
Details	53	61	hsa-miR-216b-5p
Details	54	61	hsa-miR-4313
Details	55	60	hsa-miR-335-3p
Details	56	60	hsa-miR-3201

Details	57	60	hsa-miR-7-5p
Details	58	59	hsa-miR-6853-3p
Details	59	58	hsa-miR-25-3p
Details	60	58	hsa-miR-6844
Details	61	58	hsa-miR-367-3p
Details	62	58	hsa-miR-12123
Details	63	58	hsa-miR-147b-5p
Details	64	58	hsa-miR-549a-3p
Details	65	58	hsa-miR-363-3p
Details	66	57	hsa-miR-21-5p
Details	67	57	hsa-miR-203a-3p
Details	68	57	hsa-miR-4803
Details	69	57	hsa-miR-1284
Details	70	56	hsa-miR-3912-5p
Details	71	55	hsa-miR-1185-2-3p
Details	72	55	hsa-miR-1185-1-3p
Details	73	55	hsa-miR-6728-3p
Details	74	55	hsa-miR-92a-3p
Details	75	55	hsa-miR-92b-3p
Details	76	55	hsa-miR-32-5p
Details	77	55	hsa-miR-548av-3p
Details	78	54	hsa-miR-655-3p
Details	79	54	hsa-miR-6529-5p
Details	80	54	hsa-let-7f-2-3p
Details	81	53	hsa-miR-6799-3p
Details	82	53	hsa-miR-6080
Details	83	53	hsa-miR-3173-5p
Details	84	52	hsa-miR-6884-3p
Details	85	52	hsa-miR-511-5p

Details	86	51	hsa-miR-603
Details	87	51	hsa-miR-1277-5p
Details	88	51	hsa-miR-1299
Details	89	51	hsa-miR-5701
Details	90	50	hsa-miR-548e-3p
Details	91	50	hsa-miR-548f-3p
Details	92	50	hsa-miR-6500-3p
Details	93	50	hsa-miR-548bc
Details	94	50	hsa-miR-548az-3p
Details	95	50	hsa-miR-548a-3p

Appendix XVIII: List of miRNA binding with *TLR-2* sequence in miRTarBase database

ID of miRNA	Species (Target)	miRNA	Validation methods								
			Strong evidence			Less strong evidence				Sum	# of papers
			Reporter assay	Western blot	qPCR	Microarray	NGS	pSILAC	Other		
MIRT000449	Homo sapiens	hsa-miR-146a-5p	✔	✔	✔				✔	4	1
MIRT001127	Homo sapiens	hsa-miR-105-5p	✔	✔	✔				✔	4	1
MIRT006646	Homo sapiens	hsa-miR-19a-3p	✔	✔	✔					3	2
MIRT006647	Homo sapiens	hsa-miR-19b-3p	✔	✔	✔					3	1
MIRT019178	Homo sapiens	hsa-miR-335-5p				✔				1	1
MIRT020445	Homo sapiens	hsa-miR-106b-5p				✔				1	1
MIRT052900	Homo sapiens	hsa-miR-154-5p	✔	✔	✔					3	1
MIRT734391	Homo sapiens	hsa-miR-101-5p	✔	✔	✔					3	1
MIRT734783	Homo sapiens	hsa-miR-143-3p		✔	✔					2	1
MIRT735134	Homo sapiens	hsa-miR-23a-5p	✔	✔	✔					3	1

http://mirtarbase.mbc.nctu.edu.tw/index.php

Appendix XIX: List of miRNA binding with *TLR-4* sequence in miRTarBase database

ID	miRNA	Target								Sum	Number of paper
			Reporter assay	Western blot	qPCR	Microarray	NGS	pSILAC	Other	CLIP-Seq		
MIRT735023	**hsa-miR-301a-3p**	TLR4	0	0	0	0	0	0	0	0	0	0
MIRT732637	**hsa-miR-5787**	TLR4	1	1	0	0	0	0	0	0	2	0
MIRT006530	**hsa-miR-146a-5p**	TLR4	1	1	1	1	0	0	0	0	4	1
MIRT732436	**hsa-miR-485-3p**	TLR4	1	1	1	0	0	0	0	0	3	0
MIRT735572	**hsa-miR-885-3p**	TLR4	1	1	1	0	0	0	0	0	3	0
MIRT733048	**hsa-miR-202-3p**	TLR4	0	0	1	0	0	0	0	0	1	0
MIRT722922	**hsa-miR-630**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT722924	**hsa-miR-942-5p**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT722926	**hsa-miR-548g-3p**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT722921	**hsa-miR-4279**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT722920	**hsa-miR-3658**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT722919	**hsa-miR-4659a-3p**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT722918	**hsa-miR-4659b-3p**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT722923	**hsa-miR-**	TLR4	0	0	0	0	1	0	0	1	2	2

	4738-3p											
MIRT722925	**hsa-miR-4742-3p**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT733483	**hsa-miR-4268**	TLR4	0	1	1	0	1	0	0	0	3	0
MIRT438116	**hsa-let-7b-5p**	TLR4	1	0	0	0	0	0	0	0	1	1
MIRT735623	**hsa-miR-216a-5p**	TLR4	1	1	1	1	0	0	0	0	4	0
MIRT003050	**hsa-let-7i-5p**	TLR4	1	1	1	0	0	0	1	0	4	1
MIRT732899	**hsa-miR-140-5p**	TLR4	1	1	1	0	0	0	0	0	3	0
MIRT735567	**hsa-miR-329-3p**	TLR4	1	1	1	0	0	0	0	0	3	0
MIRT053375	**hsa-miR-146b-5p**	TLR4	1	0	1	0	0	0	0	0	2	1
MIRT733771	**hsa-miR-23b-5p**	TLR4	1	1	1	0	0	0	0	0	3	0
MIRT737245	**hsa-miR-145-3p**	TLR4	1	1	1	0	0	0	0	0	3	0
MIRT737392	**hsa-miR-1178-3p**	TLR4	1	1	1	0	1	0	0	0	4	0
MIRT031004	**hsa-miR-21-5p**	TLR4	0	0	0	1	0	0	0	0	1	1
MIRT023907	**hsa-miR-1-3p**	TLR4	0	0	0	1	0	0	0	0	1	1
MIRT018411	**hsa-miR-335-5p**	TLR4	0	0	0	1	0	0	0	0	1	1
MIRT449828	**hsa-miR-7-5p**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT449826	**hsa-miR-526b-5p**	TLR4	0	0	0	0	1	0	0	1	2	2

MIRT449831	**hsa-miR-4694-3p**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT449830	**hsa-miR-4698**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT449827	**hsa-miR-6077**	TLR4	0	0	0	0	1	0	0	1	2	2
MIRT449829	**hsa-miR-1468-3p**	TLR4	0	0	1	0	1	0	0	1	3	2
MIRT736832	**hsa-miR-103a-3p**	TLR4	0	0	1	0	0	0	0	0	1	0
MIRT736833	**hsa-miR-107**	TLR4	0	0	1	0	0	0	0	0	1	0
MIRT732201	**hsa-miR-26b-5p**	TLR4	1	0	1	0	0	0	0	0	2	1
MIRT734334	**hsa-miR-146a-3p**	TLR4	0	1	1	0	0	0	0	0	2	0

Appendix XX: Genotypic and allelic frequencies of *TLR-2* gene polymorphisms in pre-obese and healthy control individuals

Polymorphisms	**Genotype frequency**			**Allele frequency**		
	Pre-obese		**Controls**	**Pre-obese**		**Controls**
Chr4:153688371T>C	**TT**[a]	80(93.0%)	324(90.7%)			
	TC	6(6.9%)	14(3.9%)	**T**[a]	166(0.96%)	662(0.92)
	CC[b]	0(0%)	19(5.3%)	**C**[b]	6(0.03%)	52(0.07)
Chr4:153702295T>C	**TT**[a]	86(100%)	301(84.3%)			
	TC	0(0%)	36(10.0%)	**T**[a]	172(1.0%)	638(0.89)
	CC[b]	0(0%)	20(5.6%)	**C**[b]	0(0.0%)	76(0.10)
Chr4:153703504T>C	**TT**[a]	71(82.5%)	335(93.8%)			
	TC	3(3.4%)	8(2.2%)	**T**[a]	145(0.84%)	678(0.94)
	CC[b]	12(13.9%)	14(3.9%)	**C**[b]	27(0.15%)	36(0.05)
Chr4:153705074C>A	**CC**[a]	78(90.6%)	332(92.9%)			
	CA	4(4.6%)	14(3.9%)	**C**[a]	160(0.93%)	678(0.94)
	AA[b]	4(4.6%)	11(3.0%)	**A**[b]	12(0.16%)	36(0.05)

[a] Reference genotype/allele

[b] Mutant genotype/allele

Appendix XXI: Genotypic and allelic frequencies of *TLR-4* gene polymorphisms in pre-obese and healthy control individuals

Polymorphisms	**Genotype frequency**			**Allele frequency**		
	Pre- obese		**Controls**	**Pre-obese**		**Controls**
Chr9:117707870	**GG[a]**	56(65.11%)	324(90.75%)			
117707870 G>A	**GA**	8(9.30%)	22(6.162%)	**G[a]**		
	AA[b]	22(25.58%)	11(3.08%)	**A[b]**	120(0.68)	670(0.93)
Chr9:117708080	**AA[a]**	75(87.20%)	322(90.19%)		52(0.29)	44(0.06)
117708080 A>G	**AG**	0(0%)	14(3.92%)	**A[a]**		
	GG[b]	11(12.79%)	21(5.88%)	**G[b]**	150(0.87)	658(0.92)
Chr9:117708777	**CC[a]**	74(86.04%)	339(94.95%)		22(0.12)	56(0.07)
117708777 C>G	**CG**	12(13.95%)	6(1.68%)	**C[a]**		
	GG[b]	0(0%)	12(3.36)	**G[b]**	160(0.93)	684(0.95)
Chr9:117708780	**GG[a]**	70(81.39%)	294(82.35%)		12(0.06)	30(0.04)
117708780 G>A	**GA**	12(13.95%)	21(5.88%)	**G[a]**		
	AA[b]	4(4.65%)	42(11.76%)	**A[b]**	152(0.88)	609(0.85)
Chr9:117713024	**AA[a]**	77(89.53%)	329(92.15%)		20(0.11)	105(0.14)
117713024 A>G	**AG**	0(0.00%)	21(5.88%)	**A[a]**		
	GG[b]	9(15.11%)	7(1.96%)	**G[b]**	154(0.89)	679(0.95)
Chr9:117715449	**TT[a]**	86(100%)	287(80.39%)		18(0.10)	35(0.04)
117715449 T>A	**TA**	0(0%)	23(6.44%)	**T[a]**		
	AA[b]	0(0%)	47(13.16%)	**A[b]**	86(1.00)	597(0.83)
Chr9:117715853	**GG[a]**	62(72.09%)	305(85.43%)		0(0.00)	117(0.16)
117715853 G>C	**GC**	12(13.95%)	35(9.80%)	**G[a]**		
	CC[b]	12(13.95%)	17(4.76%)	**C[b]**	136(0.79)	645(0.90)

[a]Reference genotype/allele

[b]Mutant genotype/allele

Appendix XXII: Association of *TLR-4* gene mutations with the pre-obese and healthy controls individuals

	Pre-obese vs controls(Genotypes)		
Polymorphisms	**Odds ratio**	**CI 95%**	***p* value**
		Lower-Upper limit	
Chr9:117707870G >A GGvs.GA	2.10	0.89-4.95	0.083
GGvs.AA	11.5	5.31-25.17	< 0.00001*
GGvs.AA+GA	5.2	2.97-9.30	< 0.00001*
Gvs.A	6.5	4.22-10.3	< 0.00001*
Chr9:117708080A>G AAvs.AG	0.14	0.00-2.49	-
AAvs.GG	2.24	1.03-4.86	0.03*
AAvs.GG+AG	1.34	0.65-2.77	0.41
Avs.G	1.72	1.02-2.91	0.013*
Chr9:117708777C>G CCvs.CG	9.16	3.33-25.19	0.00001*
CCvs.GG	0.18	0.01-3.11	-
CCvs.GG+CG	3.05	1.41-6.61	0.003*
Cvs.G	1.71	0.85-3.41	0.124
Chr9:117708780G>A GGvs.GA	2.40	1.12-5.10	0.01*
GGvs.AA	0.40	0.13-1.15	0.08
GGvs.AA+GA	1.06	0.58-1.95	0.83
Gvs.A	0.76	0.45-1.27	0.77
Chr9:117713024A>G AAvs.AG	0.09	0.00-1.65	-
AAvs.GG	5.49	1.98-15.21	0.0002*
AAvs.GG+AG	1.37	0.62-3.02	0.43
Avs.G	2.26	1.25-4.11	0.005*
Chr9:117715449T>A TTvs.TA	0.07	0.00-1.17	-
TTvs.AA	0.03	0.00-0.57	-
TTvs.AA+TA	0.02	0.00-0.38	-
Tvs.A	0.02	0.00-0.47	-
Chr9:117715853G>C GGvs.GC	1.68	0.82-3.43	0.14
GGvs.CC	3.47	1.57-7.63	0.001*
GGvs.CC+GC	2.27	1.30-3.95	0.003*
Gvs.C	2.47	1.58-3.85s	0.00004*
*P-value<0.05 considered highly significant			

Appendix XXIII: Identified Gram negative bacterial isolates from gut of pre-obese and healthy control subjects

Isolates Identified	Pre-obese n (%)	Control n (%)	*p* value	OR (CI-95%)
Escherichia coli	66 (52.8)	107 (45.1)	0.1	1.35(0.88-2.09)
Pseudomonas aeruginosa	19 (15.2)	43 (18.1)	0.4	0.80(0.44-1.45)
Klebsiella pneumonia	14 (11.2)	28 (11.8)	0.8	0.94(0.47-1.86)
Enterobacter cloacae	13 (10.4)	15 (6.3)	0.1	1.71(0.79-3.73)
Shigella spp	7 (5.6)	11 (4.6)	0.6	1.21(0.46-3.22)
Enterobacter aerogens	2 (1.6)	13 (5.4)	0.07	0.28(0.06-1.26)
Campylobacter specie	3 (2.4)	5 (2.1)	0.8	1.14(0.26-4.85)
Acinetobacter	1 (0.8)	2 (0.8)	0.9	0.96(0.08-10.55)

Appendix XXIV: Identified Gram positive bacterial isolates from gut of pre-obese and healthy control subjects

Type of bacteria	Isolates	Pre-obese n (%)	Control n (%)	*p* value	OR (CI-95%)
Gram positive cocci (aerobes)	*Staphylococcus aureus*	8 (72.7%)	18 (78.2%)	1[a]	0.45(0.008-25.16)
*Significant association *p* value >0.05 [a] *p* value by Fisher exact test					

Appendix XXV: Identified Gram negative anaerobic bacteria among pre-obese and control subjects

Isolates	**Pre-obese *n* (%)**	**Controls *n* (%)**	***p* value**	**OR (CI-95%)**
E.coli	28 (59.5)	33(34.0)	<0.01	2.59(1.25-5.33)
K. pneumonia	5 (10.6)	23(23.7)	0.04	0.35(0.12-0.99)
P. aeruginosa	5 (10.6)	12(12.3)	0.6	0.78(0.25-2.37)
E cloacae	7 (14.8)	12(12.3)	0.7	1.15(0.42-3.15)
Shigella species	2(4.2)	11(11.3)	0.1	0.32(0.06-1.52)
E aerogenes	-	6(6.1)	-	-
S. typhi	-	-	-	-

Turnitin Originality Report

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ORIGINAL ARTICLE

Detection Of TLR-2 germ line variants as a risk for obesity in local Pakistani population

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Background. Obesity is increasing rapidly affecting half billion adult's population. Pathophysiology of obesity involves low grade inflammation sustained by Toll like receptor 2 (*TLR-2*) the innate immune adapters. This study was conducted for detection and association of *TLR-2* gene mutations with obesity.

Methods. In this case-control study 228 individuals with obesity and 228 controls were enrolled based on Body Mass Index (BMI) ≥25 and 18-24 kg/m^2 respectively. The variations in *TLR-2* gene were detected by Sanger sequencing. These identified *TLR-2* variants were further analyzed *in silico* for change in miRNA binding and mRNA strucutre.

Results. Four novel single base substitutions (153688371 T >C, 153702295 T >C, 153703504 T >C and 153705074 C >A) were identified in exon 3 and 4 of *TLR-2* gene affecting splice site and poly-A tail. The genotypic and allelic frequencies of the variants were strongly associated with increasing obesity susceptibility. Only variant 153703504 T >C was significantly associated with preobesity. Despite variations in gene sequence, no change in miRNA binding except for variant 153688371 T >C of Exon 3 where a novel binding site for hsa-miR-4523 was created. Furthermore, mRNA stability and secondary structure were also compromised in identified variants.

Conclusion. All detected variants of *TLR-2* gene were significantly associated with and posed risk for development of obesity. Furthermore, *in silico* analysis revealed generation of new miRNA (hsa-miR-4523) binding site and change in mRNA structure/stability which needs to be further investigated for possible role in altering *TLR-2* gene regulation/expression in obesity.

Keywords: Inflammation, miRNA, mRNA secondary structure, Obesity, Single nucleotide polymorphism, Toll-like receptors.

Introduction

Obesity is the largest global chronic health problem, which is increasing in an alarming rate and turning into more serious problem than the malnutrition (1). Obesity develops when energy intake is surplus in comparison to energy expenditure, which causes excessive storage of triglycerides in adipose tissue (2). World Health Organization (WHO) defines obesity in individuals when their BMI is ≥30 kg/m^2 and overweight also termed as pre-obese condition if BMI is >25 kg/m^2 (3). Approximately 30% (2.1 billion) population of world is pre-obese, among them 600,000 are obese. The prevalence of obesity is considered higher in developed countries however, two-third of total obese population is also residing in developing countries (4). In Pakistan, among non-communicable diseases, prevalence of obesity is increasing in adults as well as in young pop-

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Profiling of potential pathogenic candida species in obesity

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ABSTRACT

Purpose of research: The aim of the current study was gut profiling of culturable *Candida* species and their possible pathogenic potential to asses role in obesity.
Methods: This case control study includes stool samples from 75 obese individuals and 50 controls. Isolation and identification of various *Candida* species was carried out by standard microbiological techniques. For pathogenic profiling, extracellular enzymatic assays, biofilm forming ability and resistance to azole were analyzed.
Results: Culturable gut profiling identified comparative higher abundance and diversity of *Candida* species among obese compared to controls. The most abundant specie among both groups was *C.kefyr*. A comparatively higher pathogenic potential as more hydrolases expression was detected in *C.kefyr*, *C.albicans* and *Teunomyces krusei* from obese group. Majority isolates from obese group were strong biofilm formers (47.1%) compared to control group (35.4%) suggesting it as strong risk factor for obesity. Fluconazole resistance was highest among *C.kefyr* (51%) followed by *Teunomyces krusei* and *C.albicans*. All the isolates from different species were voriconazole sensitive except *C.kefyr* displaying a 4.2% resistance in obese group only. A significant association of dominant colonizing species with meat, fruit/vegetable consumption and residence area was present ($p < 0.05$).
Conclusion: The presence of hydrolytic enzymes in gut *Candida* species showed strong association with protein's degradation and enhanced pathogenicity. *C.kefyr* and *Teunomyces krusei* has emerged as potential pathogen showing increased colonization as result of protein rich and low carb diet. Thus presenting it as a bad choice for weight loss in obese individuals.

1. Introduction

Obesity is a chronic metabolic disorder affecting 650 million population worldwide and becoming prominent cause of related complication by increasing mortality rate [1]. The pathogenic mechanism of obesity is multifactorial and difficult to understand due to involvement of complex metabolic system, multiple immune mediators, adipose tissues, macrophages and more recently involvement of microorganisms [2]. Various studies involving the gut microflora has established the possible involvement of microbes in orchestrating various metabolic diseases including obesity. The composition of gut microbiota is known to be altered in obesity as obese individuals have been characterized by decreased ratio of Bacteroidetes to Firmicutes [3], [4], [5]. Beside bacteria, fungi also resides in gastrointestinal tract (GIT) comprising less than 1% of overall gut flora and are essential part of the GIT tract which is still poorly explored [6], [7], [8]. In recent decade, mycobiome dysbiosis have been shown to be associated with many inflammatory diseases even in obesity [9], [10]. Recent advancement in field of mycology have shown the association of genera Thermomyces and Saccharomyces with metabolic disturbance and weight gain [11]. The research work on animal models have also shown association of obesity with changing gut fungal diversity [12]. Furthermore, in humans gut microbiome study have also reported a mycobiota shift in obese, where there was increase in yeast abundance among obese individuals while more filamentous fungi in healthy controls [13–15].

Among yeast, *Candida* species are well known to colonize abundantly in various body parts including mucosal lining, oral cavity, vaginal tract and GIT as a commensal. These commensal microorganisms of the gut

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