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NEWER MARKERS OF METABOLIC AND CARDIOVASCULAR DISEASES

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Dedication

This work is dedicated to my parents my husband and my two children.

For their lifelong devotion, support, selfless and unlimited presence throughout the duration of my study, training and career accomplishment I need to thank my beloved parents. My husband represented the pioneer of my development after Medical School and for this I am expressing my deepest respect and admiration and my sincere thankfulness. My two children stepped through a process of gaining gradually consciousness on how profoundly they have supported their mother in being a Paediatrician and a PhD. Always patient and thoughtful I owe them the biggest thank of all.

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Preface

The present research is structured to detect metabolic disorders and cardiovascular risk early in life, specifically in the children. More and more evidence is showing how crucial is the development of new tools in the disposition of scientists and medical practitioners that would enable them to prevent the progress of these disorders and predict their presentation from their initial stages. The aim of the study was to identify molecules that could be easily quantified in the serum of paediatric population, and that meet the criteria for constituting biomarkers of early cardiovascular signs. These signs were explored in our study, by non-invasive methods, using ultrasound techniques, easily applied to a routine visit at the medical practitioner's office. We have shown for the first time, to our knowledge, that there are such molecules and they could be used for population screening and eventually for follow up, starting early in life, in childhood.

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PART ONE – BACKGROUND AND LITERATURE REVIEW

Chapter 1.Obesity in Childhood

1.1 Definition

Obesity is defined as abnormal or excessive fat accumulation that may impair health state. Its aetiopathogenesis resides in various factors such as the demographic parameters, lifestyle and dietary routine (1). Higher parental education and socioeconomic position of the family are protective factors (2, 3). Maternal age and parental body mass index (BMI), when not optimal, have increased probabilities to negatively influence the offspring (2, 4, 5). Body fat deposition and distribution is also genetically determined. This is outlined in studies of twins indicating a potential relationship between genetic determinants and dietary choices as well as energy expenditure (6). Genome association studies have provided evidence of existing single-nucleotide polymorphisms (SNPs) associated with body fat distribution and possibly interfere with satiety responsiveness in childhood (7-9). Lifestyle is highly influencing energy expenditure; this results in being depressed in children who occupy most daily time in sedentary activities, such as television watching, personal computer and electronic game playing, leading to increased body weight. This is not only related to lower activity but also related to increased food consuming during these activities (10, 11). Dietary routine is important in shaping a growing organism. Specifically, it has been shown that lack of daily breakfast consuming may lead to increased body weight in childhood. Cereal consuming as a breakfast meal is related to lower possibilities to increased weight (12, 13). Healthier food consuming has a clear positive effect on child growth and determines body parameters; in most cases it is related to daily family habits and participation in creating and maintaining a healthy family diet.

Internationally acceptable definition for child overweight and obesity has been a durative process, requiring the definition of normal-healthy weight and the creation of growth charts, multiple times revised in order to apply to paediatric populations worldwide.

In 1977 the National Center for Health Statistics (NCHS) released reference charts that were revised by the Centers for Disease Control and Prevention (CDC) in 2000 and the World Health Organization (WHO in 2006 (14-16). Rigorously standardized methods were designed, especially for newborns and infants with detailed information on meals, and regular weighing processes. WHO used data from Multicentre Growth Reference Study (MRGS) to follow different ages of childhood and used an exclusively breast-fed population in early infancy. CDC included data partially from infants that were exclusively breast-fed and only for a few months, while infants that were partially breast-fed were also included. Consequently, charts for older children, school aged and adolescents were released by the international organisms, focusing on methods that would apply to a broader population around the world and including data from different countries (17).

To establish standard definition for child overweight and obesity, an international survey of six cross-sectional growth studies was developed in May 2000 with participation from the International Obesity Task Force, the National Center for Health Statistics in London and the CDC (18). In this study, a total of 97,876 males and 94,851 females participated from birth to 25 years of age, with different nationality coming from Brazil, Great Britain, Hong Kong, the Netherlands, Singapore, and the United States. BMI was found to be the most appropriate index to define age and sex specific cut off points for overweight and obese children at ages 2 to 18 years old. To achieve this definition, centile curves for age specific body mass index were constructed using the LMS method and these curves were designed, based on the adult cut off limits for overweight and obese, to pass respectively through BMI of 25 and 30kg/m^2 at age 18 (18, 19). The cut off limits were chosen based on the related knowledge on morbidity in adults and the corresponding morbidity in earlier stages of life and in childhood (20, 21).

BMI is not a direct measure of body fat, but it is a valid screening tool for a growing organism, as a child, due to the modality of its calculation through a ratio expressed by the consequent formula (22-24):

$$BMI = \frac{(weight\ in\ kilograms)}{height\ in\ meters^2}$$

The centile curves offer the possibility to register the BMI in specific age and sex related charts comparable within a similar population. The centile curves are defined by the consequent formula:

$$M(1+LSz)^{1/L}$$

L, M and S are values of the curves at each age and z indicates the z score for each specific centile (25).

The body mass index can be converted to z score from the L, M, S values at a specific age with the consequent formula:

$$Z = \frac{\left[\frac{BMI}{M}\right]^L - 1}{LS}$$

The Endocrine Society Clinical Practice Guideline was published in January 2017 were specific instructions were included for diagnosing overweight and obese in childhood (26). The use BMI and the CDC normative BMI percentiles to diagnose overweight or obesity in children and adolescents above 2 years of age have been recommended (26). Child or adolescent above 2 years of age should be diagnosed as overweight if the BMI is ≥85th but <95th percentile for age and sex, should be diagnosed as obese if the BMI is ≥95th percentile, and as extremely obese if the BMI is ≥120% of the 95th percentile or ≥35 kg/m^2. Calculating, plotting, and reviewing a child's and an adolescent's BMI

percentile at least annually during well-child and/or sick-child visits was suggested (26). Children <2 years of age have been suggested to be diagnosed as obese if the sex-specific weight for recumbent length is ≥97.7th percentile on the WHO charts, and the US and international pediatric groups accept this method as valid for this specific age group of children (26).

However, national data best reflect the local population and in our country, the Greek paediatric population is expressed through growth charts released by the 1st Department of Paediatrics of the University of Athens, in 2003. Five thousand boys and 5,000 girls, from birth to 18 years old, were recruited from the broader Attica area (27).

1.2 Epidemiology of Obesity

In the last fifty years, childhood obesity has increased dramatically, mostly in economically developed countries. Available information from worldwide research confirms the ongoing epidemic of obesity as well as the harming effects on health and development (28).

The latest update is provided by the Organization for Economic Co-operation and Development (OECD) on 2017, with descriptive charts from national health surveys, from participating countries all around the world, offering a global view of the ongoing epidemic of obesity in adult and paediatric population. Nearly, 1 in 6 children is overweight-obese based on the reported data.

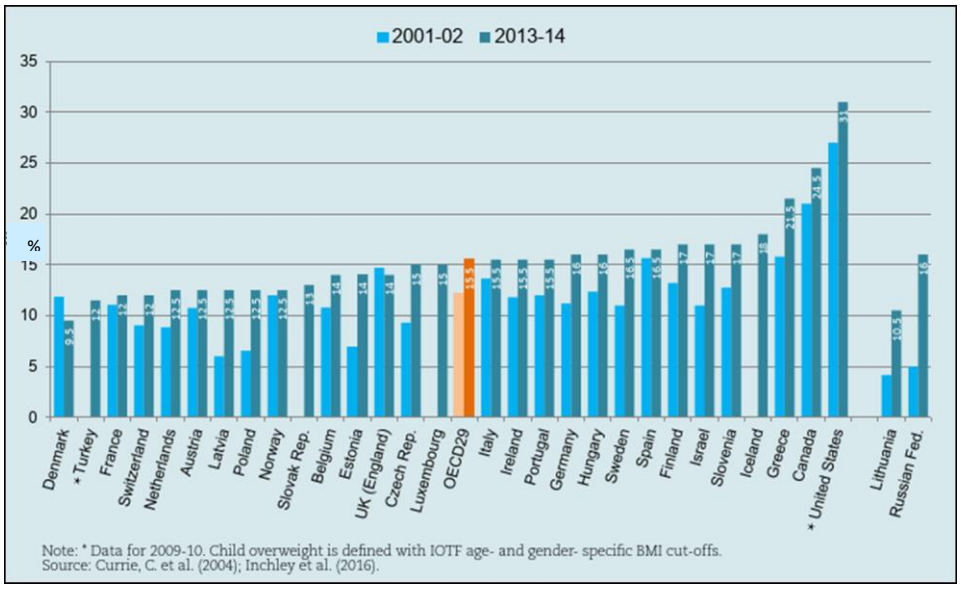

Figure 1 Self-reported overweight (including obesity) in children aged 15 years. Reproduced from (29).

It is notable that up to 2014, Greece was positioned very high in this chart, showing a 21.5% of self-reported overweight (including obesity) children aged 15 years, ranking in the third position following the United States of America and Canada (**Figure 1**). These results are consistent during the last decade as it can be noticed that Greece is proportionally high and overall in the third

position. There is an approximate 5% increase in the documented data for Greece during the last decade; this also stands out in the highest rates reported in comparison to the rest of the countries participating. Denmark stands in the lowest position in this chart.

Apparently, sex difference in obesity is inverted in childhood in comparison to adulthood and it can be noticed that boys tend to show higher rates of overweight-obese than girls, while this trend is inversed in adulthood, with women showing higher weight rates in comparison to men (**Figure 2**). This difference in adulthood is proposed to be related to the educational level (**Figure 3**) (29).

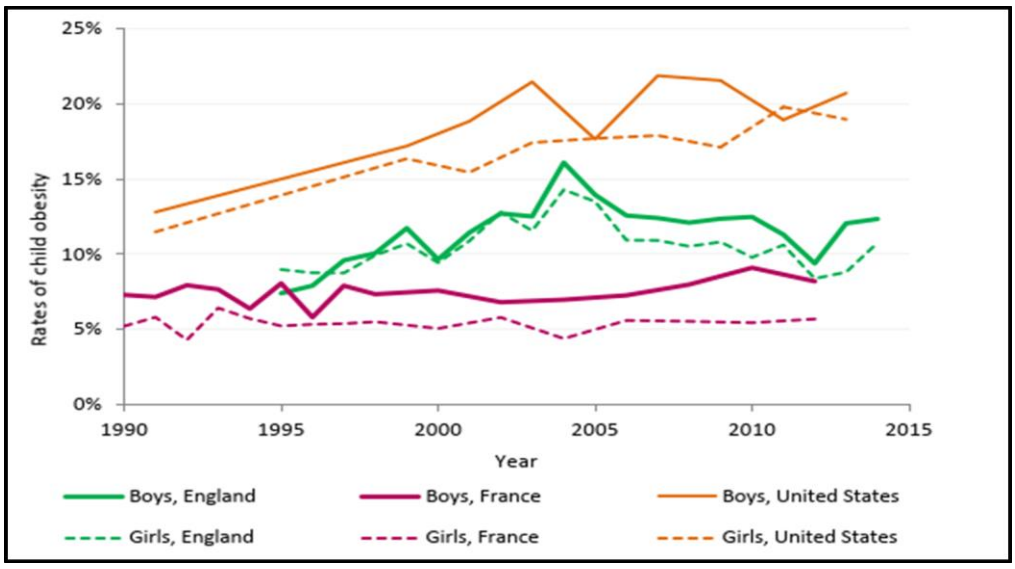

Figure 2 Obesity in children aged 3-17 years from 1999 to 2015. Reproduced from (29).

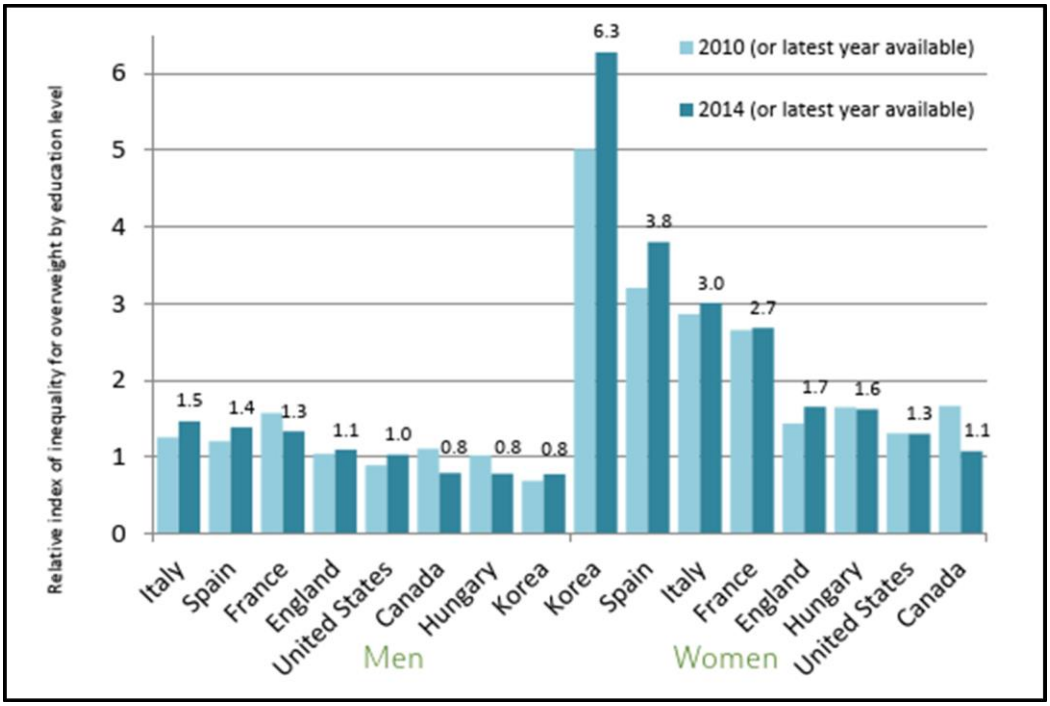

Figure 3. Education–related inequality in overweight. Reproduced from (29).

In 2018 the European Commission of OECD published the State of Health in the EU Cycle where the preliminary data by WHO is included. This data shows that nearly one in eight children aged 7-8 years are obese in EU countries (30). Within the most afflicted countries is Greece, ranking on top of the classification, side by side with Cyprus, Italy, Malta and Spain. Lowest rates of obesity in Europe in children aged 7-8 are reported for Czech Republic, Denmark, France, Ireland and Latvia. Notably, the rates of obesity among children of this age are lower in comparison to earlier years in Greece and other countries such as Italy and Portugal, but they are remaining at the top end of the classification charts. Consistently, boys are showing higher rates of obesity in comparison to girls and specifically for age 7-8 years old, 14% of the male population is obese while 10% of the same age female population is obese (30, 31).

1.3 Health Impact of Obesity

Overweight and obesity in childhood is associated with several comorbidities (32). Children with obesity have increased risk in developing hyperinsulinemia, insulin resistance and Type II diabetes mellitus (T2DM) (33). Adolescents who present T2DM are more likely to proceed in early adulthood in dyslipidemia, hypertension and cardiovascular disease (34-37). There is strong evidence that non-alcoholic fatty liver disease (NAFLD) is associated with obesity in childhood (38). In studies of children with NAFLD diagnosed by biochemical or imaging indicators, when dietary and lifestyle measures were applied for a year they were proved to be efficacious and lead to valid remission of findings related to the disease (39). A recent study has shown that children and adolescents with biopsy proven NAFLD present higher prevalence of pre-diabetes or T2DM (40).

Respiratory tissue may also be affected by obesity in childhood with presence of obstructive sleep apnea episodes (OSA). The prevalence of OSA increases proportionally with increase in BMI (41, 42). Asthma is also shown to be frequently associated with increased BMI while lifestyle interventions applied to decrease body weight and diminish BMI result to be protective in terms of developing asthma in childhood (43).

In females, obesity may be associated with earlier skeletal maturation with greater risk in developing hyperandrogenism and polycystic ovary syndrome (PCOS) (44-46). Increased body weight is also associated with musculoskeletal disorders, including increased risk for fractures involving the physis, joint pain, slipped capital femoral epiphysis and Blount’s disease (47). Some malignancies, endometrial, ovarian, breast, prostate, liver, gallbladder, kidney and colon, are also shown to be related to obesity and they could present in earlier stages of life and in adolescence. In a recent study, it was shown that physical activity, that also aims to an optimal weight control, when initiated in early stages of childhood and continued throughout lifetime, may lower the risk of developing breast, colon and endometrial cancer (48, 49).

Psychosocial distress, due to impaired quality of life, is frequently observed in children with obesity (1, 50-52). This disorder includes poor self-esteem (53), increased risk of depression and anxiety and higher-than-average risk of eating disorders and substance abuse (54-56). There is evidence that children who are heavier than their same age children have more probabilities in being teased and bullied in comparison to their thinner peers (57).

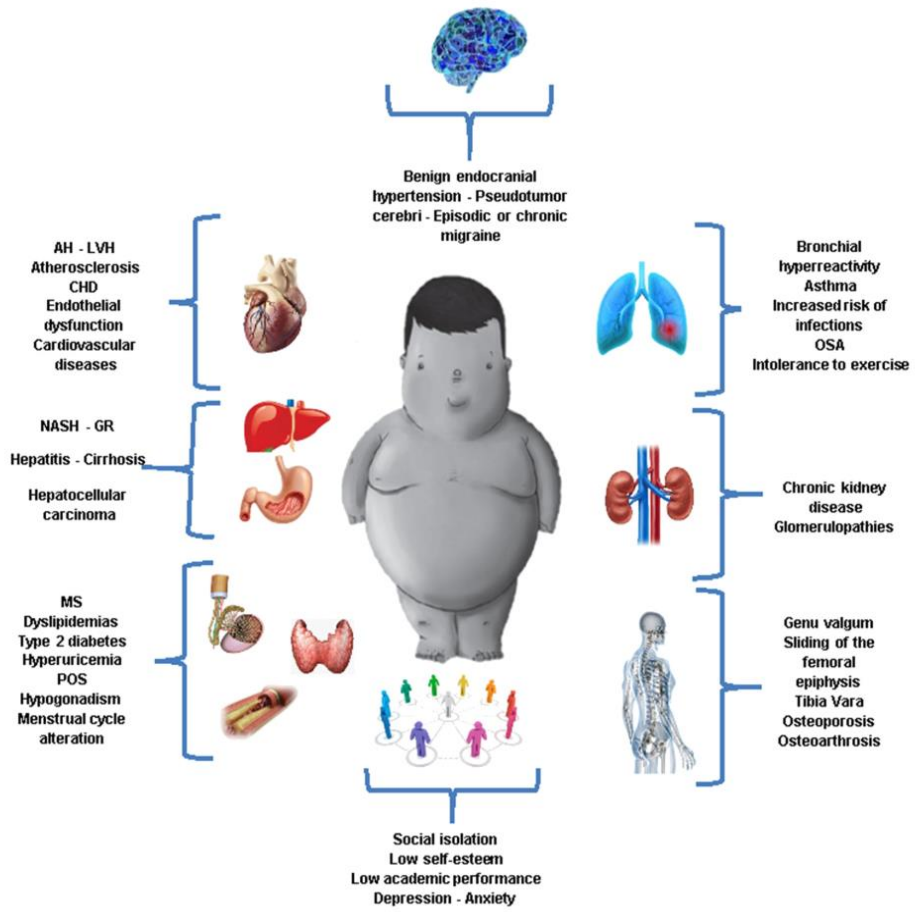

Figure 4. Comorbidities observed in children and adolescents with obesity. Reproduced from (32) with permissions from John Wiley and Sons.

Chapter 2.Metabolic Syndrome in childhood

2.1 Definition of the Metabolic Syndrome in Childhood

The metabolic syndrome (MS) is a constellation of different components participating in the development of cardiovascular disease. Historically, Gerald Reaven in 1988 was the first to define the increased risk in developing diabetes and cardiovascular disease in the presence of increased serum insulin and insulin resistance by assigning the name of Syndrome X (58-60). Later on, more risk factors were included in a revised definition, with central obesity, microalbuminuria, altered fibrinolysis and inflammation, in an effort to better describe cardiovascular risk and the syndrome was renamed as the MS (61-63). Revisions and many series of studies on the MS led the National Cholesterol Education Program (NCEP) and the Adult Treatment Panel III (ATP III) in 2001, to release guidelines in which it was specified that MS should include 5 risk factors and if anyone presented at least 3 out of those 5, would be at an increased risk for developing cardiovascular disease (64-66). The 5 risk factors were hyperglycemia, hypertriglyceridemia, central adiposity, elevated blood pressure, and low high-density lipoprotein cholesterol.

In children, there is no consistency in the definition of the MS as 3 different definitions have been proposed in the past and it is has not yet been agreed whether one of them is superior from the other in better providing risk prediction. In adults, it has been shown that when MS criteria are met, the hazard ratio for coronary heart disease mortality is 2.87 without presence of diabetes mellitus and 5.02 when diabetes mellitus is also present (67). In 2008, data from the Princeton Prevalence Study (1973-1976) and the Princeton Follow-up Study (2000-2004) were assessed and the results indicated that the pediatric population including children aged 5 to 19 years old may be screened for MS and family history for (T2DM) and identify those with increased risk of adult MS, T2DM and cardiovascular disease (68, 69). In 2003, the ATP III criteria for the pediatric MS defined MS as a constellation of metabolic derangements associated with obesity, and were published by modifying the adult criteria to the closest representative

values obtainable from pediatric reference values from the NCEP, the American Diabetes Association statement on T2DM in children and adolescents, and the Task Force report on the diagnosis and management of hypertension in childhood as well as ATP III (70). Due to lack of normal values for waist circumference, available values for waist circumference from the included population were assessed and classified. A waist circumference at or above the 90th percentile for age and sex would determine the classification of abdominal obesity. The results from this study also underlined that there may be children and adolescents that would not be obese but overweight and yet meet criteria for the MS which would be further alarming as for the importance of detecting more a cluster of risk factors more or less independent from each other and from obesity, that would underline the increased danger for cardiovascular risk (70).

In 2004, the National Health and Nutrition Examination Survey (NHANES III) released criteria for the definition of the pediatric MS by using risk factors analogous to ATP III definition for MS for adults based on data from US children from 12 to 19 years old (71). For waist circumference, percentiles comparable to the adult male cut-off point of the 70th percentile were used, and Blood Institute's recommended cut-off point of >90th percentile for age, gender, and height for the definition of normal pediatric blood pressure was also included (72, 73). The cut-off point criteria were chosen based on the ATP III adult MS definition with the perspective to be more inclusive and representative for the adolescent population (71).

There is clear preference in the usage of central obesity for the definition of the MS rather than the calculation of overweight/obese by BMI. This is based on the pathophysiology underlying MS although it is not completely understood whether obesity or insulin resistance appears first. However, it is clear that the MS describes a cluster of parameters with insulin resistance and "adiposopathy", adipose tissue dysfunction, as hallmarks that consequently trigger inflammation and cardiovascular tissue damage (74). Central obesity expresses the increased accumulation of visceral fat and it differentiates from the subcutaneous fat, as described in the broad obesity definition. In visceral obesity, there is hypertrophy of visceral tissue that becomes less responsive to insulin with inability of lipolysis suppression and this creates a viscous cycle (75). Waist circumference (WC) is a validated measurement of adult and child

central obesity (76, 77). There is evidence that in children, WC is an independent predictor of insulin resistance, lipid levels and blood pressure and that WC better predicts cardiovascular disease risk factors as compared to BMI (78-80).

There are several concerns on the presence of instability of the MS throughout childhood and especially for the adolescents who represent a highly heterogeneous group with multiple hormonal and growth parameters acting during this specific age window. By studying pre-pubertal and pubertal children via hyperinsulinemic-euglycemic clamp, decreased insulin sensitivity has been shown during puberty (81). Accordingly in 2007, the International Diabetes Federation (IDF) released a definition for the MS that requires central obesity plus at least two of the other listed abnormalities and recommends that this definition is applied to children from 10 to 16 years old, excluding from the definition those less than 10 years of age in whom MS could not be defined, and the adolescents (82). IDF confirms and underlines the lack of consistency in the available data regarding the cut-off points for the various MS components in childhood. IDF supports the promotion of research in the field focusing on developing ethnic-specific centiles for WC, initiate multicenter long-term studies that would follow child cohorts into adulthood in order to understand the persistence of MS throughout the aging process and determine the effectiveness of interventions. IDF supports also the design of studies that may detect MS predictors (82).

In 2017 the American Academy of Pediatrics (AAP) suggests shifting the concern from defining the MS in children to identifying a cluster of risk factors that predict increased cardiovascular risk as early as in childhood, allowing thus early detection of high risk population and early intervention (74). For that purpose, a series of recommendations for screening are suggested, including annual screen for obesity, glucose disorders, increased blood pressure, and dyslipidemia as well as augmenting awareness of comorbidities such as NAFLD, mental health disorders, PCOS, and OSA (74). Recommendations to treat obesity were also made based on the consensus of American Heart Association and the American Diabetes Association and on an annual screen basis. When children present a BMI greater than 95^{th} percentile, they should be referred to a comprehensive weight-management program (83, 84). Similarly, all children should be screened annually for elevated blood pressure and children aged 9 to 11 years should

be tested for lipid profile, specifically to identify metabolic disorders of genetically determined dyslipidemias (83). Table 1 resumes the three definitions of MS for children (74).

Table 1. Comparison of key published MS definitions for paediatric populations

Pediatric Definitions of MS			
	Cook et al (70)	**De Ferranti et al (71)**	**Zimmet et al (82) (IDF Definition Ages 10-16)**
Defining Criterion	≥3 criteria	≥3 criteria	Central obesity and at least 2 of remaining 4 criteria
Central Obesity	WC≥90th percentile (age and sex specific. NHANES III)	WC>75th percentile	WC≥90th percentile or adult cut off if lower
Glucose intolerance	Fasting glucose ≥110mg/dl	Fasting glucose ≥ 110mg/dl	Fasting glucose ≥ 100mg/dl or known type 2 diabetes mellitus
Dyslipidemia (TRGL)	TRGL ≥110mg/dl	TRGL≥100mg/dl	TRGL≥150mg/dl
Dyslipidemia (HDL-C)	HDL-C≤40mg/dl (all age and sexes NCEP)	HDL-C≤50mg/dl	HDL-C<40 g/dl
High BP	BP≥90th percentile (age, sex and height specific)	BP>90th percentile	Systolic BP≥130mmHg or diastolic BP≥85mmHg or treatment of previously diagnosed hypertension

MS, metabolic syndrome; WC, waist circumference; TRGL, triglycerides; HDL-C, high-density l lipoprotein cholesterol; BP, blood pressure

2.2 Epidemiological Data

Due to the lack of unanimous MS definition especially for childhood, there is variability on the reporting prevalence based on the specific criteria used. Using the definitions for MS from the NCEP ATPIII and WHO in a school based cross-sectional study of 1,513 adolescents, the prevalence of MS defined by NCEP was 4.2% and the prevalence defined by WHO 8.4%. In this study, MS was almost exclusively diagnosed among the obese children and within the obese population of this study the prevalence of MS was 19.5% for the NCEP definition and 38.9% for the WHO definition of MS. Therefore, obesity was described as a powerful risk for MS. Using the WHO definition criteria the girls are found more commonly with MS (85).

In 2004, the Yale University School of Medicine examined the effect of varying degrees of obesity on the prevalence of the MS and its relation to insulin resistance. In this study, it was reported that the prevalence of the MS increased with the severity of obesity, reaching 50% at severe obesity levels (BMI 40.6 kg/m^2) and with increasing insulin resistance. It was also reported that at this age various biomarkers of increased cardiovascular risk were also present, such as high levels of C reactive protein (CRP) and low levels of adiponectin (86).

Although both obesity and insulin resistance are factors included in the definition criteria, it is not defined whether they are independently necessary for the definition of the MS. The most accepted mechanism underlying the pathogenesis of the MS is built on insulin resistance. However, it is also known that not all individuals with insulin resistance develop MS, and non all individuals with insulin resistance are obese. Therefore it is under specific circumstances and in specific conditions such as the presence of cofactors that the MS is developed (87-89).

Available cross-sectional data from the Third National Health and Nutrition Examination Survey (1988-1994) in a population of 12 to 19 years old were analyzed using the NCEP (ATPIII) definition criteria modified for age for the MS. Based on this analysis in 2003, it was reported that the overall prevalence of the MS among adolescents was 4.2%. Specific for gender prevalence for MS was measured and found to be 6.1% for males and 2.1% for females. Based on BMI percentiles, it was found that

for BMI greater or equal to 95th percentile the MS was present in a percentage of 28.7, whereas in children with BMI corresponding to a percentile between the 85th and the 97th the percentage of children with MS was 6.8 and lastly for BMI percentile below the 85th the percentage of MS was as low as 0.1 % (70). Data from the NHANES 1999-2000 using 4 different definitions of the MS on adolescents 12 to 19 years old, showed that the prevalence of MS varied from just >9% to as low as 2% of adolescents overall. Different definitions of MS generated prevalence rates in obese adolescents that varied widely from 12% to 44% (90). However, regardless of the definition used, the prevalence of the MS is higher in older compared with younger children (83, 90).

In adults, a representation of the prevalence worldwide is shown in Figure 5 (87, 91).

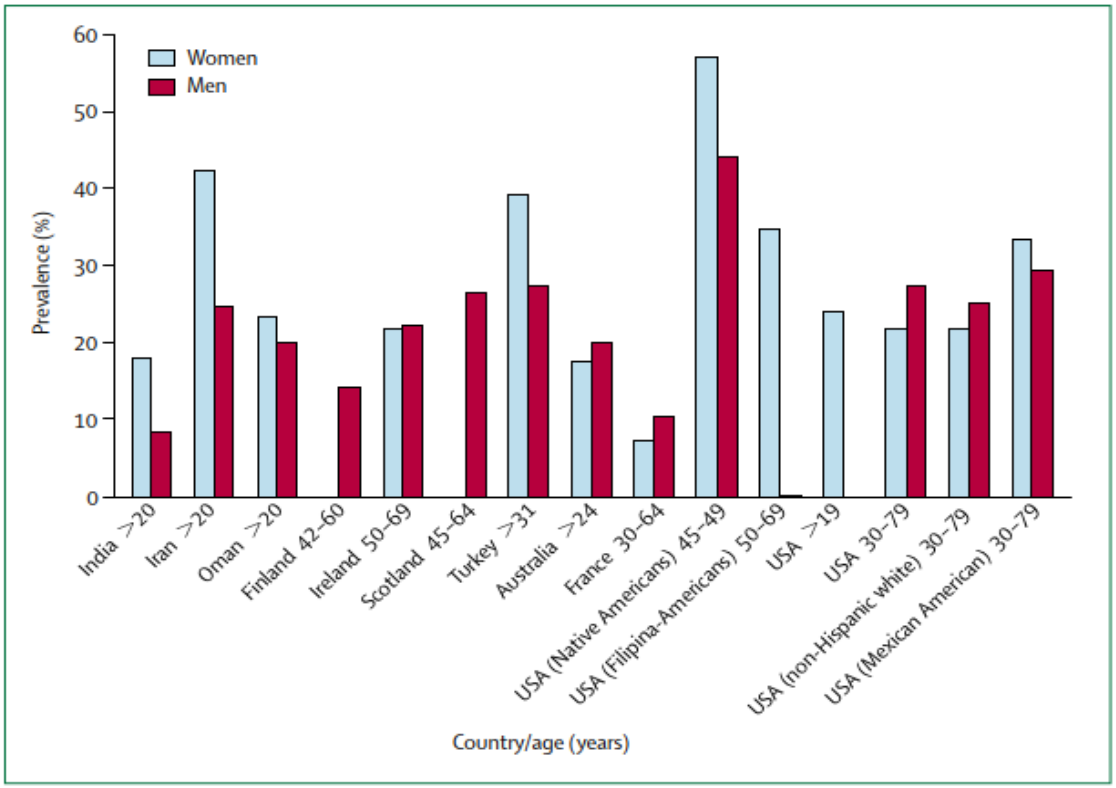

Figure 5. Prevalence of the MS. Reproduced from (87) with permissions from Elsevier.

2.3 Known Health Implications of the MS

The MS is more a tool to identify increased cardiovascular risk than a direct cause of disease. However, several known health conditions are linked to it. The major two health disorders related to MS are T2DM and cardiovascular disease (CVD).

In adults, a study from Finland in 2002, reported the association of the MS with cardiovascular and overall mortality. They showed that CVD and all-cause mortality are increased in men with the MS, even in the absence of baseline cardiovascular disease and diabetes (92). In 2003, a different study in U.S adults, over 50 years old, the NCEP criteria for MS were used to categorize by presence of MS in adults with or without diabetes. The prevalence of CVD markedly increased with the presence of MS. People with both diabetes and MS had the greatest prevalence of cardiovascular disease. People with diabetes had very high prevalence of MS (93). Further confirmation of the association of MS with cardiovascular disease, and specifically with myocardial infarction and stroke, in both male and female gender, is added by a study in 2004, by applying the NCEP-ATP III criteria at NHANES III subjects and a recent study adds the increased risk for cardiovascular disease mortality (94, 95). Diabetes is the other entity closely linked to MS and a recent study in Singapore has developed a MS severity score to detect individuals at high risk of diabetes (96).

Even though the available knowledge of the impact of childhood MS on CVD is limited, great effort has been made in determining and exploring better this relation. In 2011, the National Heart, Lung, and Blood Institute (NHLBI) and its expert panel published the integrated guidelines for cardiovascular health and risk reduction in children and adolescents where MS constitutes one of the evaluated risk factors (83). It is stated by the NHLBI that atherosclerosis begins in youth and it is consolidated by the presence of, and action of the known risk factors namely, family history, nutrition/diet, physical inactivity, tobacco exposure, blood pressure, lipid levels, overweight/obesity, diabetes mellitus and the MS.

Big studies of child autopsy offer evidence that there is early in life risk for atherosclerosis. The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study examined 2,876 subjects from 15 to 43 years old who died of external causes and undergone autopsy. In this study it is reported that intimal lesions, fatty streaks and

clinically significant raised lesions, begin in youth (97). The Bogalusa Heart Study reports on autopsies made on 204 young persons aged from 2 to 39 years who had died from various causes, principally trauma, and includes analysis on existing ante-mortem risk factors in 93 of them. Consistently with the PDAY study, the Bogalusa Heart Study confirms existence of asymptomatic coronary and aortic atherosclerosis in young people and further on specifies that as the number of cardiovascular risk factors increases, so does the severity of atherosclerotic lesions (98). Data from the NHLBI Lipid Research Clinics Princeton Prevalence Study (1973-1976) and the Princeton Follow-up Study (2000-2004) were used to assess the association of MS in childhood with adult CVD 25 years later. In this study, it was reported that pediatric MS and age at follow-up assessment were significant predictors of CVD (69). Further on, using the same data resource it was shown that pediatric MS, age at follow-up, black race, and parental diabetes were significant predictors of T2DM (68). Using data from the NHANES 1999-2002, in adolescents and adults, it was shown that low cardiorespiratory fitness, a possible precursor of CVD, is associated with all the factors of the MS (99). All of this knowledge led the NHLBI to release specific timetable for assessing each cardiovascular risk factor in childhood, all definition criteria of the MS are included in the health schedule (83).

In a Hispanic population, MS was defined using the NHANES III protocol in 126 overweight children 8 to 13 years old, with family history of T2DM. Oral glucose tolerance tests were performed to evaluate the presence of insulin resistance. In this population, increased risk for CVD and T2DM was reported, due to the presence of insulin insensitivity. Importantly, it was also shown that insulin sensitivity significantly decreased ($P < 0.001$) as the number of features of the MS increased (100).

Other conditions such as PCOS and OSA may relate to MS indirectly through their connection to obesity however, more studies need to be performed in order to prove whether direct connection of these conditions with MS exists.

The AAP in the 2017 statement, supports that the mechanism underlying the MS is insulin resistance that in turn relates to obesity (74). A schematic proposed mechanism is shown in Figure 6.

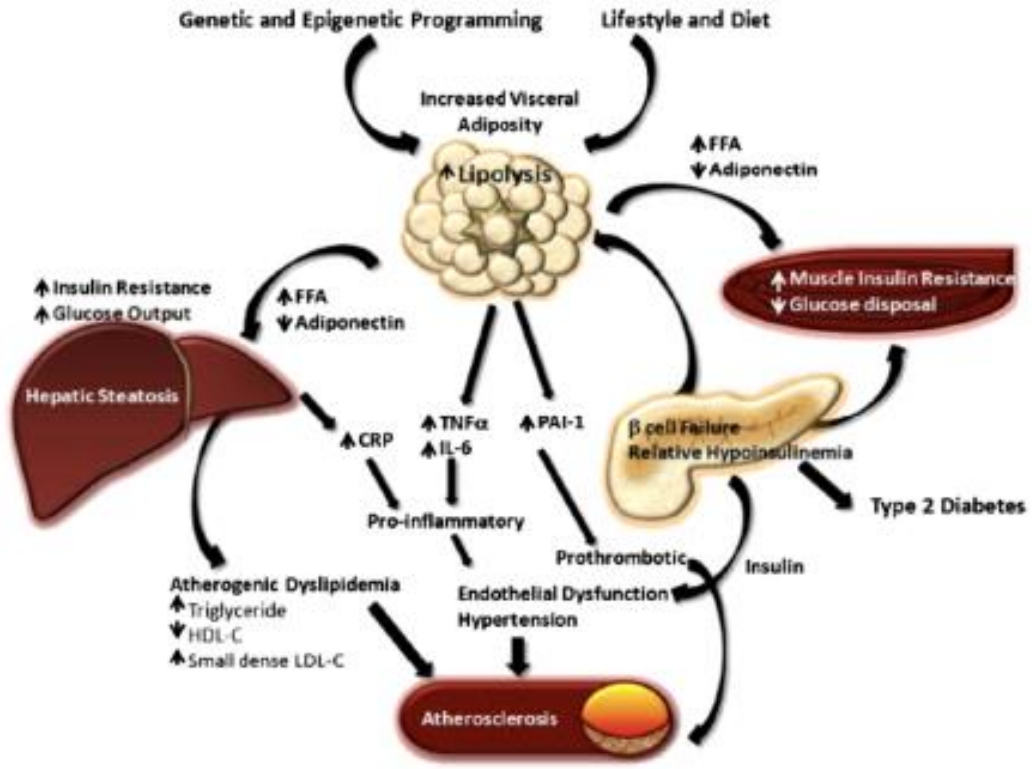

Figure 6. Proposed mechanism for the clustering of MS traits and the increased risk of T2DM in CVD. Reproduced from (74) with permissions from the American Academy of Pediatrics.

CRP, C-reactive protein; FFA, free fatty acids; IL-6, interleukin 6; LDL-C, low-density lipoprotein cholesterol; PAI-1, plasminogen activator inhibitor 1; TNF α, tumor necrosis factor α.

In normal conditions, insulin secretion begins at the level of pancreatic β cell; the hepatic insulin receptor is reached by the portal system where insulin induces two main gene transcriptions. The first action promoted by insulin in the liver is the phosphorylation of forkhead box protein O1 (FoxO1), which prevents it from entering the nucleus and thus diminishes the expression of genes required for gluconeogenesis leading to decreased glucose production (101). The other insulin action at the hepatic level is the activation of the transcription factor sterol regulatory element- binding protein (SREBP)-1c, which leads to fatty acid and triglyceride (TRG) biosynthesis and de novo lipogenesis (102). The triglycerides produced by this process are incorporated to apo-lipoprotein B (apoB) into very low-density lipoproteins (VLDL) and they are transported to peripheral tissues, endothelial cells, adipose or muscle tissues (103, 104). In the resistant state, insulin hepatic action is shifted from glucose suppression to increased free fatty acid (FFA) production. The increased production of FFA leads to dyslipidemia and ectopic adipose deposition from which the rest of the MS factors derive (74, 75).

Chapter 3: Cardiovascular Disease (CVD)

3.1 Definition of Cardiovascular Disease

CVD is the most common cause of mortality worldwide and not only in the rich countries, as evidenced recently by a rising incidence of CVD in the low-resource countries as well (105-107). The term CVD includes different conditions affecting the heart and blood vessels. The hallmark of these conditions is atherosclerosis, the build-up of fatty deposits inside the arteries, accompanied by increased inflammation and risk of blood clots (108). CVD includes damage to arteries and organs such as the brain, the heart, the kidneys and the eyes. The four main different clinical manifestations of CVD are coronary heart disease, strokes and transient ischaemic attack (TIAs), peripheral arterial disease and aortic disease.

Coronary heart disease occurs when the flow of oxygen-rich blood to the heart muscle is blocked or reduced. This condition increases the heart work and the strain effort and can lead to chest pain caused by restricted blood flow to the heart muscle (angina), or sudden block to the heart muscle blood flow (myocardial infarction or heart attack) and finally heart failure when the heart is not able to pump blood to the body in terms of maintaining normal vital organ function (108).

Strokes and TIAs represent a category of disorders where brain blood supply is permanently or transiently compromised, respectively (108).

Peripheral arterial disease is the blood flow compromise in the arteries that supply the limbs, more frequently the lower limbs (108).

Aortic disease afflicts the largest blood vessel in the human body, aorta, which supplies with blood the whole body and can be life threatening with uncontrolled bleeding in cases of ruptured or dissected aortic aneurysms (108).

The causes of CVD are multiple and include high blood pressure, high cholesterol, diabetes mellitus, overweight/obesity state, family history of CVD, ethnicity, lifestyle and mainly smoking, increased alcohol intake, unhealthy diet habits, physical inactivity, age over 50 and male gender.

Coronary heart disease is a chronic disease that indeed starts in childhood, even if the symptoms first occur in the middle age. Obesity is both an independent risk factor for CVD but is also closely related to several other risk factors.

3.2 Epidemiology of CVD

The global number of deaths from CVD has increased during the past decade by 12.5%; this may partially be explained by the increase of aging populations mostly in South and East Asia (109). CVD is responsible for approximately one third of all deaths worldwide. Importantly, age-specific death rates have actually fallen by 15.6% between 2005 and 2015 although recent data suggest that this rate of decline has been slowing and occurring most frequently in the higher income countries (110, 111).

A detailed analysis of the 10 predominant risk factors for myocardial infarction and stroke, that account for the 90% of the population attributable risk, is shown in Table 2.

Table 2. Predominant Risk Factors for Myocardial Infarction and Stroke Based on the INTERHEART and INTERSTROKE Studies. Reproduced from (112) with permissions from Wolters Kluwer Health, Inc.

	Myocardial Infarction			Stroke		
	Rank	Odds Ratio	PAR	Rank	Odds Ratio	PAR
ApoB/ApoA1 ratio	1	3.25	49.2	3	1.84	26.8
Smoking	2	2.87	35.7	7	1.67	12.4
Psychosocial factors*	3	2.67	32.5	6	2.20	17.4
Abdominal obesity	4	1.62	20.1	5	1.44	18.6
Self-report of hypertension	5	1.91	17.9	1	2.98	47.9
Healthy diet†	6	0.70	13.7	4	0.60	23.2
Physical activity	7	0.86	12.2	2	0.60	35.8
Self-report of diabetes mellitus	8	2.37	9.9	10	1.16	3.9
Regular alcohol consumption	9	0.91	6.7	9	2.09	5.8
Cardiac causes	NA	NA	NA	8	3.17	9.9

Apo indicates apolipoprotein; NA, not applicable; and PAR, population attributable risk.
*Only partly confirmed in prospective studies.
†Dietary measure in INTERHEART was daily fruit and vegetable consumption and in INTERSTROKE was measured using the modified Alternative Healthy Eating Index.

3.3 Prevention Strategies

The global action plan encompasses 9 global targets aimed at non-communicable diseases and risk factor control (112). The prevention and reduction of CVD burden is a primary target for the future worldwide. The 9 global targets are the following:

1. 25% relative reduction in the overall mortality from CVD, cancer, diabetes mellitus, or chronic respiratory diseases
2. At least 10% relative reduction in the harmful use of alcohol, as appropriate, within the national context
3. A 10% relative reduction in prevalence of insufficient physical activity
4. A 30% relative reduction in mean population intake of salt/sodium
5. A 30% relative reduction in prevalence of current tobacco use in persons aged 15+ y
6. A 25% relative reduction in the prevalence of raised blood pressure or contain the prevalence of raised blood pressure, according to national circumstances
7. Halt the rise in diabetes mellitus and obesity
8. Receive drug therapy and counseling (including glycemic control) to prevent heart attacks and strokes
9. An 80% availability of the affordable basic technologies and essential medicines in both public and private facilities.

The 9 global targets as released by the Global Action Plan clearly state the necessity of halting obesity and also address the consideration of acting in early ages and in adolescents.

Chapter 4: Newer Biomarkers of Metabolic Disorders

4.1 Leptin

Satiety and appetite are mainly regulated by the two hormones leptin and ghrelin (113). Ghrelin is a 28-amino acid lipophilic peptide initially found to be expressed in specialized enterochromaffin cells located in the mucosa of the stomach fundus (114). Ghrelin in an orexigenic hormone, secreted by the stomach, stimulates hunger, and also promotes carbohydrate metabolism while decreases lipid metabolism. This molecule also increases gastric secretion and it is shown in humans to reach peak levels before each meal and fall to lower levels immediately after meal consumption (114-116). Leptin is a 146 amino acids glycosylated protein produced predominantly by adipose tissue, although low levels of expression were also detected in the hypothalamus, pituitary, placenta, skeletal muscle, and gastric and mammary epithelia (117, 118). Conversely to ghrelin, leptin is anorexigenic, thus responsible for the regulation and modulation of satiety (119). Leptin levels in the circulation are increased proportionally to fat mass, and represents a mediator of signals from periphery to the hypothalamus regarding the amount of energy stored in adipose tissue, suppressing appetite and affecting energy expenditure (119). However, even though leptin was shown to be effective in decreasing weight end restoring energy homeostasis when administered in leptin deficient obese children, it failed to reproduce these effects and this is possibly explained by an existing phenomenon of resistance to this hormone (120-123).

Leptin shares common actions with insulin through signaling in the arcuate nucleus of hypothalamus promoting reduction of food intake and decrease in body weight (124, 125). The two hormones differentiate at level of secreting source, pancreas secrets insulin and adipose tissue secrets leptin. The two hormones link central regulation to different periphery signals. Due to this different producing source, insulin receives stimuli directly from meals that promote its secretion whereas leptin is not directly regulated by food intake, however persistent hyperinsulinemia may up-regulate leptin circulating levels.

4.2 Adiponectin

Adiponectin is a 244-amino acid protein expressed and secreted from white adipose tissue and it is an insulin-sensitizing hormone acting by increasing tissue fat oxidation which in turn reduces circulating fatty acid levels and intracellular triglyceride contents in liver and muscle (126). The circulating blood levels of adiponectin have been reported to be negatively regulated in states of obesity and T2DM (126). Adiponectin also acts on vascular endothelial cells by down-regulating the expression of adhesion molecules and cytokine production from macrophages, thus inhibiting the inflammatory processes that occur during the early phases of atherosclerosis (127). In adults, adiponectin was shown to be negatively correlated with body weight, body fat mass and insulin levels and it was proposed to be a marker of MS and cardiovascular risk as assessed by cIMT in young adults (127, 128).

In childhood obesity, the serum levels of adiponectin are only slightly down-regulated, with significant decrease described only in extreme obesity for this age group (129). In children, adiponectin serum levels have also been reported to be inversely correlated with anthropometric parameters of obesity and insulin resistance and positively correlated with HDL cholesterol levels. In the same study, signs for early endothelial damages were assessed by FMD and cIMT and no relationship was found between those vascular indices and adiponectin (130).

Figure 7 describes the known neurohormonal mechanisms that mediate cross-talk between brain and periphery in order to control food intake (131). The role of adiponectine and leptin mediating signals from periphery in to the central nervous system may be observed. Other molecules described in this Figure do not constitute the object of this thesis.

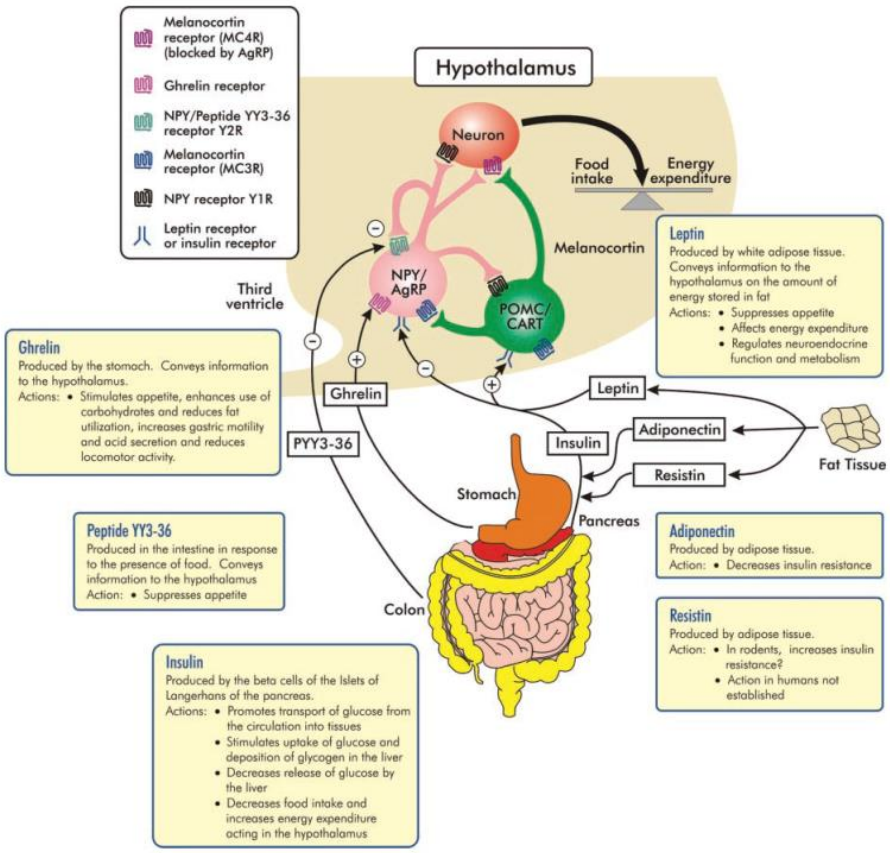

Figure 7. The link between the periphery and the brain: endocrine and neuronal interaction in the regulation of energy homeostasis and appetite. Reproduced from (131) with permissions from Oxford University Press.

4.3 Insulin-like Growth Factor-binding Protein 1 (IGFBP1)

Insulin-like growth factor binding proteins (IGFBPs) comprise a family of proteins that bind to insulin growth factors and modulate their bioactivity (132). IGFBP1 is a 30 kDa circulating protein expressed predominantly in the liver that has been implicated in reproductive physiology and metabolic homeostasis (133, 134). Increased IGFBP1 is seen in the serum of patients with T1DM and it was suggested that this can be attributed to insulin deficiency. In contrast, patients with T2DM express low levels of this protein (135). Studies of adults and children have shown that circulating IGFBP1 concentrations positively correlate with insulin sensitivity (136-139). It has been also suggested, as observed in longitudinal studies, that low circulating IGFBP1 concentrations predict the development of glucose intolerance and diabetes (140, 141). The concentrations of IGFBP1 are also inversely associated with cardiovascular risk, increased cIMT indicating thickening of carotid wall in T2DM (142). Low levels of circulating IGFBP1 are closely related with macrovascular disease and hypertension, both risk factors for diabetes while high circulating concentrations of highly phosphorylated IGFBP-1 may protect against the development of hypertension and CVD by reducing the mitogenic potential of IGFs on the vasculature (136, 143).

Chapter 5: Fibroblast Growth Factor 21 (FGF21)

5.1 FGF21 Expression

FGF21 is a protein, identified in 2000 in mouse embryos and in human cDNA, member of the vast family of 22 fibroblast growth factors, specifically assigned to the sub-family 19 comprised by fibroblast growth factor 19, 21 and 23 in humans (144, 145). The members of this sub-family have endocrine action as they are secreted in the peripheral blood circulation contrary to what happens to the rest of the fibroblast growth factors which have paracrine action (144). FGF21 is principally expressed in pancreas, testis, liver, brown and white adipose tissue and in lower amounts in other tissues including the aorta, it is released in the circulation mainly by the liver and acts through FGF receptors (FGFR) 1c and 3c and the cofactor βKlotho in target tissues such as the liver, pancreas, adipose tissue and endothelial cells (144, 146). This molecule is shown to follow a circadian secretion pattern with peak circulating levels at 2:30a.m. and nadir circulating levels at 8:30 a.m. (147). FGF21 has extended actions at tissue level and its role has been explored by multiple in vitro and in vivo studies.

Figure 8 describes major anatomical expression sites of FGF21 in mice and humans (148).

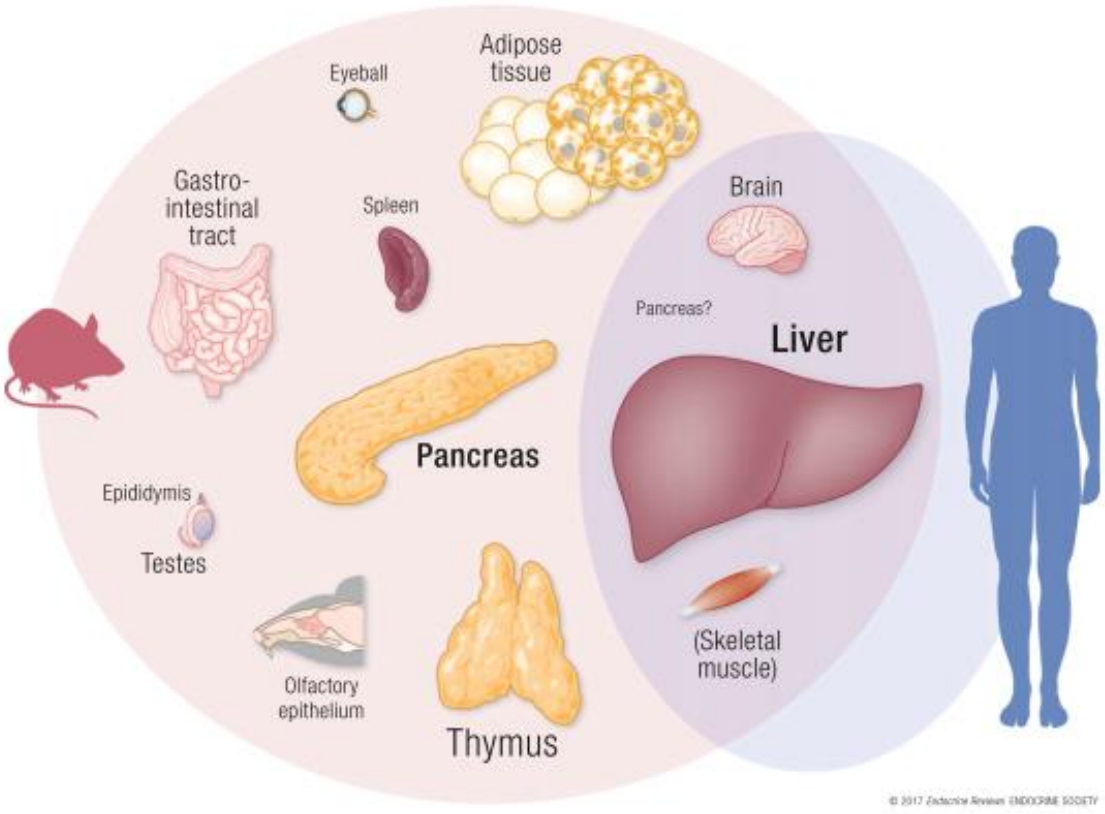

Figure 8. Comparison of major anatomical expression sites of FGF21 in mice and humans. The most prominent expression sites are highlighted by using bold fonts. The intensity of expression is indicated by font/image size. Note that skeletal muscle FGF21 expression signals were found in mice already in the basal state, in humans only upon hyperinsulinemia. Reproduced from (148) with permissions from Oxford University Press.

5.2 FGF21 Regulation

5.2.1 Regulation of FGF21 by Nutrition State

5.2.1.1 Food Deprivation

Initially it was shown that the expression of FGF21 in hepatic tissue is up-regulated after prolonged fasting in animal models, and that this action is mediated by peroxisome proliferator-activated receptor alpha (PPARα) and by cyclic AMP responsive element-binding protein H (CREBH) (149-152). In fasting states, there is increased level of circulating free fatty acids (FFA) that are shown in mice to induce PPARα and in turn lead to augmented expression of FGF21 in the liver (150, 151). On the contrary, in humans, FFAs do not lead to increased FGF21 levels but acutely diminish the circulating levels of this molecule when performing oral lipid load tests (153, 154). As shown in humans, prolonged fasting or starvation also lead to increased FGF21 circulating levels as an adaptive response (155, 156).

In pancreatic tissue, fasting induces down-regulation of FGF21 as demonstrated in mouse models. However, the expression of FGF21 in pancreatic tissue does not influence circulating levels of FGF21 as this form derives mainly from hepatic release (157). In pancreatic tissue, FGF21 is expressed mainly at the acinar part and less in the islets, and acts with paracrine manner protecting pancreas from hyperplasia and inflammation, as shown in FGF21 knock-out animal studies (157, 158).

In mouse models, FGF21 expression is also shown to be up-regulated in adipose tissue and in skeletal muscle by fasting, while it is also up-regulated in adipose tissue in the obese state (159-161). The increased expression of FGF21 in adipose tissue in the obese state is also confirmed in humans (162).

5.2.1.2. Body Mass Index (BMI) States -Nutritional State and Diets

In states of increased BMI, in genetically and diet induced obesity mouse models, FGF21 was found to be increased in peripheral blood, accompanied by respective increase in hepatic and adipose gene expression (161). In humans, obesity is linked to an increase in circulating serum levels of FGF21. In 2008, in a study of 232 Chinese subjects, it was shown that FGF21 serum levels were increased in the overweight/obese in comparison to the lean individuals and that were positively correlated to adiposity, fasting insulin, and triglycerides but negatively with HDL cholesterol, after adjusting for age and BMI (163).

It has also been demonstrated that FGF21 hepatic expression is induced through PPARα induction in newborns as a response to milk intake, further contributing to thermogenic activation of BAT (164). Consequently, it was observed that FGF21 expression was also induced when ketogenic or amino-acid deficient and low protein diets were administered in mice and this induction was also PPARα dependent (149, 165). When FGF21 was disrupted in mouse models, the response in ketogenic diet (KD) was also impaired indicating the significance of the presence of this molecule (166). KD studies in humans failed to prove agreement with mouse experimental studies and led to the conclusion that in humans, KD does not influence FGF21 expression. In a study in 76 healthy individuals, ketosis was induced by a 2-day fast or feeding a KD and this did not influence FGF21 levels (155). Importantly, when the same individuals were put on 7-day fasting, a 74% increase in the FGF21 levels occurred (155). In another study in normal and overweight obese humans, KD was consumed for 12 days and neither this nor a short fasting period altered their circulating FGF21 levels (167). When obese individuals undertook a KD for a longer period, i.e. 3 months, a decrease in FGF21 blood levels was observed (168). It is proposed that the discrepancy between mouse and human studies of the KD regulatory effect on FGF21 may in part be explained by the large variability in KD diets in humans while the murine diets are laboratory made and better controlled at the material and molecular level (148).

Hepatic FGF21 expression was further shown to be regulated in mouse and human models when fructose was administered (169, 170). Specifically, in murine knock-out models it was shown that disruption of the transcription factor carbohydrate responsive-element binding protein (ChREBP) led to inability of FGF21 regulation after fructose gavage, intact in the wild type animals, indicating a signaling axis implementing these molecules (170). In humans, a fructose load of 75gr was administered following baseline blood examinations and expression of FGF21 was monitored following a curve similar to an oral glucose tolerance test. A robust increase in FGF21 serum levels was reported in this study, following a pattern consistent to a hormonal response suggesting that it may play an important role in fructose metabolism (169). This was further confirmed in humans consuming diets with excessive content of carbohydrates, but not fat (171).

When 40 healthy sedentary individuals were put on a high fat overfeeding diet regimen for 28 days, it was shown that FGF21 and adiponectin, both considered protective hepatokines, were increased in the serum (172). Specifically FGF21 and adiponectin serum levels were increased on the 3rd day of the diet, while adiponectin levels were also found increased on the 28th day of the diet (172). The authors speculated that these findings reflect a possible role of these molecules in maintaining glucose homeostasis in a state of nutritional excess (172). In a 7-week, double-blind study of 39 healthy, lean individuals of a mean age of 27 years, 750 kcal/day were added to their habitual diets, (either containing polyunsaturated fatty acids (PUFA) or saturated fatty acids (SFA)) (173). Circulating FGF21 was found to be increased coupled to the modest weight gain and the increased markers of endothelial dysfunction and insulin resistance observed in these individuals (**Figure 9**) (173). In this study, no relevant relation was shown between FGF21 levels and the qualitatively different fatty acid consumed.

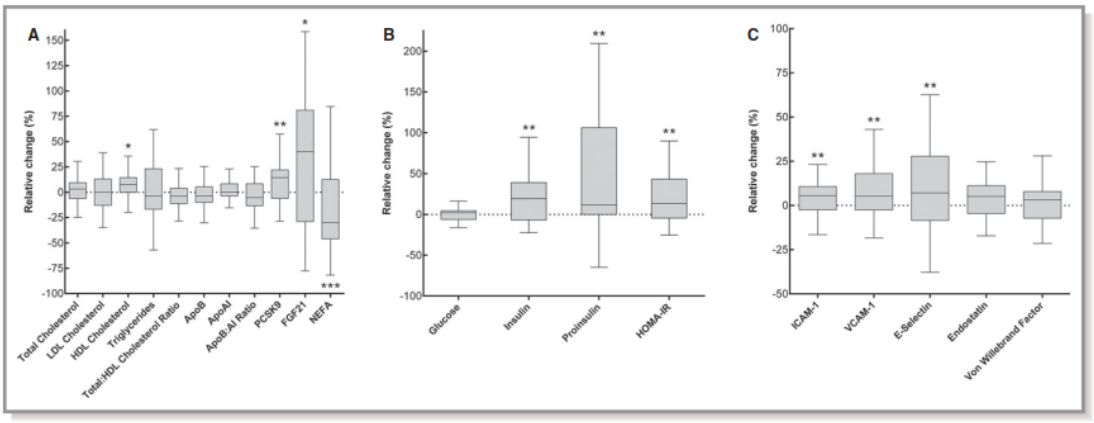

Figure 9. Relative changes during weight gain in the whole study sample (n39). A, Lipid metabolism. B, Glucose and insulin. C, Endothelial function and coagulation. Calculated as change/baseline value x 100. Boxes denote median and IQR, whiskers denote highest/lowest value within 1.5 IQR from upper/lower quartile. * P<0.05, ** P<0.01, ***P≤0.001 in paired-sample t test or Wilcoxon signed-rank test. ApoB indicates apolipoprotein B; FGF21, fibroblast growth factor 21; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; ICAM-1, intercellular adhesion molecule-1; IQR, interquartile range; LDL, low-density lipoprotein; NEFA, nonesterified fatty acids; PCSK9, proprotein convertase subtlilisin/kexin type 9; VCAM-1, vascular cell adhesion molecule-1. Reproduced from (173) under the terms of the Creative Commons Attribution Non Commercial License by publisher Wiley Blackwell

The Geometric Framework for Nutrition (GFN) is an integrating framework for taming the complexity of nutrition. It can be successfully applied to quantify simultaneous impacts of different macronutrients on energy expenditure and other vital activities of the living organisms consuming them (174). FGF21 is shown to be paradoxically regulated by various nutritional states. The expression of this molecule is up-regulated in conditions of starvation, in KD and in protein/amino acid restriction regimens while unexpectedly is also up-regulated in states of overfeeding and in obesity. In 2016, a study in Australia used the GFN to determine the role of different macronutrients and energy intake on FGF21 expression in animal models. In this study, it was demonstrated that FGF21 expression and circulation were increased in low protein diets and maximal when low protein was coupled with high carbohydrate intakes (175). By phenotypic determination of animals expressing high levels of FGF21, the presence of two contrasting phenotypes was detected. The one phenotype was hyperphagic on low protein diets showing compensatory overfeeding that led the animals to increase their

body fat, remaining however insulin sensitive , while the other phenotype was lean, energy restricted, and insulin sensitive. These results led to the suggestion that up-regulation of FGF21 may occur by both starvation and overfeeding and this may be mainly dependent on nutrient content linked to low protein intake and to a lesser extent to low protein high-carbohydrate intake ratio (**Figure 10**) (175).

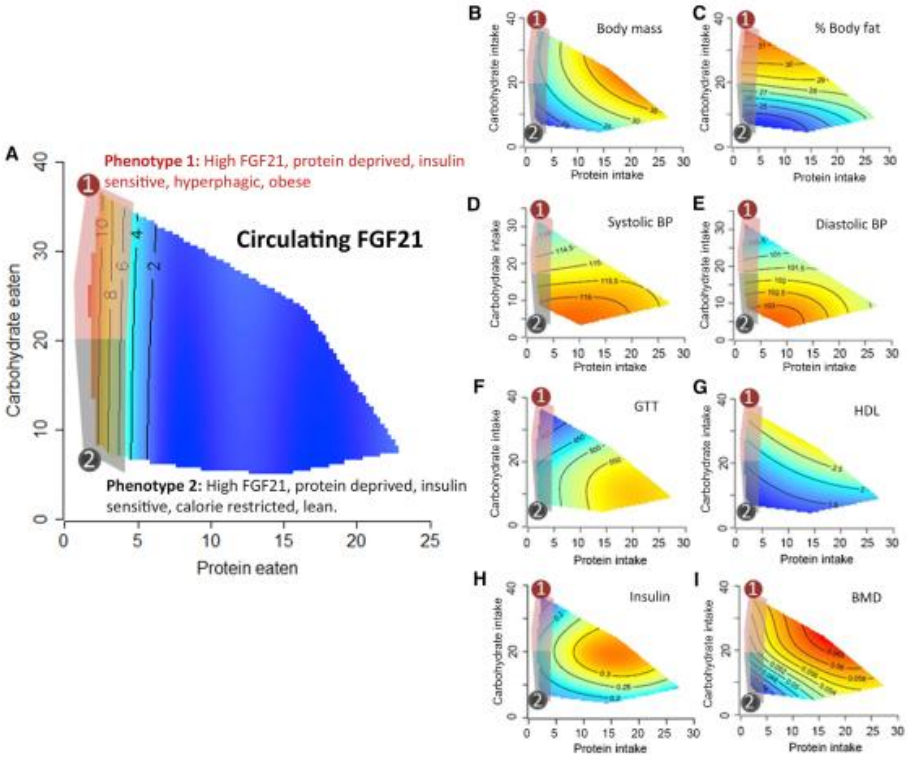

Figure 10. Elevated Circulating FGF21 Levels Are Associated with Various Metabolic Phenotypes

FGF21 levels were robustly elevated by low-protein diets, but the nutritional context in which this occurred resulted in different metabolic phenotypes. (A) Two contrasting phenotypes are superimposed onto a response surface for circulating FGF21 levels. These metabolic outcomes were previously published in Solon-Biet et al (2014). Phenotypes 1 and 2 both have high FGF21 levels, are protein deprived, and are insulin sensitive. However, phenotype 1 is hyperphagic due to compensatory for protein, resulting obesity. Phenotype 2 is calorie restricted and lean.
(B) The effects of protein and carbohydrate intakes on (B) body mass (g). (C) percent body fat. (D) systolic blood pressure (mmHg). (E) diastolic blood pressure (mmHg). (F) area under the curve from glucose tolerance test. (G) HDL (mmol/L). (H) insulin (ng/ml). And (I) bone mineral density (g/cml^2). It is clear that the two high-FGF21 phenotypic conditions are different in their metabolic profiles. Adapted from Solon-Biet et al (2014). Reproduced from (175) with permissions from Elsevier.

In the complex regulatory pathway between nutrient intake and regulation of energy homeostasis, FGF21 regulation has been proposed to drive negative signals in a feedback pathway to the brain. Liver may regulate sugar consumption through FGF21 expression and secretion, involved in this feedback pathway that in turn signals negatively for the further sugar consumption in conditions of high intake. This pathway is proposed to be located in hypothalamus (**Figure 11**) (176).

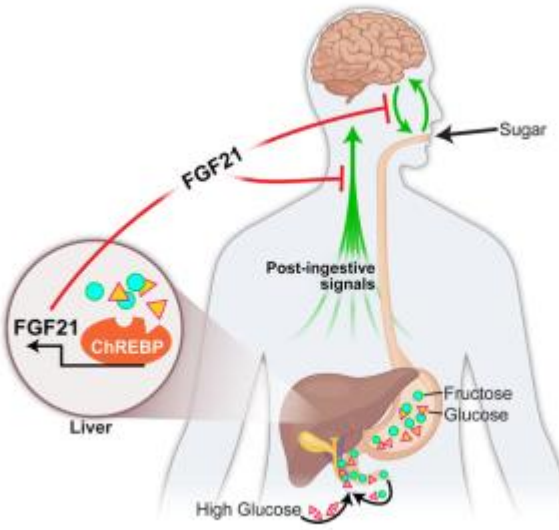

Figure 11. FGF21 mediates endocrine control of sugar intake by the liver. Reproduced from (176) with permissions from Elsevier.

This regulatory pathway extends to the adipose tissue by activation of adenosine monophosphate-activated protein kinase (AMPK) and sirtuin 1 gene (SIRT1) promoted by FGF21 (177). AMPK and SIRT1 constitute two out the four known nutrient related pathways controlling energy homeostasis together with mammalian target of rapamycin (mTOR) and insulin/IGF1 (175).

5.2.2 Regulation of FGF21 in Different Metabolic Disorders

5.2.2.1 Metabolic Syndrome

The expression of FGF21 is described to be increased in various metabolic disorders in adults. MS is independently associated with FGF21 serum levels (163, 178). The Metabolic Syndrome Berlin Potsdam (MeSyBePo) study in 2013, recruited 440 Caucasian individuals who were evaluated both at the beginning of the study and at a follow-up evaluation 5 years later. FGF21 was shown to be an independent predictor of the MS and T2DM in apparently healthy adults (179).

5.2.2.2 Non-alcoholic Fatty Liver Disease (NAFLD) and Non-alcoholic Steatohepatitis (NASH)

Positive correlation between FGF21 serum levels and obesity was also described in obese individuals with NAFLD or NASH (167). In this study, both FGF21 serum levels and hepatic mRNA expression were positively correlated with NAFLD (167). When obese humans were further discriminated into metabolically healthy (MHO) and metabolically unhealthy (MUO), based on the absence or presence respectively of concomitant insulin resistance, central obesity and hepatic steatosis, it was demonstrated that FGF21 was higher in the MUO group (180, 181).

5.2.2.3 Insulin Resistance and Type 2 Diabetes Mellitus (T2DM)

In states of insulin resistance and T2DM FGF21 is up-regulated.

In a study of obese people, obese with T2DM and healthy people it was shown that baseline FGF21 serum levels were increased in obese and obese with T2DM individuals in comparison to healthy individuals while no significant difference was shown between the obese and obese with T2DM groups (162). When the peroxisome proliferator activated receptor alpha (PPARα) agonist, fenofibrate, was administered for 3 months, or a very low calorie diet was applied for 3 consecutive weeks the levels of FGF21 in serum, increased indicating that it may promote the actions of these interventions (162). Six fold higher circulating serum FGF21 levels were observed in Japanese subjects with T2DM treated with fenofibrates (182). In contrast, when thiazolidinediones were administered, these did not affect FGF21 serum levels while they up-regulated adiponectin serum levels (182). In a different study, subjects were divided in newly diagnosed T2DM (NDDM) and with long duration of T2DM (LDDM) and in comparison to the controls, they had higher serum levels of FGF21 independent of disease duration (183). Analysis of data from the Baltimore Longitudinal Study of Aging with 700 adults of a mean age of 63.3 years participating, showed that higher serum FGF21 concentrations were associated with abnormal glucose metabolism and insulin resistance (184).

5.2.3 Other Conditions that Regulate the Expression of FGF21

5.2.3.1 Regulation of FGF21 in States of Stress

Cold and exercise are conditions in which FGF21 expression has been studied and is described to be up-regulated but in different ways and in different tissues in each condition.

With physical exercise, hepatic FGF21 gene expression is increased and the mechanism by which this is mediated may be linked to increased hepatic PPARα and activating transcription factor 4 (ATF4) expression which implies an indirect mechanism through lipolysis activation pathway and FFA release at the adipose tissue level (185). Further studies in humans aimed to elucidate the mechanism by which exercise regulates FGF21 expression and it was shown that during exercise, there is a dynamic glucagon to insulin ratio which inhibits FGF21 secretion when it is low and promotes FGF21 secretion when it is increased (186). This scheme is consistent with the concept that during exercise the body undergoes acute energy deprivation and under such conditions circulating glucagon is increased while insulin secretion is inhibited to maintain glucose homeostasis (187).

In cold exposure, FGF21 expression is up-regulated in humans and in mice at the level of the adipose tissue and not in the liver as it happens during exercise (188). On the basis of this evidence, FGF21 is proposed to participate to the thermogenic pathway in which within BAT mechanisms are activated after cold exposure. This is a cAMP-mediated beta-adrenergic pathway where protein kinase A and p38 MAPK, induces FGF21 gene transcription in BAT (189). In turn, FGF21 up-regulates brown and white adipose tissue expression of uncoupling protein 1 (UCP1) promoting thermogenesis by a mechanism of shifting white to brown adipose tissue in mouse experiments (188).

Figure 12 reviews the regulation of FGF21 in hepatic tissue as described in mice and human studies (148).

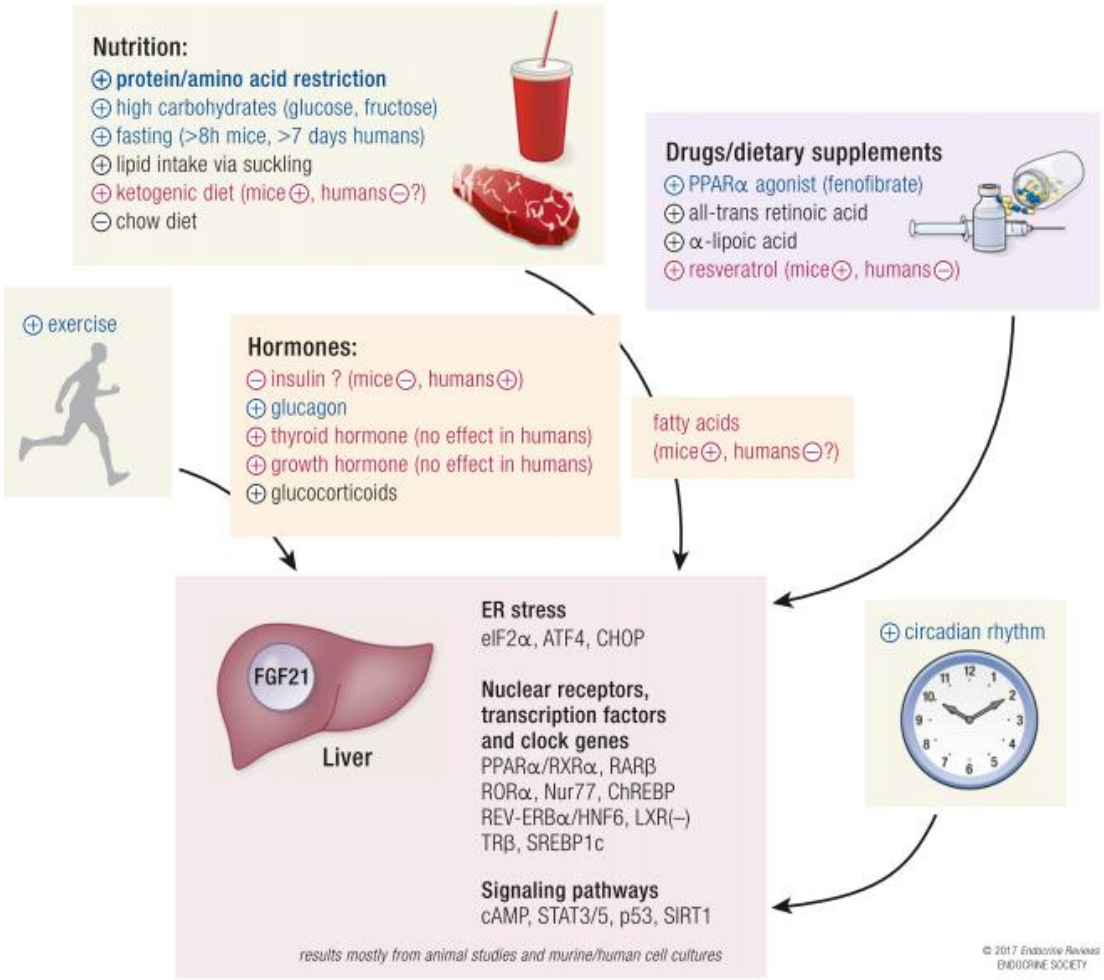

Figure 12: **Regulation of hepatic FGF21 production in mice and humans.**
Stimulatory effects are indicated by plus signs (+), the inhibitory effects by minus signs (-). Stimuli/mediators in mice and humans are indicated in blue, stimuli/mediatros that are different between mice and humans are indicated in red, and stimuli/mediators with only mice data available are indicated in black. Reproduced from (148) with permissions from Oxford University Press.

5.2.3.2 Regulation of FGF21 in Diseases

FGF21 is a molecule, as mentioned, expressed in various tissues and consequently it has been studied in respect to various tissues and organs conditions. Such studies on muscle tissue in mice indicate the influence on this molecule regulation during mitochondrial myopathy and during heart muscle damage after fasting and endoplasmic reticulum stress with accompanied increased expression of FGF21 in the skeletal muscle (190, 191). The underlying mechanism may be linked to defective lipolysis with FGF21 overexpression indicating the effort to increase energy homeostasis (191). Similarly, in human myopathies associated to enzyme cofactor deficiencies such as iron–sulfur cluster scaffold homolog (ISCU) myopathy, by global changes in gene expression, microarray analysis from muscle biopsies, an overexpression of FGF21 gene was shown, interestingly accompanied to increased circulating levels of this molecule. Concomitant increase of the ketogenic enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) indicates activation of a pathway possibly leading to an effort to restore capacity of channeling debris of mitochondrial dysfunction. This pathway is closely linked to conditions such as starvation (192).

Mechanistic insight came recently from a study showing that the increase in FGF21 leads to beneficial effects by enhancing mitochondrial function through an mTOR-Yin Yang 1 (YY1)-peroxisome proliferator activated receptor coactivator 1alpha (PGC1alpha-dependent pathway in skeletal muscle (193). Further human studies on mitochondrial disorders confirmed the usefulness of this molecule as it consistently shows an up-regulation possibly indicating that FGF21 may constitute a new biomarker in adults and children in the screening process for muscle biopsy in addition to other classical serum indicators such as creatine kinase, lactate, pyruvate, and the lactate to pyruvate ratio (194, 195).

Figure 13 reviews the regulation of FGF21 in extra-hepatic tissues as described in mice and human studies (148).

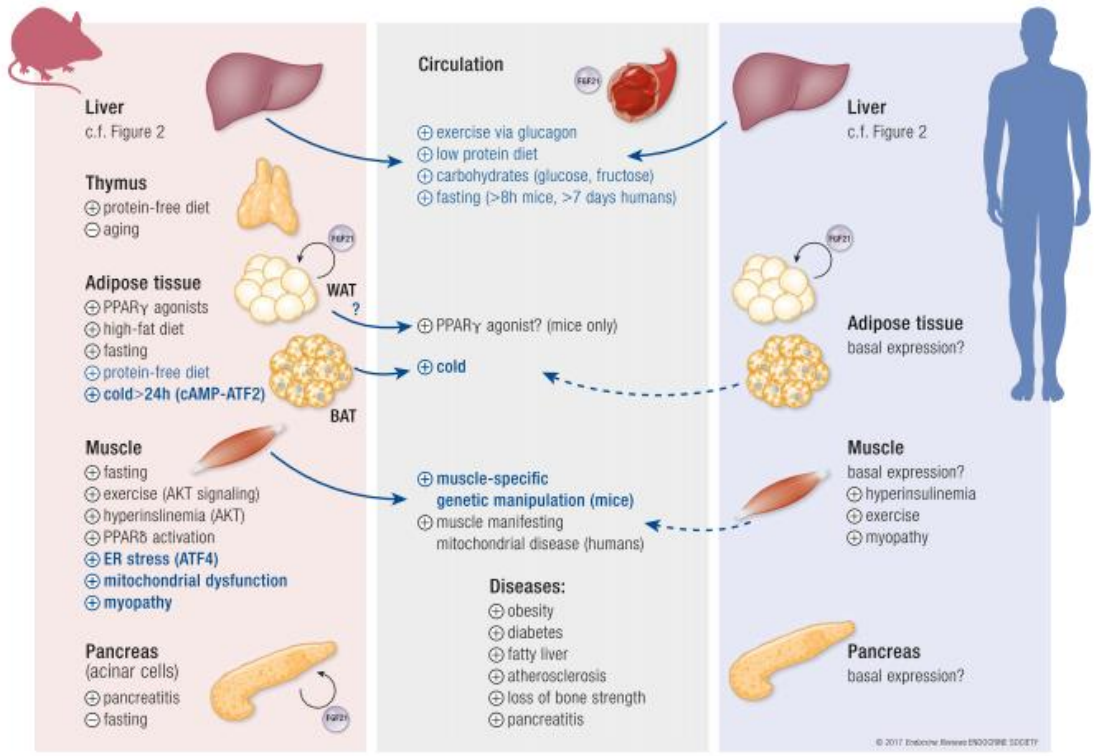

Figure 13. Regulation of extrahepatic production and circulating FGF21 in mice and humans. Stimulatory effects are indicated by plus signs (+), the inhibitory effects by a minus sign (-). Stimuli rendering extrahepatic tissues as a source of circulating FGF21 levels are indicated in blue. Reproduced from (148) with permissions form Oxford University Press.

5.2.3 Regulation of FGF21 in Children

FGF21 has multiple metabolic actions and it has been intensively studied in adults. However, there are not many studies describing the role of this molecule in children. In 2012, 60 obese and 40 lean children of the same age, gender, and pubertal stage were enrolled in a 1-year interventional study (196). The study included assessment of body weight and fat mass, lipid profile, insulin homeostasis, and FGF21 and leptin serum levels. For a year they followed a program based on exercise, behavior and nutrition therapy. In this study, FGF21 was shown to be increased in the serum of obese children accompanied by increased leptin and HOMA levels and it was further shown that FGF21 serum levels correlated with BMI, free fatty acids and leptin levels in both cross sectional and longitudinal analysis. In this study, there was no difference in FGF21 levels between children with MS (defined by the IDF criteria) or with NAFLD in comparison to the controls. Importantly, a decrease in BMI in the interventional study was accompanied with a significant decrease in serum FGF21 levels (196). The BCAMS study in Chinese children, reported an association of FGF21 with obesity with an unexpected decrease in FGF21 circulating levels in the obese group when compared to lean children (197). In this study, a strong association between circulating FGF21 levels, insulin resistance and MS was observed (197). The authors stated in the limitations of the study that the kit used for the quantification of FGF21 serum levels was different from the one used by the other published studies. Further information on FGF21 in paediatric MS was provided in 2014, in a study of 9-year old children. In this study, the paediatric MS was defined by the National Cholesterol Education Program's Adult Treatment Panel III criteria and no significant association were noted between FGF21 and any defining parameters of the MS (198).

In a different study in 2016, obese adolescents with and without T2DM were recruited and it was reported that in the absence of difference in BMI between the two groups there was significant increase in FGF21 serum levels in the obese group with T2DM (199). In this study, FGF21 correlated to hs-CRP and leptin with TNF-α. Interestingly, leptin levels were shown to be decreased in the obese adolescents with T2DM. The

authors of these studies have suggested that T2DM is a resistance state for FGF21 and they also have argued against leptin resistance in T2DM since the levels of this molecule were decreased in their group of adolescents (200, 201). They agreed on the fact that both FGF21 and leptin are protective in terms of insulin resistance since they improve it (200). They also reported on the role of adiponectin in T2DM and supported that it is not related to T2DM in obese adolescents since the circulating levels of this molecule are not different in these children when compared to obese adolescents without T2DM indicating a strong link to the BMI state (201).

In 2014, based on data from the Copenhagen Puberty Study, the relation of oral glucose tolerance, insulin, FGF21, adiponectin, and leptin to body fat percentage as measured by dual energy X-ray absorptiometry (DXA) scans was explored (202). The results of this study showed that girls had a significantly higher level of FGF21 compared with boys. The baseline levels of FGF21 positively correlated with TRG, but not with the BMI, fat percentage, LDL, HDL or total cholesterol, leptin, or adiponectin. There was no correlation between the baseline FGF21 levels and dynamic changes in glucose and insulin resulted by the glucose tolerance test. The authors concluded that FGF21 is independent of adiposity in children and its metabolic effect seems to be limited to pathological conditions associated with insulin resistance (202). In contrast to this study, in 2013 in obese adolescents it was shown that the increase in circulating FGF21 levels positively correlated to hepatic fat content and markers of hepatic apoptosis (203). In 2018, the BCAMS study in Chinese obese children has shown that the metabolic health is related to circulating adipokine profile and thus circulating osteonectin, leptin, FGF21, adiponectin, resistin and retinol binding protein 4 (204). The metabolic health was defined as the absence of insulin resistance and/or any metabolic syndrome components (204).

5.3 FGF21 actions

FGF21 is expressed by different tissues in different conditions mostly linked by energy homeostasis and stress. This expression is not only a passive phenomenon, but FGF21 has been shown to have potentials in promoting other gene expressions and initiating metabolic pathways in order to maintain energy balance. The most important known effects of FGF21 have been described on metabolic pathways of glucose homeostasis and lipid balance through hepatic, adipose and pancreatic actions, but it is also described to have limited actions in brain and bone tissue. FGF21 appears as a prominent thermogenic, anti-hyperglycemic and anti-hyperlipidemic factor (205).

5.3.1 Effects on Glucose and Lipid Homeostasis in Humans and in Mice

Initially loss and gain action experiments were designed to explore the effects of FGF21. When FGF21 was genetically deprived in FGF21 knockout animal models with concomitant daily administration of KD, the induction of glucose intolerance and the increase in body weight combined to histologically proved hepatic steatosis was observed (166).

Administration of FGF21 was initially designed in-vitro to 3T3-L1 and primary human adipocytes, and it was described to have effects on glucose molecule by inducing increased glucose uptake (206). Loss of FGF21 action experiments and exogenous administration of FGF21 in in-vitro studies have shown a specific action of FGF21 versus stimulation of hepatic fatty acid oxidation and ketogenesis in the fasting state, with suppression of hepatic de novo lipogenesis (150, 166). These actions were described to be possibly mediated by (SREBP1c) in human liver-derived HepG2 cells (207).

In vivo experiments of FGF21 administration in leptin deficient (db/db) and genetically induced obese (ob/ob) mice, both models of diabetes, have shown that FGF21 acts as a metabolic regulator reducing plasma glucose and triglycerides to near normal levels, while transgenic animals overexpressing FGF21 were similar to the control animals

(206). Furthermore, in two different models of insulin resistance, genetically determined obese ob/ob mice and diet induced obese (DIO) mice, FGF21 acute administration declined blood glucose levels and immediately improved insulin sensitivity (208). These effects were mediated in liver and adipose tissue by inhibiting glucose release from liver and stimulating glucose uptake in adipose tissue (208). The effects of FGF21 in adipose tissue are, at least partially, extracellular signal-regulated kinase (ERK) and serum response factor (SRF)/Elk1-mediated; this specific pathway has been shown to be impaired in DIO mouse models suggesting the strong interaction with the diet (209). As expected, when FGF21 was chronically administered in high fat diet mice, the hepatic and the peripheral insulin sensitivity improved (210). The actions of FGF21 on hepatic tissue are not clear. However, there is evidence that FGF21 cholesterol lowering effect is via a direct action on hepatic tissue through βKlotho and FGFR2, which is the second most expressed FGFR in the liver (211, 212).

To test whether adipose tissue mediates the effects of FGF21, this was administered to mice with reduced body fat in a lipodystrophy model; mice were found to be refractory to the beneficial effects of this molecule as compared to the control animals. To further assess the importance of adipose tissue in mediating the beneficial effects of exogenously administered FGF21, white adipose tissue was transplanted to the lipodystrophic mice and a complete restoration of the effects was recorded in these animals (213). There is conflicting data on how FGF21 may act on adipose tissue and lipid metabolism. There is possibly conditioning based on the tissue expansion, body weight as well as the duration of FGF21 administration. Obesity is a state where circulating FGF21 is found to be increased, although there is evidence that this elevation is incapable to promote weight lowering or improve lipid and glucose homeostasis. In DIO mice, acute administration of low dose FGF21 could not induce metabolic response confirmed also by reduced ERK1/2 phosphorylation in both liver and adipose tissue. This condition was described as a state of resistance (161).

In terms of lipid regulation, FGF21 administration both in vitro and in vivo, acutely reduces levels of circulating FFA consistently and dose dependently to a reduction of glucose circulating levels and inhibits adipocyte lipolysis possibly through lipase

inhibition (214). Chronic administration in human adipocytes and in experiments including transgenic mouse models and exogenous administration of FGF21 in mice, have shown an induction of a lipolytic effect possibly by promoting pancreatic lipases in the liver evidenced by microarray analysis of hepatic tissue (150, 215). These lipases are normally expressed in very low levels in the liver and this finding was linked to an overall increased ketogenic effect of FGF21 observed in these experiments (150).

5.3.2 Obesity Reversal in Animals and Humans by FGF21

When FGF21 was administered for two weeks in genetically induced (ob/ob) obese and diabetic mice, it managed to reduce their adiposity and overall body weight by 20%. These effects were linked to increased energy expenditure with no observed increased activity of the animals and concomitant amelioration of insulin sensitivity and lipid profile (216). Further research in diet-induced models of mouse obesity and administration of FGF21 confirmed the observations mentioned above, while an influence of FGF21 on increased physical activity was also observed (217).

In humans, the most prominent effect produced by FGF21 analog (LY2405319) administration was a significant improvement of dyslipidemia, including a decrease in LDL cholesterol and TRG and an increase in HDL cholesterol accompanied by a less atherogenic apolipoprotein concentration profile (218). Improved body weight with increased insulin sensitivity was also observed in this clinical trial although the glucose lowering effect was hardly detectable (218). Administration of a different FGF21 analogue (PF-05231023) in overweight/obese patients with T2DM led to a consequent significant decrease in body weight, improved plasma lipoprotein profile, and increased adiponectin levels (219). However, when the same analogue was administered in obese patients with and without T2DM, treated with atorvastatin for increased TRG, showed beneficial effects on lowering TRG but no effects were observed on body weight or glucose homeostasis (220).

5.3.3 Endothelial Protection by FGF21

In a study in a Chinese population, 253 patients were screened by coronary angiography for coronary artery disease, and by ultrasonography for NAFLD. The study showed that FGF21 serum levels were higher in patients with NAFLD and with coronary artery disease regardless of NAFLD state, and levels correlated with increased total cholesterol and TRG (221). Another study in China provided further insight showing that high levels of circulating FGF21 are associated with adverse lipid profiles in people with coronary artery disease and this may represent a compensatory response or resistance to FGF21 (222). To explore whether increased FGF21 serum levels are independently associated with atherosclerotic disease further studies were designed. A study in 670 Chinese subjects, assessing carotid atherosclerosis by ultrasonography carotid intima media thickness (cIMT) showed that FGF21 serum levels are associated with carotid atherosclerosis independent of established risk factors such as adverse lipid profiles and CRP (223). An effort to explore whether the association of FGF21 with atherosclerosis may involve a protective role or the opposite, studies on cultured cardiac microvascular endothelial cells (CMECs) were designed adding oxidized low density lipoprotein (ox-LDL). In this study, addition of ox-LDL induced apoptosis of CMECs and FGF21 mRNA and protein expression was increased in a dose-dependent injury-related manner (224). When bezafibrate was added to the culture, it further increased FGF21 expression and this in turn protected CMECS from apoptosis (224).

The protective effects of FGF21 on cardiac cell apoptosis were also shown in vivo, in a mouse model of diabetes where FGF21 prevented palmitate-induced cardiac apoptosis via up-regulating the ERK1/2-dependent p38 MAPK-AMPK signaling pathway (225). In the same study, it was shown that FGF21 knockout mice were more susceptible to diabetes-induced cardiac apoptosis, while the administration of FGF21 could prevent it (225). In streptozotocin-induced diabetes in FGF21 knockout mice, it was shown that FGF21 is protective with regards to cardiac lipid accumulation and diabetic cardiomyopathy (226). Furthermore, in an ex vivo Langendorff isolated heart perfusion system, it was shown that FGF21-induced cardiac protection and restoration of cardiac function involving autocrine-paracrine pathways (227). This effect was reduced in obesity. (227).

5.3.4 Effects of FGF21 on Central Nervous System - Brain

A role of FGF21 has been described in the central nervous system. Firstly, FGF21 has been shown to be able to pass through the murine blood-brain barrier and this has been described in the human cerebrospinal fluid (CSF) in both studies of serum and CSF of adults, where FGF21 in both biological liquids increased with the rising of fat mass and BMI (228, 229). In the murine brain, there are regions including substantia nigra, striatum and cerebellar neurons that express FGF21 (230, 231). Importantly, the brain expresses also receptors for FGF21, specifically FGFR1 and FGFR3 as well as the β-klotho cofactor expressed in the hypothalamus. These receptors and the cofactor permit FGF21 to act centrally by increasing systemic glucocorticoid levels, suppressing physical activity and altering circadian behavior, which are all features of the adaptive starvation response (232).

In 2014, by intra-cerebroventricular injection of FGF21 in mice, it was shown that FGF21 maintains glucose homeostasis by mediating the cross talk between liver and brain during prolonged fasting (233). Two years later, proof for further action of FGF21 in the brain was provided by a study in mice, where a liver-to-brain hormonal axis was described regulating micronutrient intake through FGF21. Specifically, a negative feedback to reduce sweet-seeking behavior is transmitted from the liver to the hypothalamus by expressing and secreting FGF21 (**Figure 11**) (176).

5.3.5 Effects of FGF21 on Bone metabolism

FGF21 is described to interfere with bone metabolism by decreasing bone mass, inhibiting osteoblastogenesis and stimulating adipogenesis in bone marrow–derived mesenchymal stem cells. This is possible as bone marrow mesenchymal stem cells may differentiate either to osteoblasts or to adipocytes (234). FGF21 is also described to indirectly enhance bone resorption. This function of FGF21 is mediated through stimulating IGFBP1 release from the liver, and by promoting IGFBP1 stimulation of

bone resorption in vivo (235). No direct effect of FGF21 on osteoclasts has been described.

Figure 14 reviews the multiple effects of FGF21 on various tissues (236).

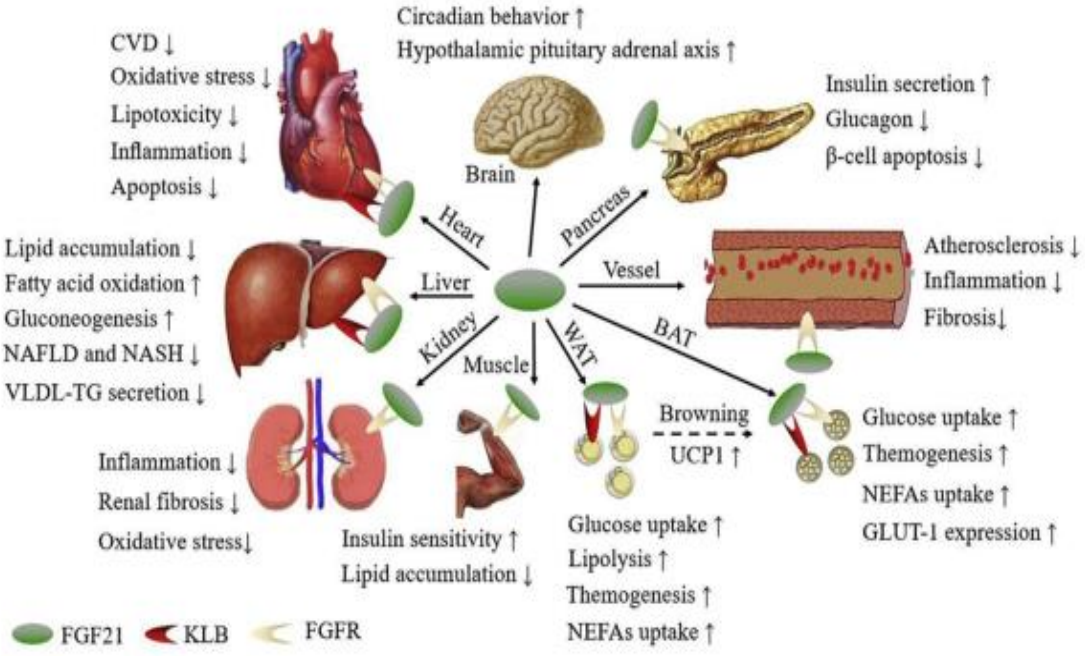

Figure 14. The relationship and the multiple effects of FGF21 on different organs and tissues. Abbreviations: FGF21, fibroblast growth factor 21; FGFR, fibroblast growth factor receptor; KLB, β-klotho; , white adipose tissue; BAT, brown adipose tissue; UCP1, uncoupling protein-1; CVD, cardiovascular disease; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; TG, triglyceride; VLDL, very low density lipoprotein; GLUT-1, glucose transporter-1; NEFA, non-esterified fatty acids. Reproduced from (236) with permissions from Elsevier.

5.4 Possible Administration in Humans - Clinical Trials

The pharmacological effects of recombinant FGF21 were widely tested in animal models; recombinant FGF21 was clearly shown to be a potent metabolic regulator with beneficial effects on body weight lowering, glucose homeostasis and lipid profile improving. In the recent years, research is focusing in improving the properties of human FGF21 protein to a more suitable drug for use in humans. This improvement concerns its bioavailability (half-life, renal clearance) stability (rapid proteolysis of FGF21 human protein has been described), and immunogenicity.

At least nine FGF21-class molecules have been tested in humans, and several are still active in different stages of clinical development as a T2DM or NASH therapeutic. Namely, the FGF21 molecules used sofar are LY2405319, LY3025876, LY3084077, BMS986036, BMS986171, PF05231023, AMG876, BFKB8488A and NGM313 (218, 219, 237). Although more studies have to be performed, the results have been promising sofar. Further to that, combination therapies are also proposed using fibrate and fibroblast activation protein (FAP) inhibitor which may produce a busting effect on FGF21 action (238, 239).

Chapter 6: Markers of Endothelial Dysfunction Applied in the Diagnosis of CVD

6.1 Flow-mediated Dilation (FMD)

6.1.1 FMD definition and regulation in adults' metabolic and CV disease

Arterial function and elasticity requires a balance between vasodilation and vasoconstriction. This function is regulated by the release of substances acting on the vessel wall at the endothelial cells (240). FMD is an ultrasound-based technique, introduced in 1992, as a method of non-invasive detection of endothelial dysfunction, and it is the most well established method in assessing the brachial reactivity in adults (241). FMD assessed with this technique measures the nitric oxide–mediated vasodilation produced as a response to increased flow after a period of ischemia applied to the brachial artery. Briefly, FMD technique examines, by ultrasound, the changes in brachial artery diameter in response to ischemia induced by inflating a blood pressure cuff distal to the imaged artery, around the forearm, to supra-systolic level (**Figure 15)** (241). When there is endothelial damage, the vessel loses the ability to dilate and thus FMD is decreased. Non–endothelium-dependent dilation (NEDD) measures the arterial changes induced by administration of a sublingual dose of nitroglycerin, which reflects predominantly the smooth muscle response (242).

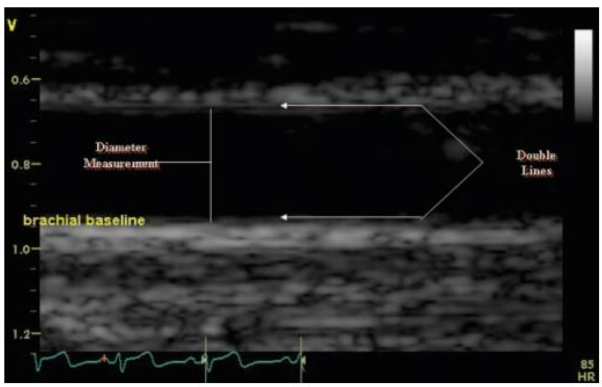

Figure 15 This image demonstrates the typical image of the brachial artery obtained at baseline. The double-layered appearance of the vessel wall can be appreciated and the vessel lumen easily measured. Reproduced from (242) with permissions from Wolters Kluwer Health, Inc.

FMD is affected by cardiovascular risk factors, has been related to the presence of coronary artery disease (CAD) and endothelial function and independently predicts CVD outcomes (243). FMD has been reported to be associated with cIMT progression in a population free of CVD (244). Despite concerns about its reproducibility, the available evidence shows that this method is reliable when the operator is well trained and follows standardized protocols (243). Several studies and meta-analyses have explored and confirmed, the prognostic value of brachial FMD for cardiovascular events (245-248). From these meta-analyses, a significantly lower risk of cardiovascular events (by 8-13%) has been reported for every percent point increase in brachial artery FMD (243).

In patients with CAD, FMD was shown to be an independent predictor of coronary events. (249, 250). Both FMD and LDL levels have been observed to be significantly improved after 6 months of cardiovascular risk factor management. In healthy adults, low FMD is correlated with HDL cholesterol and blood pressure and when resistance exercise was applied to healthy young adults, peripheral arterial remodeling occurred as indicated by an increase in mean brachial artery diameter post-occlusion (251, 252). Hormonal influence also may dysregulate endothelial function and this may be assessed by FMD as shown in a study of postmenopausal women (253).

In interventional studies in patients with impaired endothelial function, FMD shows relative modification with treatment. In patients with T1DM without overt angiopathy, bicycle exercise training was initiated and an amelioration of FMD was observed, accompanied with increased peak oxygen uptake (254). When obese subjects with insulin resistance were put on a program of a 6-month physical training and weight reduction, improvement of endothelial function was observed using FMD evaluation (255).

Dietary interventions and their effect on endothelial function have also been investigated. In subjects with T2DM, a beneficial effect on insulin resistance and endothelial function was observed using FMD, by modifying diet from polyunsaturated to monounsaturated fat (256). When patients with ischemic heart disease and increased cholesterol where put on Mediterranean diet plus initiated statin treatment, a greater

improvement in FMD was observed compared to the treatment with statin alone (257). These results indicate the valuable information provided by the FMD technique on the effect of different interventions in various CVD conditions, indicating the great value of the method.

6.1.2 FMD in Children

In children, there is less information on FMD as compared to adults, although the available data confirm the validity of this method in detecting early endothelial dysfunction in this age group. FMD has been independently associated with BMI and it has been negatively correlated with fasting insulin and apolipoprotein A-I (258-260). In contrast, a recent observational study in obese children explored the relation of FMD with excess weight and traditional cardiovascular risk factors including anthropometric features and blood pressure and no association was found (261). In another study in children with increased BMI ($\geq 85^{th}$ percentile), a higher risk of hypertension and left ventricular hypertrophy was reported with decreased FMD (262). When FMD was evaluated in 35 obese children of a mean age of 9 years, no relation was found to any clinical and biochemical characteristics in contrast to another study in 252 children aged 9 to 18 years in whom FMD was positively associated with high adiposity (263, 264). It has been described that fasting glucose and BMI are independent predictive variables of decreased FMD (265).

It has been speculated that conflicting data may result from unhomogeneous conditions applied to the studies and mainly sample size, as well as to some extent from the variation of FMD results among the different age groups. Table 3 summarizes the studies, published until 2012, that have investigated the effects of obesity on vascular structure and function in children and adolescents. FMD is the most used method followed by cIMT, while other methods have only been sporadically applied (266).

Table 3. Studies investigating the effects of obesity on vascular structure and function in children and adolescents. Reproduced from (266) with permissions from Elsevier.

	Obese			Control										
First Author, Year (Ref. #)	n	Female	Age (yrs)	n	Female	Age (yrs)	Tanner*	FMD	CIMT	AC	CAC	CAS	AS	PWVc	PWVp
Tounian, 2001 (59)	48	58%	12.6	27	41%	12.0	Yes	↓	↔		↑	↑			
Iannuzzi, 2004 (53)	100	39%	10.0	47	21%	10.0	No		↑			↑			
Woo, 2004 (55)	36	58%	10.3	36	58%	10.3	Yes	↓	↑						
Zhu, 2005 (56)	43	46%	12.0	28	40%	12.0	No	↓	↑						
Kapiotis, 2006 (54)	145	48%	12.0	54	44%	12.0	No	↓	↑						
Meyer, 2006 (50)	32	53%	13.7	20	60%	14.7	Yes	↓	↑						
Meyer, 2006b (91)	96	51%	14.2	35	51%	14.7	Yes	↓							
Pena, 2006 (95)	58	48%	13.3	53	55%	14.1	No	↓							
Reinehr, 2006 (57)	96	54%	11.0	25	56%	11.0	Yes		↑						
Lee, 2007 (75)	126	0%	14.6	130	0%	14.9	No							↑	
Aggoun, 2008 (58)	48	67%	8.9	23	69%	8.3	Yes	↓	↔						
Dangardt, 2008 (80)	33	61%	13.9	18	61%	14.3	No								↓
Karpoff, 2009 (51)	12	0%	11.6	13	0%	11.6	No	↓	↑		↔				
Mahmud, 2009 (94)	26	39%	13.4	51	41%	14.0	Yes	↓							
Sakuragi, 2009 (76)	191	49%	10.1	0			Yes†							↑	
Bhattacharjee, 2010 (93)	55	51%	8.6	50	40%	8.0	No	↓							
Pac, 2010 (77)	37	41%	13.9	30	43%	13.1	No						↑		
Urbina, 2010 (74)	234	70%	18.1	241	61%	17.8	No							↑	↔
Yilmazer, 2010 (52)	77	47%	11.5	40	43%	9.8	Yes†	↓	↑		↓				
Chalmers, 2011 (81)	34	53%	13.6	33	39%	13.2	Yes†			↑					
Ciccone, 2011 (92)	93	47%	10.9				No	↓							
Harris, 2012 (17)	61	44%	13.8	55	60%	13.8	No						↑		
Koopman, 2012 (13)	21	19%	14.2	27	19%	13.9	No	↔	↑					↑	
Lurbe, 2012 (79)	284	44%	12.0	79	56%	13.4	No							↓	
Mahfouz, 2012 (36)	56	30%	13.8	40	38%	13.4	No						↑		
Ozcetin, 2012 (18)	42	57%	10.1	36	61%	9.8	No		↑			↑			
Tryggestad, 2012 (82)	63	52%	13.9	61	49%	13.3	Yes†	↔		↑					

LVd=left ventricular dimension; LVt=left ventricular function; LVM= left ventricular mass; NR= not reported; ↑=greater compared to control; ↓ = lower compared to control; ↔ = no difference between control and obese.

Obesity is a main field of application of FMD although however other metabolic conditions are potentially linked to early endothelial damage such as diabetes, where FMD was found to be decreased as expected (267). Early endothelial dysfunction was found in adolescents with a positive family history of cardiovascular events with further reduction seen in children with familial hypercholesterolemia and also in familial combined hypercholesterolemia (268-270).

FMD in childhood has been explored under different conditions and found to be impaired in peadiatric patients with human immunodeficiency virus, Kawasaki disease,

juvenile idiopathic arthritis and systemic inflammation, leading to the hypothesis that CRP may be involved in the pathogenesis of early atherosclerosis (271-274). Chronic renal failure is also described as a state associated with impaired FMD both before and after renal transplantation due to alterations in the arterial wall (275, 276). Interestingly, in a large study with 402 children aged 11 years, reduced FMD was shown in children with elevated serum levels of cotinine as a result of exposure to passive smoking (277).

6.2 Carotid Intima-media Thickness (cIMT)

Introduced in the early 90s, cIMT is a method based on ultrasound, quantifying the part of the carotid wall constituted by the intima and the media tunic (**Figure 16)**. This thickness is shown to be increased in processes of vascular damage, plaque formation and atherosclerosis (278, 279).

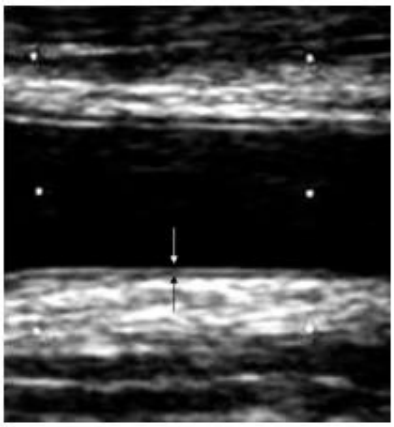

Figure 16 Image taken from the distal common carotid artery demonstrating the intimal-medial complex (between arrows). The intimal-medial thickness is measured from the border between the echolucent vessel lumen and the echogenic intima (white arrow) and the border between the echolucent media and echogenic adventitia (black arrow). Reproduced from (242) with permissions from Wolters Kluwer Health, Inc.

In adults, cIMT is shown to be associated with cardiovascular risk factors such as age, race, total , LDL or HDL cholesterol, higher systolic blood pressure, and smoking (280, 281). Increased cIMT is associated with CAD and is predictive for future cardiovascular

events (282-284). In patients with CVD treated with anti-hypertensive and lipid lowering medications, an amelioration of cIMThas been consistently reported (285).

In children, cIMT has been demonstrated to be increased in the presence of familial hypercholesterolemia, hypertension, obesity and T1DM (286-289). Various diseases, Kawasaki disease, renal disease and renal transplantation and systemic lupus erythematosus have been shown to influence the thickness of the carotid wall, and in these diseases, cIMT has also been applied for treatment monitoring (290-293).

In children, IMT has also been applied to the aorta (aIMT) especially in neonates and young children. Increased aIMT has been associated with low birth weight, intrauterine growth restriction, maternal smoking and familial hypercholesterolemia (286, 294-296).

6.3 Other markers of Endothelial Dysfunction

6.3.1 Pulse Wave Velocity (PWV)

In 1922, the recognition of PWV in men was first described by Bramwell and Hill (297). PWV is a measure of arterial stiffness and is based on the principle that the pressure pulse, generated by the left ventricular ejection, is propagated along the arterial tree at a speed determined by the geometric and elastic properties of the arterial wall (298). PWV is defined by the Moens-Korteweg equation as follows:

$$PWV = \sqrt{(Eh/2\rho R)}$$

where E is Young's modulus of the arterial wall, h is wall thickness, ρ is blood density, and R is arterial radius at the end of diastole (242, 298).

The arterial pulse wave is measured at both a proximal and a distal artery, such as the common carotid and femoral arteries respectively. The pulse waveform is recorded at each site, and the time delay between the arrival of a predefined point on the waveform (typically the "foot") at the 2 sites is obtained by gating to the peak of the R wave on the ECG. Measurement of distances on the body surface, a practice somewhat dependent on the body habitus, allows an estimate of the distance traveled. PWV is then calculated as distance/time (m/s) (242).

In adults, PWV is increased in the presence of arterial stiffness and vascular damage and is strongly associated with the presence and extent of atherosclerosis (299, 300). PWV is also found to be increased in conditions associated with increased cardiovascular risk such as diabetes, hypertension, end-stage renal disease, hyperlipidemia, increasing age, and sedentary lifestyle (301-304).

In paediatric populations, PWV has been previously described to be increased in diseases such as T1DM, Kawasaki disease (305-307).

6.3.2 Augmentation Index

Augmentation index represents another method used to assess arterial stiffness and is one of the parameters that have also been tested in paediatric populations. This parameter is derived from the pulse contour analysis, and has been found to be elevated indicating increased stiffness in children with conditions that predispose to CVD, such as diabetes, as compared to healthy control subjects (308).

Part Two - Original Research

Chapter 7: Aim of the Study

In 2013, WHO published the Global Action Plan where it is very clear how important the decrease of mortality from non-communicable diseases such as CVD and diabetes may be, as well as how crucial the halting of diabetes and obesity is (112). Monitoring the worldwide prevalence of obesity has shown the increasingly high rates in adults but also in the paediatric population (29). The MS is also another grey area especially in children as it constitutes a clear indicator of cardiovascular risk in adults, while concerns arise when applied to younger adults and children. Questions have been raised towards the applicability of MS in children and the alternative use of MS criteria as clustering components to indicate increased cardiovascular risk (74). There is a definite lack of biomarkers that could easily and accurately detect cardiovascular risk and metabolic disorders especially early in life and in childhood.

The aim of our study was to focus on detecting new molecules that could be used as biomarkers of such disorders in childhood. Thus, we explored the association of FGF21, a new protein with hormonal and regulatory effects on metabolism, as well as leptin and adiponectin that have been only partially studied sofar, with indices of vascular structure and function in order to investigate whether these molecules could represent potential biomarkers of MS and other disorders in childhood. IGFBP1 was also investigated in our research as this has been shown to be involved in metabolic pathways and have protective role on vascular tissue. We aimed to use non-invasive techniques to assess arterial structure and function in our research, as these methods can be easily applied at an outpatient environment in paediatric populations. With the current research, we aim to better understand metabolic disorders and the potentially associated cardiovascular insult in childhood. We also hope that the results of this research could provide a useful matrix on which to build tools for timely and successful prevention of the progression of metabolic disorders and their effects on the vasculature, as early as in a paediatric population.

Chapter 8: Population and Methods

8.1 Population and Study Protocol

One hundred children consented to participate in our protocol, 9% waived participation, while 22 were excluded due to incomplete and/or unavailable data series. Our study population finally consisted of 78 children, from northwestern Greece, aged 7 to 16 years, boys and girls that visited the Pediatric Department for a check-up prior to initiation of sport activities. Children were generally healthy, had no fever or signs of illness, had not undertaken any therapy, while they had not been ill for the last 3 months.

The study was conducted according to the Declaration of Helsinki and each participant's guardian provided written informed consent. The study protocol was approved by the Medical Ethics Committee of the University Hospital of Ioannina.

On the morning of the study, the personal and family health history were recorded, demographic and anthropometric data were assessed and blood sampling was performed at 8:00am after an overnight fast.

Standing height was measured to the nearest 0.1cm on a wall-mounted stadiometer. Body weight was measured in the morning, with light clothing and no shoes on, on an electronic scale. Waist circumference was measured in a horizontal plane, midway between the inferior margin of the ribs and the superior border of the iliac crest, based on guidelines in the IDF definition for MS for adults and for children (82, 309). The BMI was calculated as weight/height^2 (kg/m^2) and z-scores were derived using the National Greek growth charts. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were assessed with electronic sphygmomanometer at a basal state before any further procedures were initiated, in the morning after overnight fasting and in a calm and quiet environment.

8.2 Methods and Statistical analysis

Serum Analysis

Serum samples were collected after a standard centrifuge procedure and they were either directly assessed or stored at -80 degrees laboratory freezer until their assaying.

The lipid profile analysis included total cholesterol (T Chol), triglycerides (TRG) and high density lipoprotein (HDL) analysis on the analyzer Beckman Coulter AU5800 Clinical Chemistry and low density lipoprotein (LDL) was derived from the Friedewald formula. It included also Apolipoprotein A (apoA), apolipoprotein B (apoB), lipoprotein A [Lp(a)] analysis on the nephelometer Siemens BM ProSpec.

The thyroid function was assessed by determination of free thyroxin (fT4) and thyroid stimulating hormone (TSH), 3rd generation (HYPERsensitive hTSH) by ACCESS IMMUNOASSAYS on Beckman Coulter Dx1800 analyzer. The liver function was assessed by the transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT) on the Beckman Coulter AU5800 Clinical Chemistry analyzer.

Glucose homeostasis was assessed by HOMA-IR as defined by the ratio fasting blood glucose (mg/dl) x fasting blood insulin (μU/mL)) /405 and the glycosylated hemoglobin (HbA1c) (310, 311). Glucose was analyzed on Beckman Coulter AU5800 Clinical Chemistry analyzer, insulin was measured by ACCESS IMMUNOASSAYS on Beckman Coulter Dx1800 analyzer and HbA1c was measured on Biorad Variant II, HPLC analyzer.

Inflammation was assessed by C reactive protein (CRP) using turbidimetry, IMMAGE Immunochemistry Systems and Calibrator 5 Plus, Bechman Coulter.

The measurement of the biomarkers FGF21, adiponectin, leptin and IGFBP1 was carried out by enzyme-linked immune-assays (ELISA). The commercial kit used for FGF21 was purchased from R&D Systems, MN, USA (Human FGF21 Quantikine ELISA kit). The sensitivity of the method was 4.67 pg/ml and the CVs were 3.9% and 10.9% for intra and inter assays respectively. For adiponectin (Human adiponectin

ELISA) was used the kit by Biovendor (Research and Diagnostic Products, Brno Czech Republic). The sensitivity of the method was 26 ng/ml and the respective CVs were 5.9% and 6.3%. For leptin (Leptin Sandwich ELISA) the kit used was by DRG Instrs GmbH, Germany). The sensitivity of the method was 1.0 ng/ml and the CVs 6% and 9% respectively. IGFBP1 was measured by using the kit by DIA Source, (IGFBP-1 ELISA KARME 01, Belgium) respectively. The sensitivity of the method was 0.02 ng/ml and the respective CVs 5.2% and 5.9%.

Ultrasound studies

All studies were performed with the participants fasted for at least 6 hours and all measurements were taken in the supine position in a quiet environment, and in a temperature controlled room (~22ºC). An Echo-Doppler ultrasound (Ultrasound ATL, HDI 5000, Bothell, WA, USA) and a 5-12 MHz transducer was used for optimal imaging of the brachial and common carotid arteries.

FMD Protocol: Endothelial function was assessed in all participants by measurement of endothelium-dependent vasodilation in the right brachial artery in response to hand hyperemia, based on previously described methodology according to recently published recommendations in children and adolescents (242, 274). Images were acquired at baseline and every 30 seconds, from the first to the third minute after deflation of a wrist cuff inflated to 250 mmHg for 4 min for measurement of FMD. Brachial artery blood flow was measured by continuous wave Doppler at baseline and 15 seconds after cuff release. FMD was calculated as the maximum percent increase in arterial diameter during the first 3 minutes of hyperemia compared with the diameter at rest.

Carotid artery protocol: Common carotid artery (CCA) IMT measurement was performed in all participants using a standardized protocol published previously based on recently published recommendations in children and adolescents (242, 274). Three consecutive longitudinal images of each CCA 1–2 cm proximal to the bifurcation were acquired. Measurements were always made at the far wall of the artery. The mean value of IMT for right and left CCA was obtained by averaging the three measurements at

each artery. Finally, the mean carotid IMT of the CCAs was determined. Off-line analysis and measurement of brachial artery end-diastolic diameter and IMT were performed by another blinded operator by means of the software QLAB (Philips Ultrasound, Bothell, WA, USA) with manual and automatic (for brachial and carotid artery respectively) determination of the relative vascular wall margins. Measurements were made at end-diastole coincident with the R-wave on electrocardiogram. In studies performed on two separate days (7-10 days apart) in 10 subjects by a single operator, the within-subject coefficient of variation of FMD and IMT were 6.9% and 1.5% respectively.

Shear stimulus and normalised FMD

Shear rate is an estimate of shear stress without viscosity and was used to quantify the stimulus for FMD. Shear rate was calculated as mean blood flow velocity/vessel diameter. The peak of the shear stress stimulus created with reactive hyperemia occurs within the first seconds post cuff-release, and thus, the brachial artery mean blood flow velocity at 15 sec post cuff-release was used for calculating the peak shear rate (312).

The magnitude of imposed shear stress stimulus has great importance on influencing the magnitude of FMD (313, 314). Thus, to compare the FMD response between subjects and groups taking into account any variations in the magnitude of the shear stress stimulus, the FMD response was normalized to the magnitude of the shear stimulus (315). For that purpose, peak %FMD during the first 90 sec (i.e. within the time that the peak vessel diameter adaptation is typically observed) was divided by the peak shear rate computed a few seconds post cuff-release as described above.

Statistical analysis

The normality of the data was tested using the Kolmogorov-Smirnov test and most variables of interest (including FGF21, leptin, HOMA-IR, IGFBP1 and normalized FMD) were found to deviate significantly from the normal distribution. Accordingly, continuous variables are presented as median [interquartile range (IQR)]. Non-parametric analysis with the Mann-Whitney test was employed for comparing

continuous variables between two groups. The correlation between continuous variables was studied using the Spearman's rho (ρ) correlation coefficient. Receiver-operating curve analysis was performed to identify the best cut-offs (Youden index: max [Sensitivity and Specificity]) of biomarkers (FGF21 and leptin) for the diagnosis of MS. Univariable and multivariable logistic regression analysis was performed to investigate the association of biomarkers (FGF21 and leptin) with MS as well as the independent predictive value of the biomarkers for the presence of MS. The level of significance was set at $p<0.05$. The SPSS statistical software package (version 17.0 for Windows, SPSS Inc., USA) was used for the analysis.

8.3 Results

8.3.1 Descriptives

Seventy eight children, mean age 12.4 (IQR 10.3-14.9) years were included in the study. The weight median was 54.3 (IQR 40.1-70.3) kg and the height median was 157.0 (IQR 142.0-166.2) cm. The WC was calculated in our children with a median value 75.0 (IQR 65.7-88.3) cm and the median value for the BMI was 23.0 (IQR 18.1-27.7) kg/m^2 (**Table 4)**.

Table 4. Population Demographics

VARIBALE	
TOTAL NUMBER	78
AGE (years)	12.4 (10.3-14.9)
WEIGHT (kilograms)	54.3 (40.1-70.3)
HEIGHT (cm)	157.0 (142.0-166.2)
WAIST CIRC (cm)	75.0 (65.7-88.3)
BMI (kg/m^2)	23.0 (18.1-27.7)

8.3.2. Glucose and Lipid Profile

From the serum analysis in our population, an overall normal lipid profile was observed with a possible presence of insulin resistance within the population as the median values for HbA1c/Hb (x100) %, insulin and HOMA-IR were increased.

Lipid profile: T Chol 162.2 (Interquartile range (IQR) 136.5-193.5) mg/dl, TRG 68.0 (IQR 45.6-127.5) mg/dl, HDL 46.0 (IQR 38.5-55.5) mg/dl, LDL 99.0 (IQR77.7-125.0) mg/dl, Lp(a) 7.4 (IQR 4.2-21.2) mg/dl, ApoA 139.0 (IQR 127.0-151.2) mg/dl, ApoB 71.7 (IQR 55.5-89.7) mg/dl.

Glucose metabolism: Glu 85.0 (IQR 79.0-92.0) mg/dl, HbA1c/Hb (x100) 5.4 (IQR 5.0-5.5)%, Insulin 9.0 (IQR 5.4-18.3)µU/ml, HOMA-IR 2.0 (IQR 1.1-3.9) (**Table 5**).

Table 5. Lipid and glucose profile in the study population.

Variable	Median	Interquartile range
T Chol (mg/dl)	162.2	136.5-193.5
TRG (mg/dl)	68.0	46.5-127.5
HDL (mg/dl)	46.0	38.5-55.5
LDL (mg/dl)	99.0	77.7-125.0
Lp(a) (mg/dl)	7.4	4.2- 1.2
ApoA (mg/dl)	139.0	127.0-151.2
ApoB (mg/dl)	71.7	55.5-89.7
ApoB/ApoA	0.55	0.39-0.65
Glu (mg/dl)	85.0	79.0-92.0
HbA1c/Hb (x100) %	5.4	5.0-5.5
Insulin (µU/ml)	9.0	5.4-18.3
HOMA-IR	2.0	1.1-3.9

8.3.3 Biochemical Function and Serum Biomarkers

To assure the hepatic and thyroid intact function, we measured serum transaminases ALT, AST, T4 and TSH level in all our children and verified they were normal. To assess for inflammation, serum CRP was determined and was found within the normal range. Median values for these parameters were as follows: AST 21.0 (IQR 18.0-28.5) IU/l, ALT 16.0 (IQR 12.0-22.0) IU/l, TSH 2.0 (IQR 1.5-2.6) μIU/ml, fT4 0.8 (IQR 0.8-0.9) ng/dl, CRP 2 (IQR 1.8-5.0) mg/l (**Table 5**).

Furthermore, serum biomarkers of metabolic relevance were assessed: FGF21, leptin, adiponectin and IGFBP1. For leptin, increased overall levels were observed 9.4 (IQR 2.4-18.0) ng/ml, based on expected normal values suggested by the kit provider (males 3.84 ±1.79 and females 7.36 ± 3.73 ng/mL). However, higher levels (3.3 up to 18.3 ng/mL) have been described elsewhere as normally expected for females, while pediatric reference values are not specified (316). IGFBP1 values measured in the serum of our population were within the normal expected levels 2.1 (IQR 1.3-5.2) ng/ml, described by the kit provider (males 0.42-17.94 and females 0.23-16.7 ng/ml). As with leptin, no specified pediatric reference values are reported.

The range of normal values for adiponectin, varies depending on the BMI. The levels found in our population, 8.3 (IQR 6.4-9.2) μg/ml, were within the normal low male reference range for all BMI categories, as suggested by the provider of the kit. These levels were lower than expected for the healthy BMI (<25 kg/m^2) female category. Interestingly, the levels of adiponectin observed in our population were within the normal range for the overweight female BMI category, while they were borderline low for the obese female BMI category, based on the reference values provided by the kit manufacturer. However, elsewhere defined reference ranges position the overall levels circulating adiponectin in our population within the normal for gender and BMI category (**Table 6**) (316).

Table 6. Adiponectin Reference Range Values (Reproduced from reference 316)

Variable	BMI (kg/m^2)	n	Mean (μg/ml)	SD (μg/ml)
Men	<25	41	10.9	4.0
	25-30	52	8.8	4.0
	>30	23	8.3	2.8
	Total	115	9.5	3.9
Women	<25	92	13.6	5.4
	25-30	56	13.9	8.6
	>30	57	11.4	3.8
	Total	220	13.2	6.1

FGF21 circulating levels, 72.5 (IQR 27.4-114.3) pg/ml, were within the expected detectable levels suggested by the manufacturer of the kit, however there is little information on normal range and much less on relation to gender or age (199, 317). Human studies suggest that FGF21 levels have small diurnal variation (155). It is suggested that in healthy humans, FGF21 serum concentrations show marked inter-individual variation (148, 155).

The ratios between leptin and adiponectin and between FGF21 and adiponectin were calculated. We sought these on the insight that the ratio leptin / adiponectin has been suggested in healthy subjects and in patients with T2DM, as a clinical biomarker of atherosclerosis assessed by PWV and/or cIMT (318, 319). Leptin levels are increased in the serum of obese people and correlate strongly to BMI and fat tissue (123). Increased serum levels of leptin have been proposed as a CVD risk factor (320). On the other hand, adiponectin serum levels are low in obese people and have been shown to be associated with increased risk for CVD (321-323). We further speculated that there may be a similar ratio between FGF21 and adiponectin, since FGF21 is a potent lipid and glucose regulator similar to leptin, albeit secreted from liver as opposed to leptin which is an adipokine. FGF21 is shown to be increased in the serum of obese people and the aim of our study was to explore the possible role of this molecule as a biomarker of

atherosclerosis. In our population, the ratios in the overall population studied were for leptin/adiponectin 1.34 and for FGF21/adiponectin 8.0.

The results are shown in Table 7.

Table 7. Biomarkers and hormones in the study population.

Variable	Median	Interquartile Range
AST (IU/l)	21.0	18.0-28.5
ALT (IU/l)	16.0	12.0-22.0
CRP (mg/L)	2.0	1.8-5.0
fT4 (ng/dl)	0.8	0.8-0.9
TSH (µIU/ml)	1.9	1.5-2.6
FGF21 (pg/ml)	72.5	27.4-114.3
Leptin (ng/ml)	9.4	2.4-18.0
Leptin/Adiponectin	1.3	0.3-2.4
FGF21/Adiponectin	8.0	3-17
Adiponectin (µg/ml)	8.3	6.4-9.2
IGFBP1 (ng/ml)	2.1	1.3-5.2

Chapter 9: Gender differences

9.1 Descriptives by Gender

The population included 42 boys and 36 girls, and the mean age was calculated to be 11.9 (9.8-14.2) years for boys and 13.4 (11.2-15.3) years for girls. The females showed a tendency for higher mean weight values and higher BMI in comparison to the boys (**Table 8**).

Table 8. Descriptives by gender

Variable	BOYS (n=42)	GIRLS (n=36)
AGE (years)	11.9 (9.8-14.2)	13.4 (11.2-15.3)
WEIGHT (kilograms)	51 (36.1-62.2)	61.5 (44.7-74.0)
HEIGHT (cm)	153.5 (136.6-168.0)	158.0 (150.5-164.7)
WAIST CIRC (cm)	74.0 (63.0-87.6)	75.5 (70.0-90.7)
BMI (kg/m^2)	20.2 (17.1-25.6)	24.4 (20.2-29.2)

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9.2 Assessment of BMI Category by Gender

For the 2 genders, the normal BMI, overweight and obese subjects are shown in Figure 17. The male population included 23 normal BMI, 8 overweight and 11 obese boys. The female population consisted of 15 normal BMI, 5 overweight and 16 obese girls (**Figure 17**).

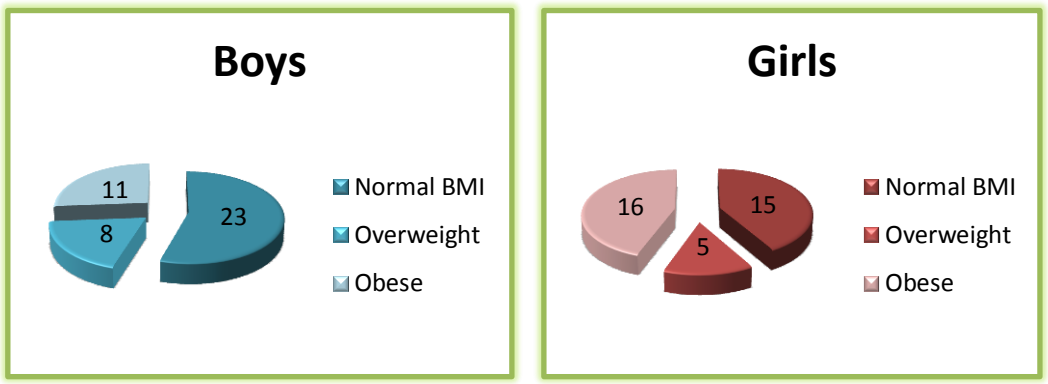

Figure 17. Normal BMI, overweight and obese children in the two genders.

9.3 BMI by Age and Gender

The two genders were enrolled in our protocol in a random fashion. Therefore, their weight was assessed separately for age by year and a consistently higher weight was observed in the females. Exemption to this was evidenced for the age of 12 years for which males were found to have a higher weight compared to females (**Figure 18)**.

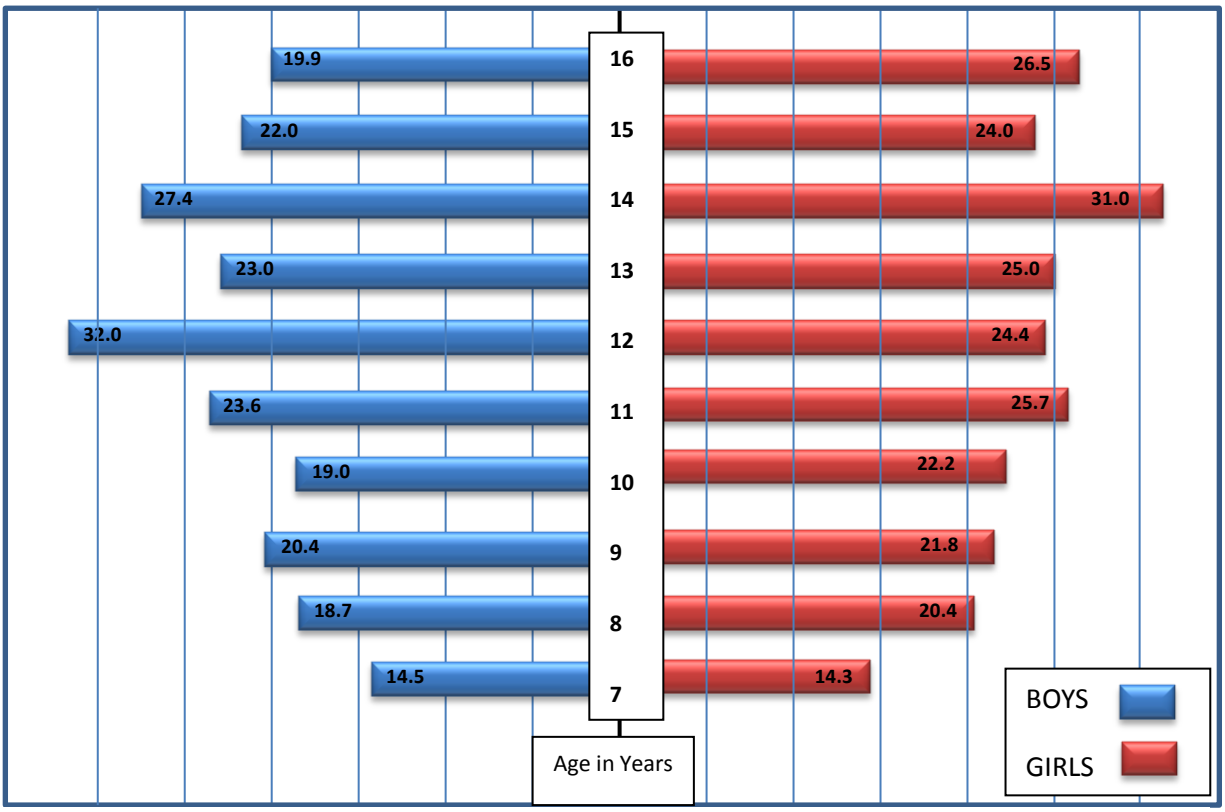

Figure 18. BMI by age and gender

9.4 Obesity by Gender

When the obesity rate, based on age and gender specific BMI percentiles, was assessed, the following were observed: the overweight/obesity rate was 45% of the male group and 58.3% of the female group. The overweight/obesity rate in the total population (both genders included) was 24.35% for the boys and 26.9% for the girls and the overall overweight/obesity rate in our population independently of gender was 51.3% (**Figure 19)**.

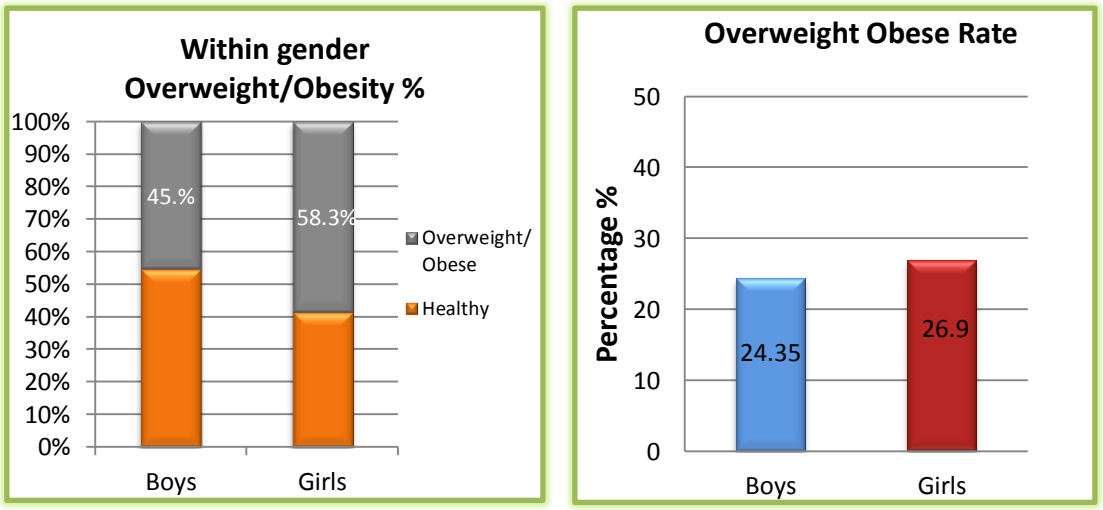

Figure 19. Obesity rate by gender.

Chapter 10: BMI differences: Obese/overweight vs. Normal BMI children.

10.1 Assessment of Paediatric Population Based on BMI

Our population was divided in groups based on the BMI category. Percentile curves specific for age and gender were used to define the normal BMI, the overweight and the obese children. We then analyzed the data collected based on the BMI category. The two groups will be described as normal BMI group and high BMI group (overweight/obese group).

There was no difference in age [normal BMI 13.1 (IQR 10.4-15.3) years vs. high BMI 12.3 (10.2-14.3) years, p=0.5] or in height [normal BMI 155.0 (138.0-165.5) cm vs. 158.0 (145.5-167.0) cm, p=0.39] between the two groups.

The liver function [AST: normal BMI 21.5 (17.5-28.5) IU/l vs. high BMI 21.0 (18.0-28.5) IU/l, p=0.9 and ALT: normal BMI 14.5 (11.0-18.0) IU/l vs. high BMI 17.0 (12.0-27.0) IU/l, p=0.08] and the thyroid function (fT4: normal BMI 0.8 (0.8-1.0) ng/dl vs. high BMI 0.8 (0.7-0.9) ng/dl, p=0.15 and TSH: normal BMI 1.8 (1.5-2.6) μIU/ml vs. high BMI 2.0 (1.5-2.6) μIU/ml, p=0.99] were normal in both groups. However, ALT levels ahowed a trend to be higher in the high BMI group remaining although within normal limits. CRP was significantly higher in the high BMI group [normal BMI 2.0 (1.0-2.8) mg/l vs. high BMI 3.5 (2.0-7.0) mg/l, p<0.001], consistent with an expected degree of subclinical inflammation in this group. However, CRP values did not exceed the higher normal limits. Results are shown in Table 9.

Table 9. Anthropometrics, liver and thyroid function tests and CRP in the 2 BMI subgroups of our study population.

Variable	Normal BMI (n=37)	High BMI (n=41)	P value
DESCRIPTIVES			
Age (years)	13.1 (10.4-15.3)	12.3 (10.2-14.3)	0.5
Weight (kg)	41.0 (31.0-57.5)	68.0 (52.0-86.0)	<0.001
Height (cm)	155.0 (138.0-165.5)	158.0 (145.5-167.0)	0.39
BMI (kg/m^2)	18.0 (16.6-20.3)	27.4 (23.9-29.9)	<0.001
WC (cm)	67.0 (59.5-72.5)	88.0 (78.3-95.5)	<0.001
LIVER FUNCTION TESTS			
AST (IU/l)	21.5 (17.5-28.5)	21.0 (18.0-28.5)	0.9
ALT (IU/l)	14.5 (11.0-18.0)	17.0 (12.0-27.0)	0.08
INFLAMMATION MARKER			
CRP (mg/l)	2.0 (1.0-2.8)	3.5 (2.0-7.0)	<0.001
THYROID FUNCTION TESTS			
fT4 (ng/dl)	0.8 (0.8-1.0)	0.8 (0.7-0.9)	0.15
TSH μIU/ml)	1.8 (1.5-2.6)	2.0 (1.5-2.6)	0.99

Regarding glucose homeostasis, the following were observed (results also shown in **Table 10**).

Glucose serum levels did not differ between normal BMI and high BMI groups (normal BMI 84.0 (81.0-93.0)mg/dl vs. high BMI 87.0 (79.0-90.5)mg/dl, p=0.88). Insulin levels were significantly higher in the high BMI group (normal BMI 6.4 (4.6-9.2) μU/ml vs. high BMI 16.3 (7.6-27.7) μU/ml, p<0.001). Consistently with insulin levels HOMAIR was also significantly increased in the high BMI group (normal BMI 1.3 (0.9-2.1) vs. high BMI 3.1 (1.7-6.2), p<0.001). HbA1c/Hb(x100)% did not differ between the two groups (normal BMI 5.3 (4.9-5.4) vs. high BMI 5.3 (5.0-5.5), p=0.5) (**Figure 20)**.

Table 10. Glucose homeostasis and lipid profile in the 2 BMI subgroups of our study population.

Variable	Normal BMI (n=37)	High BMI (n=41)	P value
GLUCOSE HOMEOSTASIS			
Glucose (mg/dl)	84.0 (81.0-93.0)	87.0 (79.0-90.5)	0.88
Insulin (µU/ml)	6.4 (4.6-9.2)	16.3 (7.6-27.7)	<0.001
HOMAIR	1.3 (0.9-2.1)	3.1 (1.7-6.2)	<0.001
HbA1c/Hb(x100)%	5.3 (4.9-5.4)	5.3 (50-5.5)	0.5
LIPID PROFILE			
T Chol (mg/dl)	162.0 (134.2-193.0)	164.0 (138.5-201.0)	0.8
TRG (mg/dl)	58.0 (41.3-73.3)	117.0 (62.6-154.0)	<0.001
HDL (mg/dl)	51.5 (44.3-57.0)	41.0 (36.0-49.5)	<0.001
LDL (mg/dl)	97.0 (77.5-120.0)	97.0 (74.5-130.5)	0.7
Lp(a) (mg/dl)	9.3 (4.8-25.4)	5.9 (2.6-15.3)	0.09
ApoA (mg/dl)	145.0 (134.0-155.0)	130.0 (123.0-143.2)	0.006
ApoB (mg/dl)	71.0 (52.9-81.1)	71.7 (58.3-96.1)	0.1
ApoB/ApoA	0.47 (0.37-0.60)	0.59 (0.46-0.74)	0.01

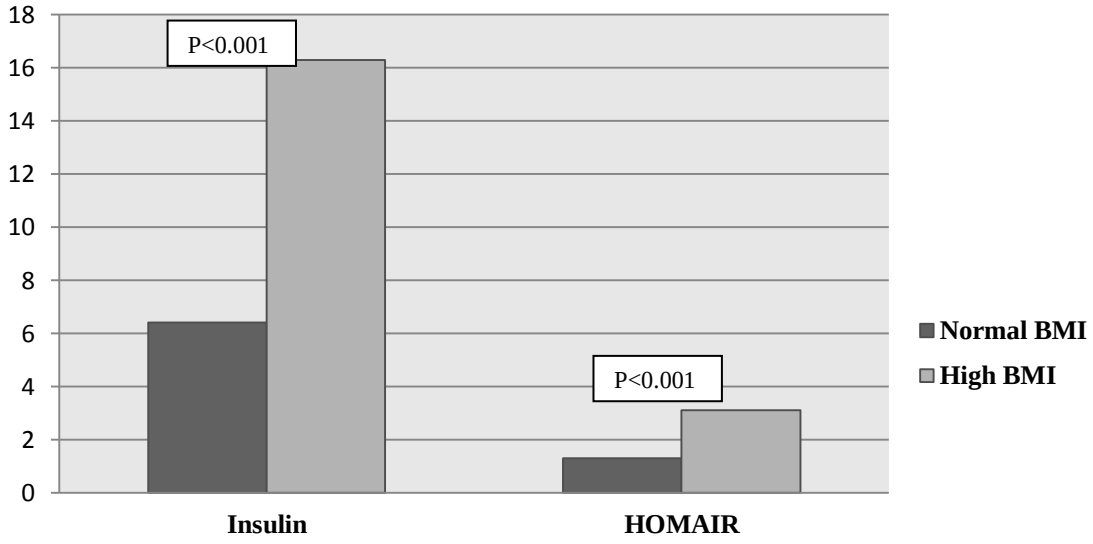

Figure 20 Insulin and HOMAIR in the normal BMI vs. the high BMI group

Regarding the lipid profile in our population, the following were observed (results also shown in **Table 10**).

T Chol did not differ between the two groups [normal BMI 162.0 (134.2-193.0) mg/dl vs. high BMI 164.0 (138.5-201.0) mg/dl, p=0.8]. Serum TRG were significantly higher in the high BMI compared to the normal BMI group [normal BMI 58.0 (41.3-73.3) mg/dl vs. high BMI 117.0 (62.6-154.0) mg/dl, p<0.001] accompanied by a significant decrease in HDL cholesterol in the high BMI compared to the normal BMI group [normal BMI 51.5 (44.3-57.0) mg/dl vs. high BMI 41.0 (36.0-49.5) mg/dl, p<0.001]. The LDL serum levels did not differ between the two groups [normal BMI 97.0 (77.5-120.0) mg/dl vs. high BMI 97.0 (74.5-130.5) mg/dl, p=0.07]. Serum lipoprotein levels shifted to a more atherogenic profile with a trend to decrease in Lp(a) and a trend to increase in ApoB in the high BMI group [Lp(a): normal BMI 9.3 (4.8-25.4) mg/dl vs. high BMI 5.9 (2.6-15.3) mg/dl, p=0.09 and ApoB: normal BMI 71.0 (52.9-81.1) mg/dl vs. high BMI 71.7 (58.3-96.1) mg/dl, p=0.1]. ApoA showed a significant decrease and ApoB/ApoA ratio showed a significant increase in the high BMI compared to the normal BMI group [ApoA: normal BMI 145.0 (134.0-155.0) vs. high BMI 130.0

(123.0-143.2), p=0.006 and ApoB/ApoA: normal BMI 0.47 (0.37-0.60) vs. high BMI 0.59 (0.46-0.74), p=0.01] (**Figure 21)**.

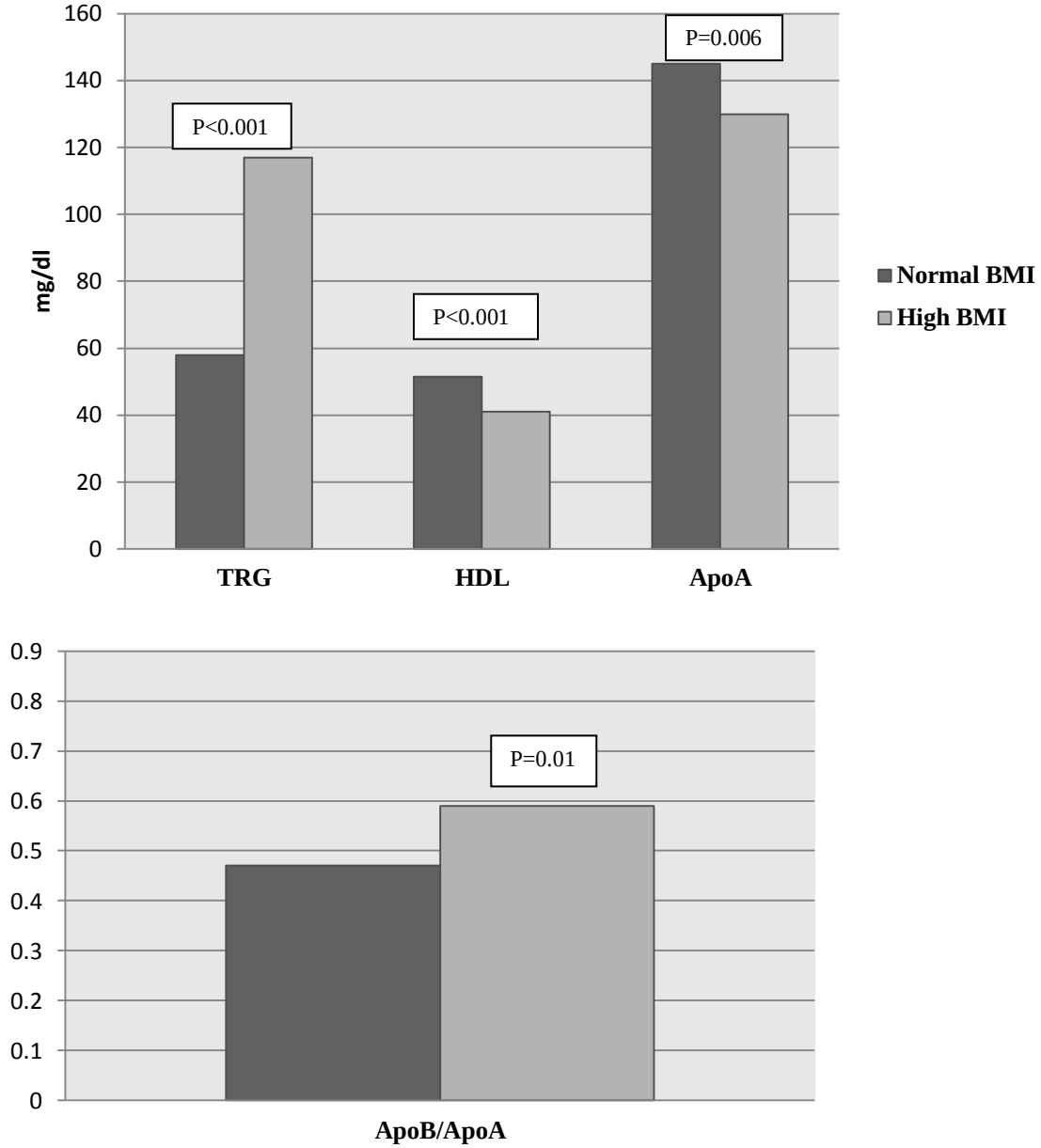

Figure 21 Serum TRG, HDL, ApoA levels and ApoB/ApoA ratio in the normal BMI compared to the high BMI group

Table 11. Newer metabolic biomarkers in the 2 BMI subgroups of our study population

Variable	Normal BMI (n=37)	High BMI (n=41)	P value
NEWER METABOLIC MARKERS			
FGF21 (pg/ml)	30.4 (13.8-92.6)	73.6 (41.6-127.8)	0.02
Leptin (ng/ml)	2.8 (1.1-8.7)	16.8 (9.6-30.4)	<0.001
Adiponectin (μg/ml)	8.5 (6.8-9.6)	7.6 (5.9-8.9)	0.16
IGFBP1 (ng/ml)	2.8 (1.8-7.9)	1.8 (1.1-3.0)	0.002
Leptin/Adiponectin	0.37 (0.13-1.43)	2.21 (1.1-4.1)	<0.001
FGF21/Adiponectin	4.5 (1.6-12.0)	11.1 (5.2-26.5)	0.006

FGF21 serum levels were significantly increased in the high BMI compared to the normal BMI group [normal BMI 30.4 (13.8-92.6) pg/ml vs. high BMI 73.6 (41.6-127.8) pg/ml, p=0.02]. Leptin serum levels were significantly increased in the high BMI compared to the normal BMI group [normal BMI 2.8 (1.1-8.7) ng/ml vs. high BMI 16.8 (9.6-30.4) ng/ml, p<0.001]. Adiponectin serum levels show a weak trend to decrease in the high BMI group compared to the normal BMI group [normal BMI 8.5 (6.8-9.6) μg/ml vs. high BMI 7.6 (5.9-8.9) μg/ml, p=0.16]. IGFBP1 serum levels significantly decreased in the high BMI versus the normal BMI group [normal BMI 2.8 (1.8-7.9) ng/ml vs. high BMI 1.8 (1.1-3.0) ng/ml, p=0.002]. Both leptin / adiponectin and FGF21 / adiponectin ratios showed a significant increase in the high BMI versus the normal BMI group [leptin/adiponectin: normal BMI 0.37 (0.13-1.43) vs. high BMI 2.21 (1.1-4.1), p<0.001 and FGF21/adiponectin: normal BMI 4.5 (1.6-12.0) vs. high BMI 11.1 (5.2-26.5), p=0.006] (**Figure 22)**.

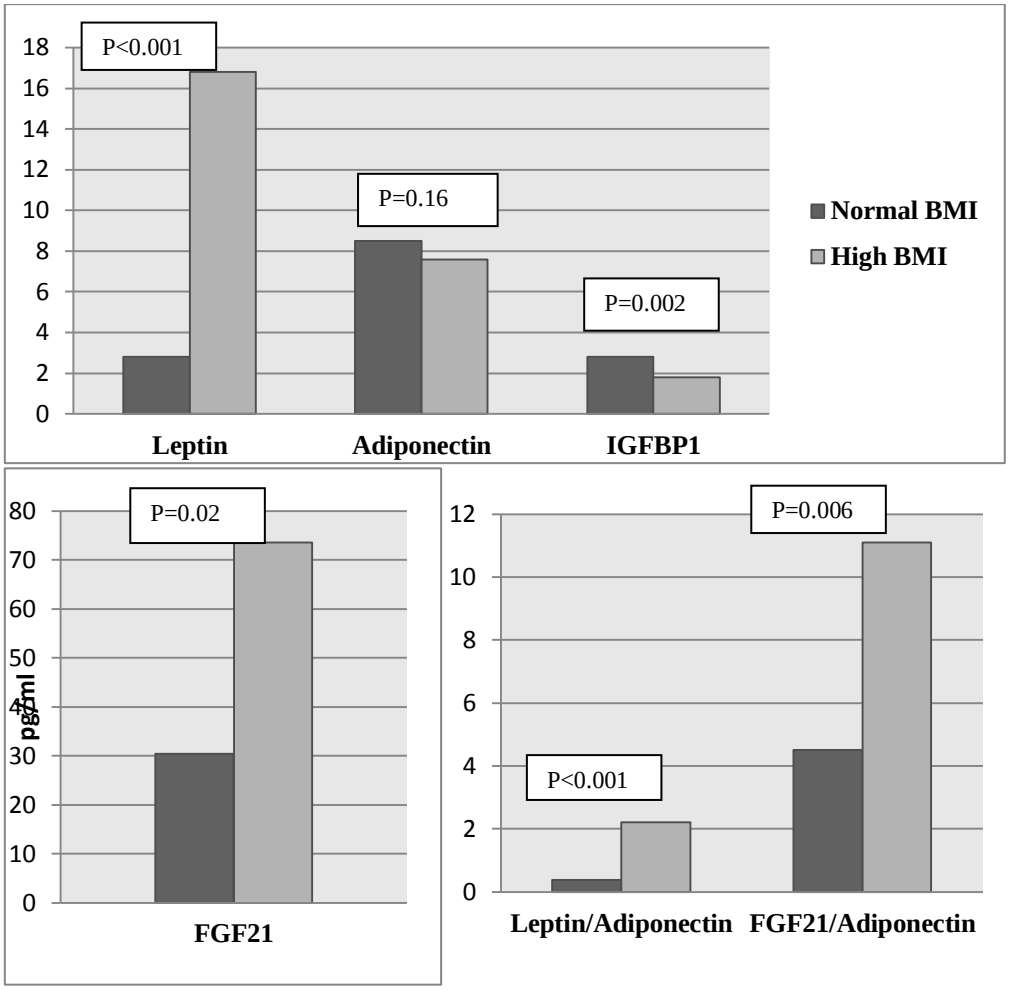

Figure 22. Leptin, adiponectin, IGFBP1, FGF21 levels and leptin/adiponectin and FGF21/adiponectin ratios in the normal BMI versus the high BMI group.

10.2 Endothelial function in Obese/overweight vs. Normal BMI Children

To assess the endothelial function in our pediatric population, we used FMD and cIMT. We observed a significant decrease of normalized FMD in the high BMI group [0.09 (0.04-0.12) vs. 0.06 (0.04-0.09), p=0.04] indicating early signs of endothelial damage in obese/overweight children. cIMT was not significantly different between the two groups (**Figure 23)**.

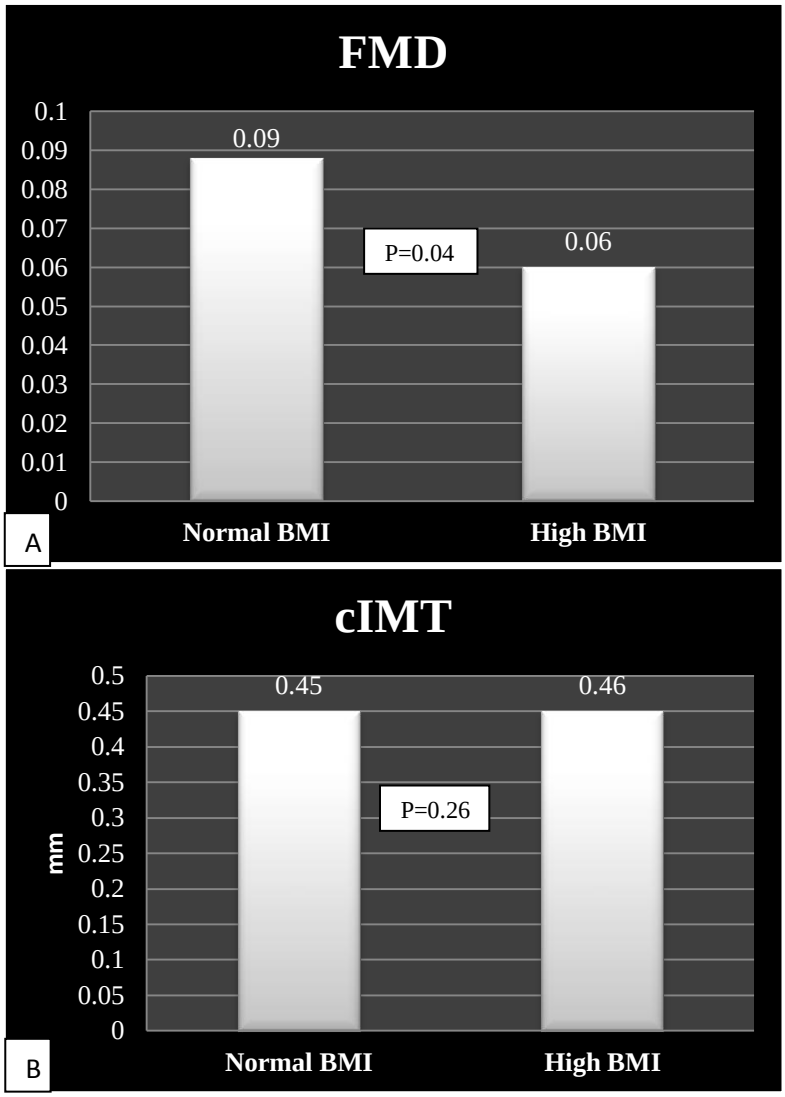

Figure 23. Endothelial function in obese/overweight versus normal BMI children. Panel A: FMD in normal BMI vs. High BMI children (0.09 vs. 0.06), p=0.04. Panel B: cIMT in normal BMI vs. High BMI children (0.45mm vs. 0.46mm), p=0.26.

Chapter 11: Metabolic Health Assessment

11.1 Definition and Descriptives

Even though obesity is linked to adverse metabolic consequences for the living organism, a benign type of obesity has been described in adults which differentiates from the malignant obesity in lacking adverse metabolic effects (324, 325). Metabolically benign obesity is not accompanied by insulin resistance and early atherosclerosis as assessed by cIMT in adults (180). This type of obesity may represent as much as 20% of the obese population (326-328). Furthermore, in the differentiation of this phenotype of obesity, ectopic fat in the liver may be more important than visceral fat.

We based our definition of metabolically unhealthy on the following criteria (204):

- HOMAIR ≥3
- TRG ≥110mg/dl
- HDL ≤40mg/dl
- fasting blood glucose ≥ 100mg/dl
- SBP ≥130mmHg and
- DBP ≥85 mmHg

In our population 4 groups were formed based on BMI and presence of criteria for metabolically unhealthy status as follows (**Figure 24)**:

1. Children with normal weight without any of the criteria therefore metabolically healthy (NMH)
2. Children with normal weight with at least one of the criteria therefore metabolically unhealthy (NMU)
3. Children with obesity without any criteria (OMH)
4. Children with obesity with at least one of the criteria (OMU)

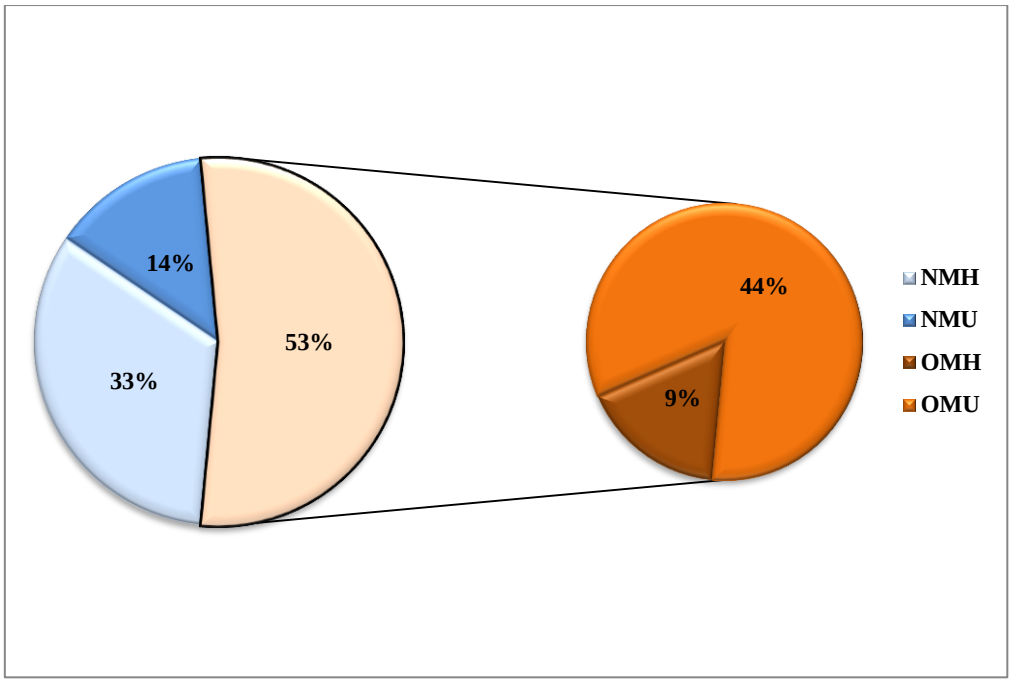

Figure 24. Subgroups in our population based on BMI and presence of criteria for metabolically unhealthy status.

11.2 Results

Comparison within the formed groups of all available data indicated the following (**Tables 12 - 17**).

Table 12. Laboratory investigations in the children with normal weight considered to be metabolically healthy (NMH) and children with normal weight considered to be metabolically unhealthy (NMU).

Variable*	NMH (n=26)	NMU (n=11)	p VALUE
GLUCOSE HOMEOSTASIS			
Glucose (mg/dl)	84.0 (78.0-.91.0)	90.0 (83.0-99.0)	p=0.06
Insulin (μU/ml)	5.8 (4.3-7.4)	9.5 (5.6-18.5)	p=0.03
HOMAIR	1.27 (0.89-1.6)	2.22 (1.14-4.10)	p=0.04
THYROID FUNCTION TESTS			
TSH (μIU/ml)	1.6 (1.4-2.4)	2.5 (1.8-2.7)	p=0.03
fT4 (ng/dl)	0.88 (0.81-1.0)	0.80 (0.75-0.93)	p=0.07
LIPID PROFILE			
Lp(α) (mg/dl)	11.0 (4.6-24.1)	8.1 (5.3-37.4)	p=1.0
ApoA (mg/dl)	145.0 (137.0-155.5)	136.0 (118.0-157.7)	p=0.2
ApoB (mg/dl)	67.4 (51.7-78.5)	79.8 (57.8-92.7)	p=0.1
ApoB/ApoA	0.44 (0.35-0.56)	0.54 (0.46-0.63)	p=0.04
TRGL (mg/dl)	55.0 (39.5-67.5)	71.0 (44.0-112.0)	p=0.09
HDL (mg/dl)	53.0 (47.5-57.5)	45.0 (39-57)	p=0.08
NEWER METABOLIC MARKERS			
FGF21 (pg/ml)	28.7 (11.0-91.0)	86.2 (28.2-107.9)	p=0.07
Leptin (ng/ml)	2.8 (1.0-9.9)	3.9 (2.2-8.1)	p=0.7
IGFBP1 (ng/ml)	3.0 (1.8-7.2)	2.3 (1.5-9.9)	p=0.8
Adiponectin (μg/ml)	8.5 (7.0-9.5)	8.0 (5.5-9.8)	p=0.6
FGF21/Adiponectin	3.1 (1.5-10.3)	8.3 (3.7-13.3)	p=0.05

*Comparisons for all other variables did not show statistical significance or trend (data not shown)

When **NMU** children were compared to **NMH,** significant differences were observed with an increase in serum insulin [9.5 (5.6-18.5) vs. 5.8 (4.3-7.4) μU/ml, p=0.03], in HOMAIR [2.22 (1.14-4.10) vs. 1.27 (0.89-1.6), p=0.04] and surprisingly also in TSH [2.5 (1.8-2.7) vs. 1.6 (1.4-2.4) μIU/ml, p=0.03] with a concomitant trend to decrease in fT4 [0.80 (0.75-0.93) vs. 0.88 (0.81-1.0) ng/dl, p=0.07] in NMU vs NMH respectively. A trend for an increase was also found for serum Glu [90.0 (83.0-99.0) vs. 84.0 (78.0-91.0) mg/dl, p=0.06], TRG [71 (44.0-112.0) vs. 55 (39.5-67.5) mg/dl, p=0.09], and FGF21 [86.2 (28.2-107.9) vs. 28.7 (11.0-91.0) pg/ml, p=0.07] in NMU vs NMH respectively. A trend for a decrease was observed for HDL [45.0 (39.0-57.0) vs. 53.0 (47.5-57.5) mg/dl, p=0.08], while ApoB/ApoA ratio was increased [0.54 (0.46-0.63) vs. 0.44 (0.35-0.56), p=0.04] in NMU vs. NMH respectively. FGF21/adiponectin ratio was increased [8.3 (3.7-13.3) vs. 3.1 (1.5-10.3), p=0.05] in NMU vs. NMH respectively (**Figure 25**).No differences were found in vascular indices between the 2 groups, FMD in NMU vs. NMH [0.09 (0.04-0.21) vs. 0.07 (0.04-0.12), p=0.66] and cIMT [0.43 (0.38-0.48) vs. 0.45 (0.42-0.48)mm, p=0.38] respectively.

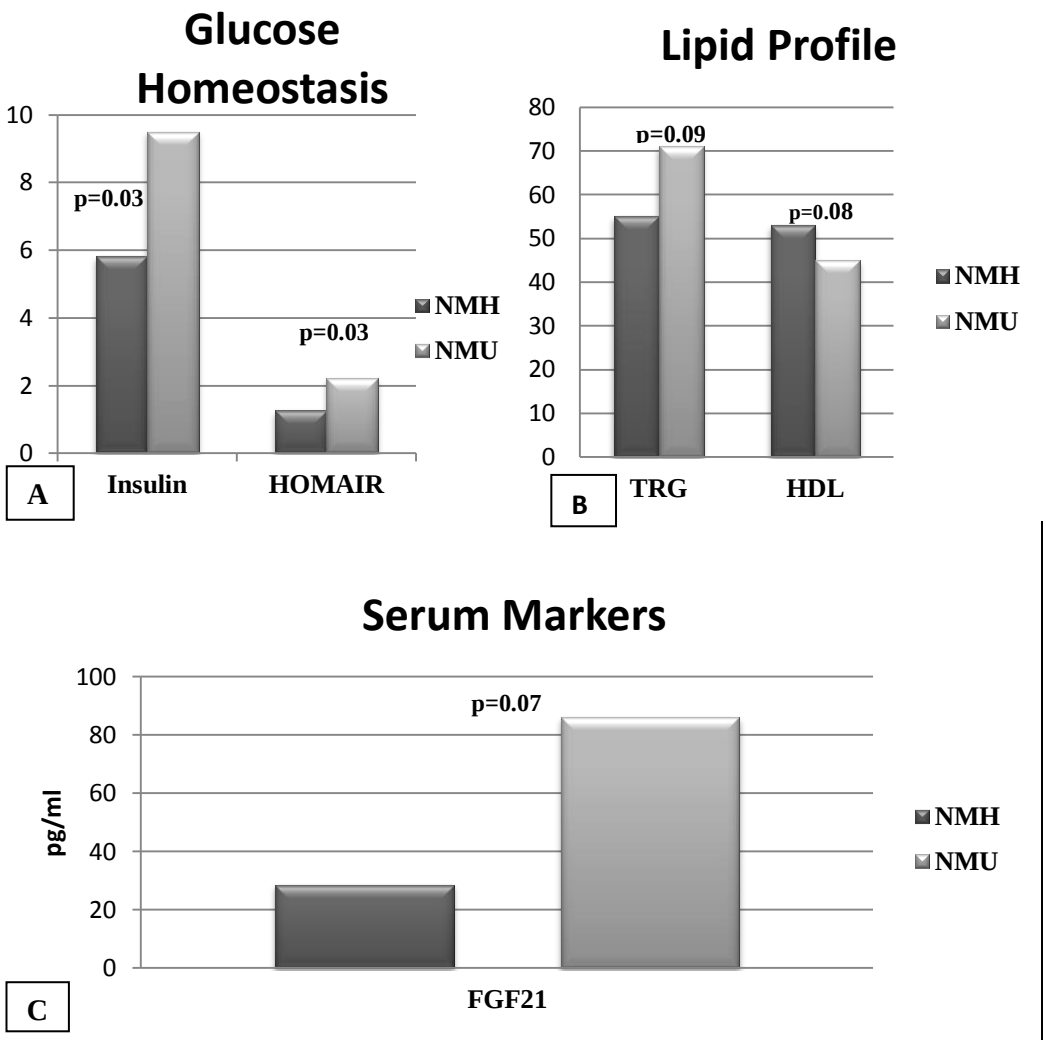

Figure 25. Biochemical profile in normal BMI children based on their metabolic health status. Panel A: Glucose homeostasis with insulin levels and HOMAIR significantly higher in the NMU compared to NMH group. Panel B: Lipid profile with borderline higher TRG serum levels in the NMU vs. the NMH group and borderline lower HDL cholesterol levels in the NMU vs. the NMH group. Panel C: FGF21 borderline higher serum levels in the NMU vs. the NMH group.

Table 13. Laboratory investigations in the children with obesity considered to be metabolically healthy (OMH) and children with obesity considered to be metabolically unhealthy (OMU). No differences were found in FGF21, adiponectin and IGFBP1 or the other serum analysis between the 2 groups.

Variable*	OMH (n=7)	OMU (n=34)	p VALUE
Age	9.9 (9.5-13.8)	12.4 (11.4-14.6)	p=0.05
GLUCOSE HOMEOSTASIS			
Insulin (µU/ml)	5.2 (3.9-9.2)	17.7 (10.4-28.3)	p=0.002
HOMAIR	1.1 (0.8-2.0)	3.7 (2.1-6.5)	P=0.002
LIPID PROFILE			
TRG (mg/dl)	53.0 (43.0-94.0)	130.0 (69.5-157.7)	p=0.007
ApoA (mg/dl)	142 (129.0-155.0)	129 (114.0-141.0)	p=0.1
HDL (mg/dl)	48 (42.0-58.0)	38.5 (34.7-49.0)	p=0.02
NEWER METABOLIC MARKERS			
Leptin (ng/ml)	10.3 (5.8-13.8)	18.1 (10.7-31.5)	p=0.09
FGF21 (pg/ml)	97.7 (8.1-120.0)	73.55 (48.7-136.6)	p=0.7
IGFBP1 (ng/ml)	2.6 (1.0-5.0)	1.5 (1.1-2.9)	p=0.4
Adiponectin (µg/ml)	8.5 (3.5-11.0)	7.3 (5.9-8.9)	p=0.7

*Comparisons for all other variables did not show statistical significance or trend (data not shown)

Within the obese groups, a difference was observed in age with **OMU** being older in comparison to the **OMH** children [12.4 (11.4-14.6) vs. 9.9 (9.5-13.8) years, p=0.05]. In the OMU compared to OMH, an increase of serum insulin [17.7 (10.4-28.3) vs. 5.2 (3.9-9.2) µU/ml, p=0.002], TRG [130.0 (69.5-157.7) vs. 53.0 (43.0-94.0) mg/dl, p=0.007], HOMAIR [3.7 (2.1-6.5) vs. 1.1 (0.8-2.0), p=0.002] and a trend for an increase in serum leptin concentrations [18.1 (10.7-31.5) vs. 10.3 (5.8-13.8) ng/ml,

p=0.09] was observed. A significant decrease in serum HDL [38.5 (34.7-49.0) vs. 48 (42.0-58.0) mg/dl, p=0.02] and a trend for a decrease in serum ApoA [129 (114.0-141.0) vs. 142 (129.0-155.0) mg/dl, p=0.1] was observed in OMU vs OMH respectively (**Figure 26)**. No differences were found in vascular indices between the 2 groups. FMD in OMU compared to OMH [0.05 (0.03-0.08) vs. 0.06 (0.05-0.10), p=0.4] and cIMT [0.46 (0.43-0.49) vs. 0.48 (0.46-0.49)mm, p=0.2], respectively.

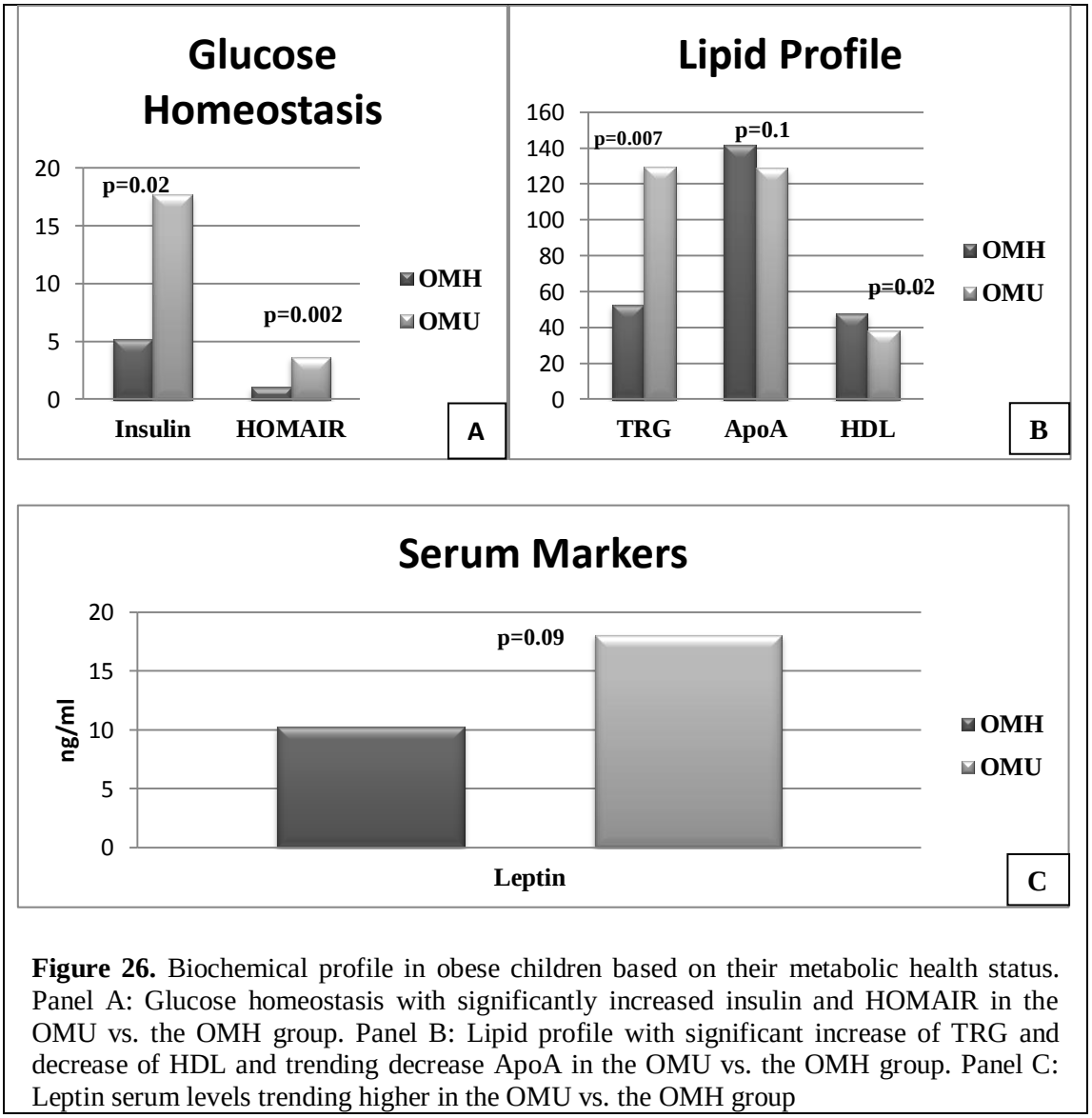

Figure 26. Biochemical profile in obese children based on their metabolic health status. Panel A: Glucose homeostasis with significantly increased insulin and HOMAIR in the OMU vs. the OMH group. Panel B: Lipid profile with significant increase of TRG and decrease of HDL and trending decrease ApoA in the OMU vs. the OMH group. Panel C: Leptin serum levels trending higher in the OMU vs. the OMH group

Finally, a comparison between the **NMH** and **OMU** groups resulted as the most meaningful comparison between the 2 far ends of the whole obesity-metabolic health spectrum (results are shown in **Table 14**). This comparison showed that except for the difference in various metabolic markers, there was a trend towards reduced FMD [0.06 (0.04-0.09) vs. 0.07 (0.04-0.12), p=0.09] and thus early endothelial dysfunction, while cIMT did not differ significantly between the 2 groups.

A significant increase was observed when OMU was compared to NMH in CRP [3 (2-7) vs. 2 (1-2) mg/dl, p=0.001)] insulin [17.7 (10.4-28.3) vs. 5.8 (4.3-7.3) μU/ml, p<0.001], ApoB/ApoA ratio [0.60 (0.40-0.75) vs. 0.44 (0.35-0.57). p=0.005], TRG [130.0 (69.5-157.7) vs. 55.0 (39.5-67.5) mg/dl, p<0.001)], WC [87.7 (57.2-72.2) vs. 65.5 (78.8-98.8 cm), p<0.001], HOMAIR [3.7 (2.1-6.5) vs. 1.3 (0.9-1.6), p<0.001], leptin [18.1 (10.7-31.5) vs. 2.8 (1.0-10.0) ng/ml, p<0.001], FGF21 [73.5 (48.7-136.6) vs. 28.7 (11.0-91.0) pg/ml, p=0.003], and a trend towards increase in ApoB [71.7 (57.8-96.1) vs. 67.4 (51.7-78.4) mg/dl, p=0.07] and ALT [17.5 (12.7-28.2) vs. 14.0 (10.5-18.5) IU/l, p=0.06). A decrease was observed for serum fT4 [0.80 (0.75-0.90) vs. 0.88 (0.82-1.0) ng/dl, p=0.03], ApoA [129.0 (114.0-141.0) vs. 145.0 (137.0-145.5) mg/dl, p=0.001], HDL [38.5 (34.7-49.0) vs. 53.0 (47.5-57.5) mg/dl, p<0.001], IGFBP1 [1.5 (1.1-2.8) vs. 3.0 (1.8-7.2) ng/ml, p=0.002], and a trend for reduced adiponectin [7.3 (5.9-8.8) vs. 8.5 (7.0-9.5) μg/ml, p=0.08] (**Figures 27** and **28)**.

Table 14. Laboratory and vascular investigations in children with normal BMI considered to be metabolically healthy (NMH) and children with obesity considered to be metabolically unhealthy (OMU).

Variables*	NMH (n=26)	OMU (n=34)	p VALUE
WC (cm)	65.5 (78.8-98.8)	87.7 (57.2-72.2)	p<0.001
GLUCOSE HOMEOSTASIS			
Insulin (µU/ml)	5.8 (4.3-7.3)	17.7 (10.4-28.3)	p<0.001
HOMAIR	1.3 (0.9-1.6)	3.7 (2.1-6.5)	p<0.001
LIPID PROFILE			
TRG (mg/dl)	55.0 (39.5-67.5)	130.0 (69.5-157.7)	p<0.001
HDL (mg/dl)	53.0 (47.5-57.5)	38.5 (34.7-49.0)	p<0.001
ApoA (mg/dl)	145.0 (137.0-145.5)	129.0 (114.0-141.0)	p=0.001
ApoB (mg/dl)	67.4 (51.7-78.4)	71.7 (57.8-96.1)	p=0.07
ApoB/ApoA ratio	0.44 (0.35-0.57)	0.60 (0.40-0.75)	p=0.005
LIVER FUNCTION TESTS			
ALT (IU/l)	14.0 (10.5-18.5)	17.5 (12.7-28.2)	p=0.06
THYROID FUNCTION TESTS			
fT4 (ng/dl)	0.88 (0.82-1.0)	0.80 (0.75-0.90)	p=0.03
NEWER METABOLIC MARKERS			
CRP (mg/dl)	2 (1-2)	3 (2-7)	p=0.001
FGF21 (pg/ml)	28.7 (11.0-91.0)	73.5 (48.7-136.6)	p=0.003
Leptin (ng/ml)	2.8 (1.0-10.0)	18.1 (10.7-31.5)	p<0.001
Adiponectin (µg/ml)	8.5 (7.0-9.5)	7.3 (5.9-8.8)	p=0.08
IGFBP1(ng/ml)	3.0 (1.8-7.2)	1.5 (1.1-2.8)	p=0.002
ENDOTHELIAL FUNCTION			
FMD	0.07 (0.04-0.12)	0.06 (0.04-0.09)	p=0.09
cIMT (mm)	0.45 (0.43-0.48)	0.46 (0.43-0.49)	p=0.77

*Comparisons for all other variables did not show statistical significance or trend (data not shown)

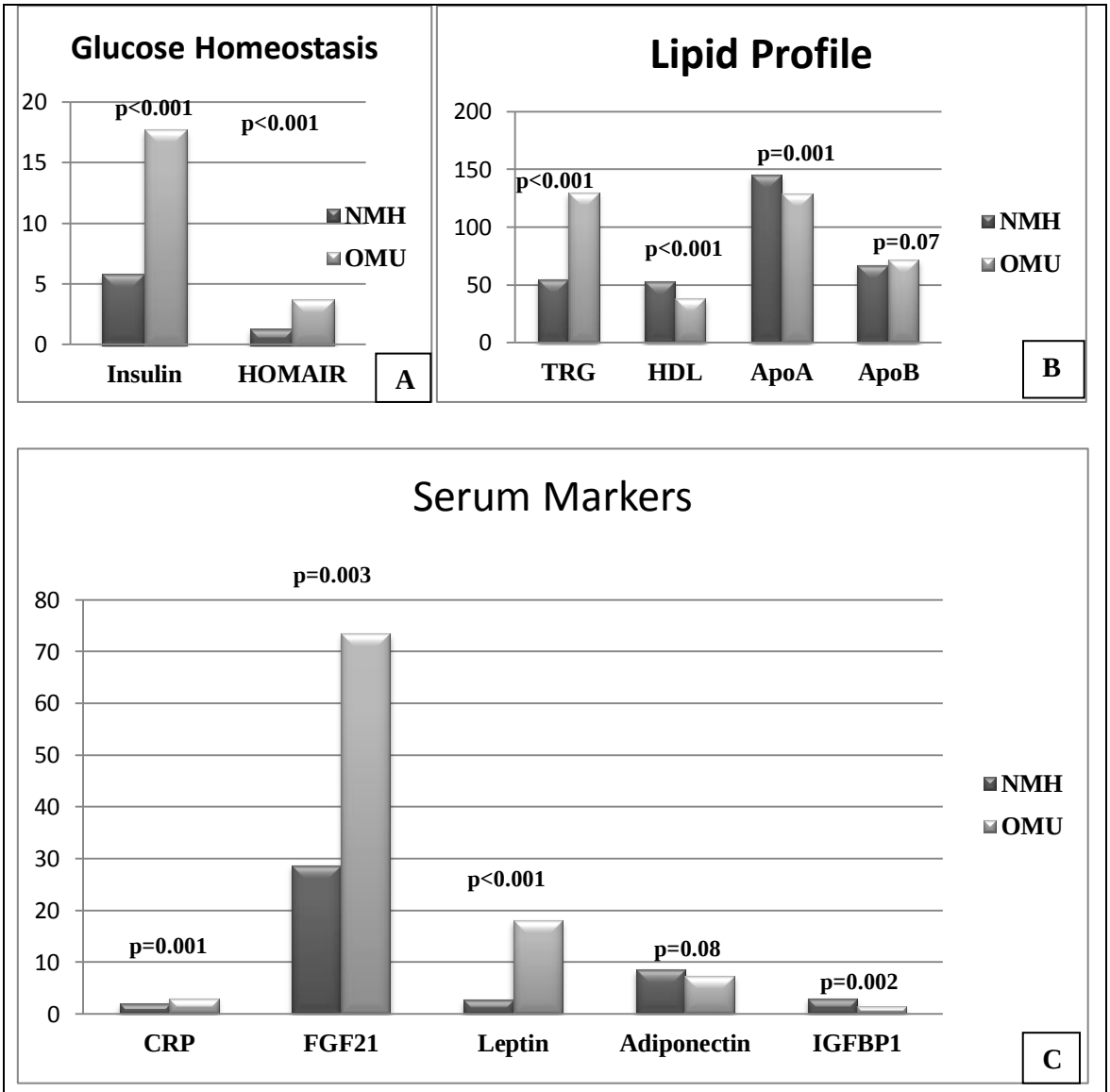

Figure 27. Biochemical profile in normal BMI metabolically healthy (NMH) children compared to obese children metabolically unhealthy (OMU). Panel A: Glucose homeostasis with significant increase in insulin and HOMAIR levels in OMU vs. NMH group. Panel B: Lipid profile with significant increase in serum TRG and a trend towards an increase in serum ApoB, significant decrease in serum HDL and ApoA in OMU vs. NMH group. Panel C: significant increase in CRP, FGF21and leptin serum levels in the OMU vs. the NMH group, significant decrease in serum IGFBP1 levels and a trend towards a decrease in serum adiponectin levels in the OMU vs. the NMH group.

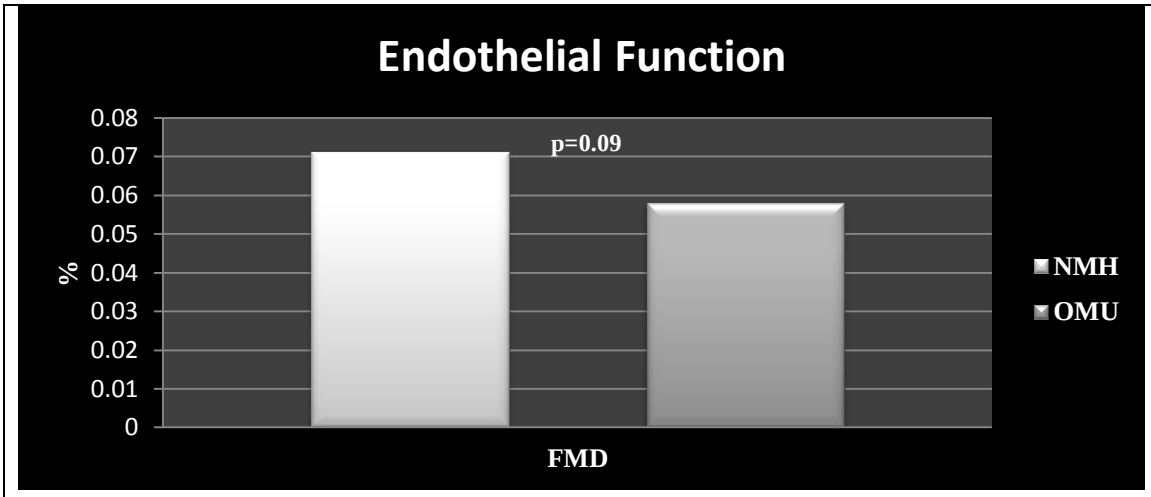

Figure 28. Endothelial function assessed using FMD; a trend towards reduced FMD in the OMU vs. the NMH group is observed.

Furthermore, we evaluated the remaining comparisons between the NMU and the OMH between the NMU and the OMU and finally between the NMU and the OMH. Results are shown in **Tables 15-17.**

Table 15. Laboratory investigations in the children with normal BMI considered to be metabolically healthy (NMH) and children with obesity considered to be metabolically healthy (OMH).

Variable*	NMH (n=26)	OMH (n=7)	p VALUE
WC (cm)	65.5 (57.3-72.3)	88.8 (68.0-95.0)	p=0.01
NEWER METABOLIC MARKERS			
Leptin (ng/ml)	2.8 (1.0-10.0)	10.3 (5.8-13.8)	p=0.09
Leptin/Adiponectin	0.36 (0.1-1.4)	2.0 (0.65-3.5)	p=0.04
Adiponectin (μg/ml)	8.5 (7.0-9.5)	8.5 (3.5-11.0)	p=0.85

*Comparisons for all other variables did not show statistical significance or trend (data not shown)

Between NMH and OMH there was a difference in WC [65.5 (57.3-72.3) vs. 88.8 (68.0-95.0) cm, p=0.01] and there was really not much other difference in any of the parameters studied except for a weak trend for increased leptin and a significant increase in the ratio leptin/adiponectin in the obese group [2.8 (1.0-10.0) vs. 10.3 (5.8-13.8)ng/ml, p=0.09 for leptin and 0.36 (0.1-1.4) vs. 2.0 (0.65-3.5), p=0.04] for leptin/adiponectin respectively). Vascular indices did not differ significantly between the two groups (data not shown).

Table 16. Laboratory investigations in the children with normal BMI considered to be metabolically unhealthy (NMU) and children with obesity considered to be metabolically unhealthy (OMU).

Variable*	NMU (n=11)	OMU (n=34)	p VALUE
WC (cm)	70.0 (65.0-73.0)	87.7 (78.8-96.8)	p<0.001
GLUCOSE HOMEOSTASIS			
Insulin (µU/ml)	9.5 (5.6-18.5)	17.7 (10.4-28.3)	p=0.06
LIPID PROFILE			
TRG (mg/dl)	71.0 (44.0-112.0)	130.0 (69.5-157.7)	p=0.02
NEWER METABOLIC MARKERS			
CRP (mg/dl)	2 (1.0-3.0)	3 (2.0-7.0)	p=0.03
Leptin (ng/ml)	3.9 (2.2-8.1)	18.1 (10.7-31.5)	p<0.001
Adiponectin (µg/ml)	8.0 (5.5-9.8)	7.3 (5.9-8.8)	p=0.6
Leptin/Adiponectin	0.37 (0.27-1.5)	2.4 (1.2-4.5)	p<0.001
IGFBP1(ng/ml)	2.3 (1.5-10.0)	1.5 (1.1-2.8)	p=0.03
ENDOTHELIAL FUNCTION			
FMD	0.09 (0.04-0.21)	0.06 (0.04-0.09)	p=0.05
cIMT (mm)	0.43 (0.38-0.49)	0.46 (0.43-0.49)	p=0.42

*Comparisons for all other variables did not show statistical significance or trend (data not shown)

The metabolically unhealthy normal weight and obese groups showed significant difference in WC [70.0 (65.0-73.0) vs. 87.7 (78.8-96.8) cm, p<0.001]. Insulin serum level was higher in the OMU vs. NMU [17.7 (10.4-28.3) vs. 9.5 (5.6-18.5) μU/ml, p=0.06]. Serum TRG levels were higher in the OMU, remaining however within the normal limits, when compared to NMU [130.0 (69.5-157.7) vs. 71.0 (44.0-112.0) mg/dl, p=0.02]. CRP was higher in the OMU vs. NMU [3 (2.0-7.0) vs. 2 (1.0-3.0) mg/dl, p=0.03]. From the biomarkers studied, leptin as well as leptin/adiponectin ratio were significantly increased in the OMU vs. the NMU group [18.1 (10.7-31.5) vs. 3.9 (2.2-8.1) ng/ml, p<0.001 and 2.4 (1.2-4.5) vs. 0.37 (0.27-1.5), p<0.001] respectively. IGFBP1 was significantly decreased in the OMU vs. the NMU [1.5 (1.1-2.8) vs. 2.3 (1.5-10.0) ng/ml, p=0.03]. FMD was decreased in the OMU vs. the NMU group [0.06 (0.04-0.09) vs. 0.09 (0.04-0.21), p=0.05] (results shown in **Table 16).**

Table 17. Laboratory investigations in the children with normal BMI considered to be metabolically unhealthy (NMU) and children with obesity considered to be metabolically healthy (OMH).

Variable*	NMU (n=11)	OMH (n=7)	p VALUE
Age (years)	13.1 (10.8-15.1)	9.8 (9.4-13.8)	p=0.05
GLUCOSE HOMEOSTASIS			
Glucose (mg/dl)	90.0 (83.0-99.0)	81.0 (74.0-88.0)	p=0.03
NEWER METABOLIC MARKERS			
Leptin (ng/ml)	3.9 (2.2-8.1)	10.3 (5.8-13.8)	p=0.08
Adiponectin (μg/ml)	8.0 (5.5-9.8)	8.5 (3.5-11.0)	p=0.96
Leptin/Adiponectin	0.37 (0.3-1.5)	2.0 (0.6-3.5)	p=0.06

*Comparisons for all other variables did not show statistical significance or trend (data not shown)

Between NMU and OMH children, it was observed that the NMU were significantly older in comparison to the OMH children [13.1 (10.8-15.1) vs. 9.8 (9.4-13.8) years, p=0.05]. Glucose serum levels were significantly higher in the NMU vs. the OMH group [90.0 (83.0-99.0) vs. 81.0 (74.0-88.0) mg/dl, p=0.03]. Serum leptin and leptin/adiponectin ratio showed a trend towards an increase in the OMH group compared to the NMU group [10.3 (5.8-13.8) vs. 3.9 (2.2-8.1) ng/ml, p=0.08 and 2.0 (0.6-3.5) vs. 0.37 (0.3-1.5], p=0.06 respectively) (results shown in **Table 17**).

Chapter 12: Metabolic Syndrome in Children

12.1 Definition

The IDF definition criteria were used to determine the diagnosis of Metabolic Syndrome MS) in our population. Children aged 10-16 years were diagnosed with MS if they had a WC ≥90th percentile, AND ≥2 from the listed criteria:

- serum TRG ≥ 150mg/dl,
- HDL <40mg/dl,
- fasting blood glucose ≥100mg/dl or known T2DM,
- SBP ≥130mmHg or DBP ≥85mmHg or treatment of previously diagnosed hypertension (82).

12.2 Results

In our population, 12 out of 78 children were diagnosed with MS; they represent 15.4% of the population studied (**Figure 29)**.

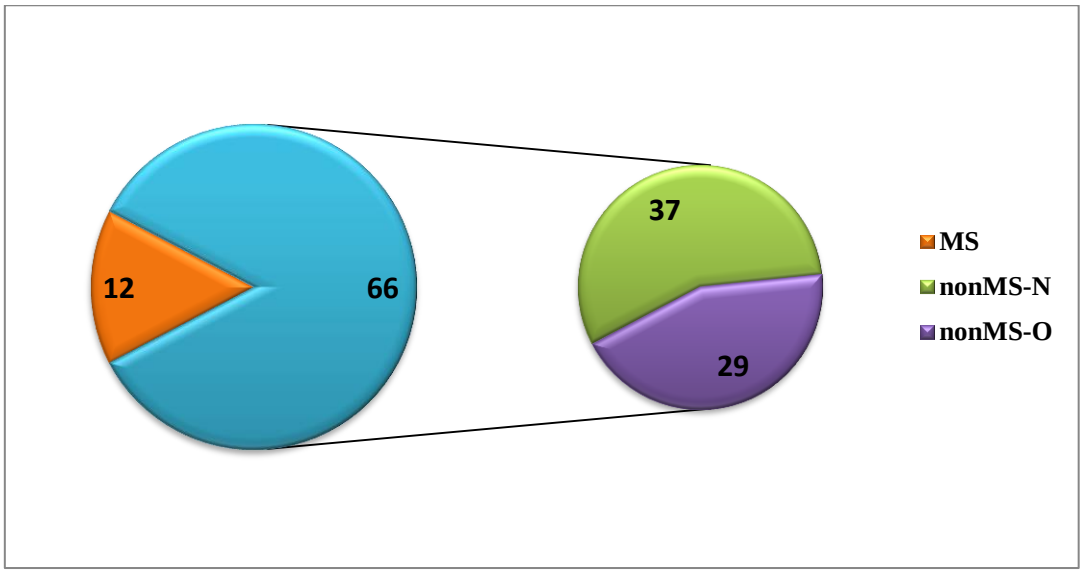

Figure 29. Twelve children from our population were diagnosed as having MS. Children without MS (nonMS) were further categorized into children with normal BMI (nonMS-N) and with increased BMI (nonMS-O).

Importantly, all of the children diagnosed with MS were obese as described elsewhere (85).

Comparisons were made in respect to the classification in four groups as follows:

1. Children with MS (MS; in our population 100% obese children)
2. Children without MS independent of BMI category (nonMS)
3. Children without MS with normal BMI, (nonMS-N)
4. Children without MS with increased BMI-Overweight/obese, (nonMS-O)

This classification was made with the specific aim to differentiate the effect of general obesity to that of central obesity as included in the definition criteria in the general population. The results are reported in Tables 18 and 19 (p values are expressed for each group in comparison to the MS group).

Table 18. Laboratory investigations in the children with MS, without MS independent of BMI (nonMS), without MS and normal BMI (nonMS-N) and without MS but overweight/obese (nonMS-O).

Variable*	MS (n=12)	nonMS (n=66)	nonMS-N (n=37)	nonMS-O (n=29)
Age (years)	12 (11.9-13.4)	13 (10.2-15.0) p=0.9	13.1 (10.4-15.3) p=0.8	12.4 (9.8-14.6) p=1.0
GLUCOSE HOMEOSTASIS				
Insulin (µU/ml)	7.4 (5.2-16.0)	23.0 (15.6-46.3) p<0.001	6.4 (4.6-9.2) p<0.001	10.2 (5.9-23.8) p=0.003
HOMAIR	4.7 (3.3-12.0)	1.6 (1.0-2.9) p<0.001	1.3 (0.9-2.1) p<0.001	2.1 (1.2-5.4) p=0.002
LIPID PROFILE				
T Chol (mg/dl)	188.0 (154.0-217.0)	161.0 (134.5-163.0) p=0.09	162.0 (134.2-193.0) p=0.1	154.0 (132.0-195.5) p=0.1
LDL (mg/dl)	119.0 (92.0-146.7)	97.0 (77.5-126..0) p=0.1	100.5 (78.0-127.0) p=0.2	95.0 (69.5-124.0) p=0.1
ApoA (mg/dl)	130.0 (112.0-139.0)	140.0 (127.7-152.5) p=0.04	145.0 (134.0-155.0) p=0.007	130.0 (126.0-134.0) p=0.2
ApoB (mg/dl)	96.1 (74.7-112.0)	70.5 (52.9-81.7) p=0.002	71.0 (52.0-81.1) p=0.001	68.8 (55.4-89.1) p=0.008
ApoB/ ApoA	0.75 (0.65-0.87)	0.49 (0.38-0.62) p<0.001	0.47 (0.37-0.60) p<0.001	0.51 (0.38-0.68) p=0.002
Lp(a) (mg/dl)	2.5 (2.5-16.5)	8.4 (4.7-21.6) p=0.04	9.3 (4.8-25.4) p=0.02	6.8 (4.8-15-9) p=0.1
LIVER FUNCTION TESTS				
ALT (IU/l)	23.5 (16.2-48.7)	15.0 (11.0-19.0) p=0.009	14.5 (11.0-18.0) p=0.005	16.0 (12.0-23.0) p=0.05

*Comparisons for all other variables did not show statistical significance or trend (data not shown)

Blood biochemistry showed a dysregulation of glucose homeostasis in MS compared to the other three groups. HOMAIR in the children with MS vs. the children without MS was found to be 4.7 (3.3-12.0) vs. 1.6 (1.0-2.9) respectively, p<0.001 (**Figure 30)**. A dysregulation in the lipid profile was also observed with lipoproteins being more affected. A significant increase was observed in ApoB and ApoB/ApoA ratio in the MS compared to the nonMS group [ApoB 96.1 (74.7-112.0) vs. 70.5 (52.9-81.7) mg/dl, p<0.001 and ApoB/ApoA ratio 0.75 (0.65-0.87) vs. 0.49 (0.38-0.62), p<0.001]. Liver assessment showed an increase in serum ALT in the MS vs. nonMS group [23.5 (16.2-48.7) vs. 15.0 (11.0-19.0) IU/l, p=0.009] (**Table 18)**. All other comparisons between parameters analyzed were not statistically significant neither presented a trend (data not shown)

Table 18. Newer metabolic markers in the children, with MS, without MS independent of BMI (nonMS), without MS with normal BMI (nonMS-N), and without MS but overweight/obese (nonMS-O).

Variable*	MS (n=12)	nonMS (n=66)	nonMS-N (n=37)	nonMS-O (n=29)
NEWER METABOLIC MARKERS				
FGF21 (pg/ml)	127.7(75.9-188.5)	60.1 (20.0-97.6) p=0.003	30.4 (13.9-92.6) p=0.002	72.3 (29.3-115.2) p=0.02
Leptin (ng/ml)	18.1 (11.0-34.8)	7.5 (1.8-16.5) p=0.003	2.8 (1.1-8.8) p<0.001	16.4 (8.1-27.6) p=0.3
Adiponectin (μg/ml)	6.4 (5.5-8.7)	8.4 (6.5-9.3) p=0.2	8.5 (6.8-9.6) p=0.1	8.0 (6.0-9.1) p=0.4
Leptin/ Adiponectin	3 2 (1.2-6.0)	1.0 (0.2-2.2) p=0.005	0.4 (0.1-1.4) p<0.001	2.2 (1.0-3.5) p=0.3
FGF21/ Adiponectin	18.5 (8.0-33.5)	6.8 (2.2-14.2) p=0.007	4.5 (1.6-12.0) p=0.003	8.2 (4.1-20.8) p=0.06
IGFBP1 (ng/ml)	1.5 (1.2-2.0)	2.3 (1.5-6.0) p=0.03	2.8 (1.8-7.9) p=0.004	2.0 (1.1-3.6) p=0.3

*Comparisons for all other variables did not show statistical significance or trend (data not shown)

Assessment of newer metabolic markers in the serum of MS vs. nonMS group showed an increase in FGF21 and leptin levels [FGF21; 127.7 (75.9-188.5) vs. 60.1 (20.0-97.6) pg/ml, p=0.003 and leptin; 18.1 (11.0-34.8) vs. 7.5 (1.8-16.5) ng/ml, p=0.003]. Adiponectin levels showed a trend to decrease (6.4 (5.5-8.7) vs. 8.5 (6.5-9.3) μg/ml, p=0.2), while IGFBP1 was found to be significantly decreased in the MS group in comparison to the nonMS group [1.5 (1.2-2.0) vs. 2.3 (1.5-6.0) ng/ml, p=0.03] (**Table 19** and **Figure 30)**.

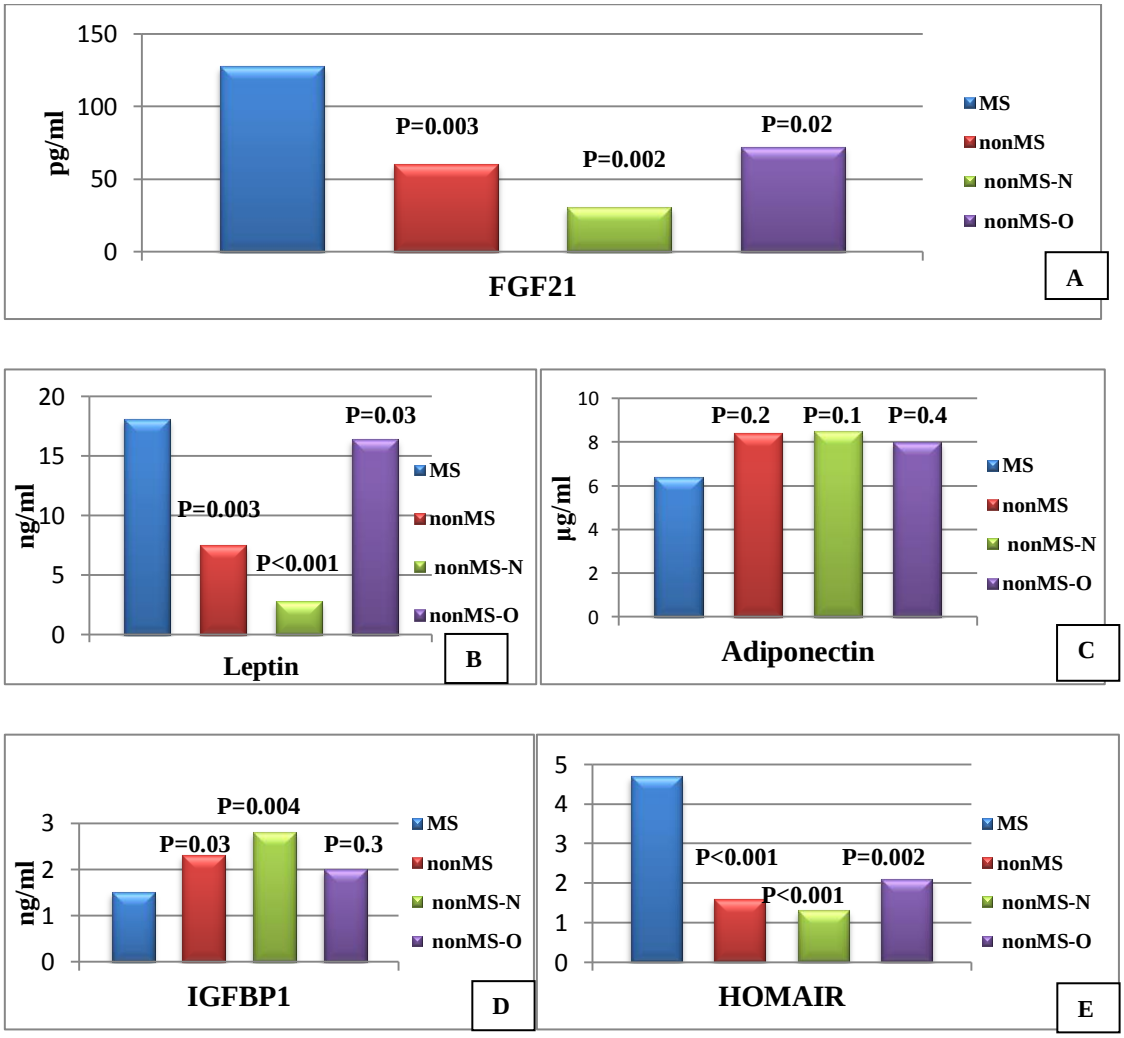

Figure 30. Serum biomarkers and HOMAIR in the MS compared to nonMS, nonMS-N and nonMS-O groups. Panel A: FGF21 is significantly increased in the MS group. Panel B: Leptin is significantly increased in the MS group. Panel C: Adiponectin shows a trend to decrease in the MS group. Panel D: IGFBP1 is decreased in the MS group. Panel E: HOMAIR is significantly increased in the MS group.

FMD was decreased in children with MS in comparison to the nonMS group [0.06 (0.03-0.07) vs. 0.07 (0.04-0.1), p=0.06]. cIMT did not show any difference between the groups. cIMT in MS vs. nonMS [0.46 (0.43-0.51) vs. 0.45 (0.42-0.48), p=0.5] (**Figure 31)**.

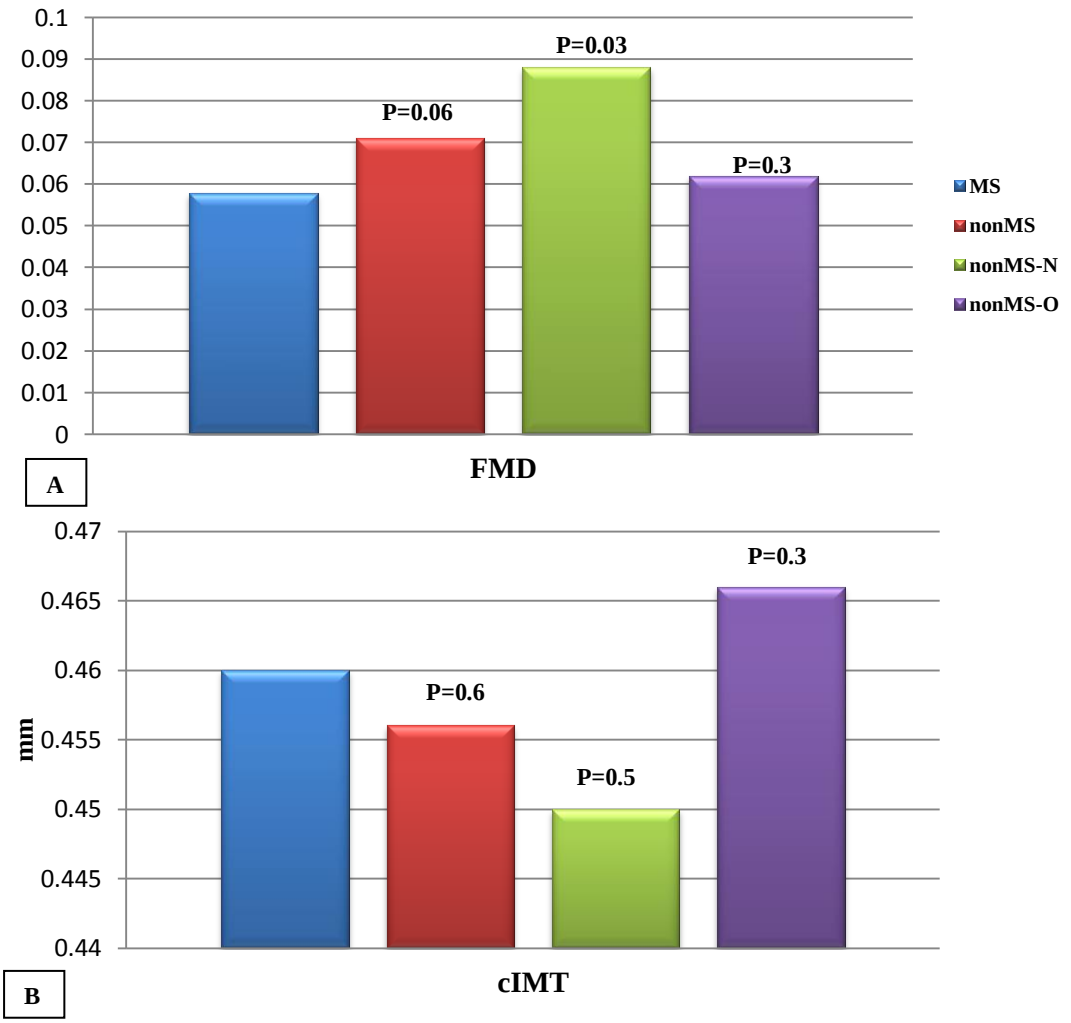

Figure 31. Endothelial function in the MS group compared to the nonMS, nonMS-N and nonMS-O groups. Panel A: FMD was significantly decreased in the MS compared to the nonMS-N group and was borderline reduced when compared to the nonMS group. Panel B: cIMT did not differ between the groups.

Chapter 13: Correlations Among Biomarkers and FMD

13.1 Definitions

To assess the strength of the relationship between our measured serum markers, endothelial function and known metabolic parameters we created scattered plots and Spearman's rho correlation coefficient was employed for this purpose.

It was proposed that MS as a dichotomous designation is not a reliable predictor of adults' MS. A cluster score was suggested to be a more powerful predictor of cardiovascular risk (329-332). Based on this cluster score it is possible to assess the MS at any age as follows (333):

1/5*((WC-77.7)/11.4 - (HDL-T.Chol-44.6)/10.2 + (TRGL-90.5)/52.9 + (SBP-107.6)/9.2 + (Glu-88.2)/7.4)

13.2 Results

In our population there was a correlation between the following parameters.

Normalized FMD

Normalized FMD negatively correlated with FGF21 (corr. Coef. 0.2, p<0.03), leptin (corr. Coef. 0.3, p=0.002) and with HOMAIR (corr. Coef. 0.3, p<0.001) (**Figure 32)**.

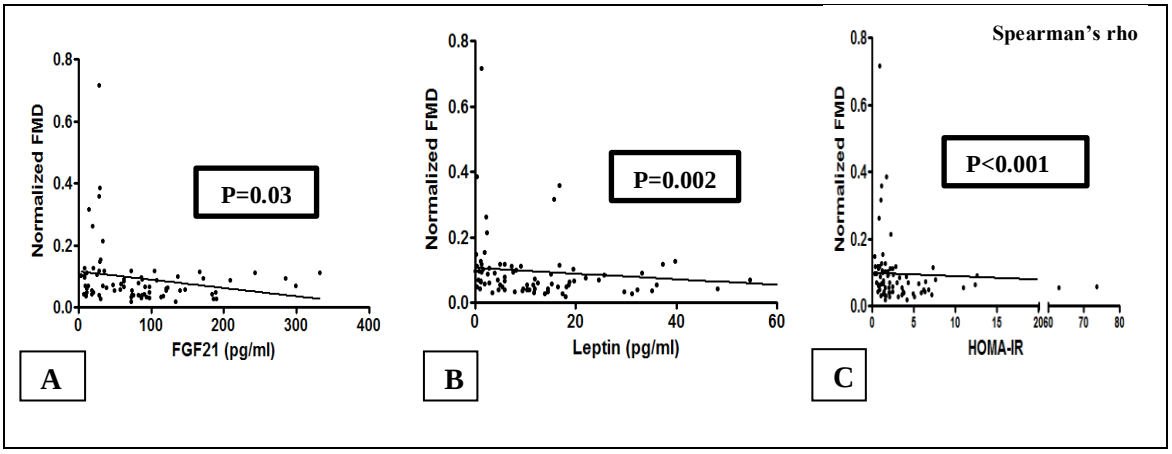

Figure 32 Correlations of normalized FMD with studied parameters. Panel A, B and C negative correlations of normalized FMD with FGF21, leptin and HOMAIR respectively

MS Cluster Score

MS cluster score positively correlated with FGF21 (corr. Coef. 0.3, p=0.01), with leptin (corr. Coef. 0.3, p=0.02) and with HOMAIR (corr. Coef. 0.5, p<0.001) (**Figure 33)**.

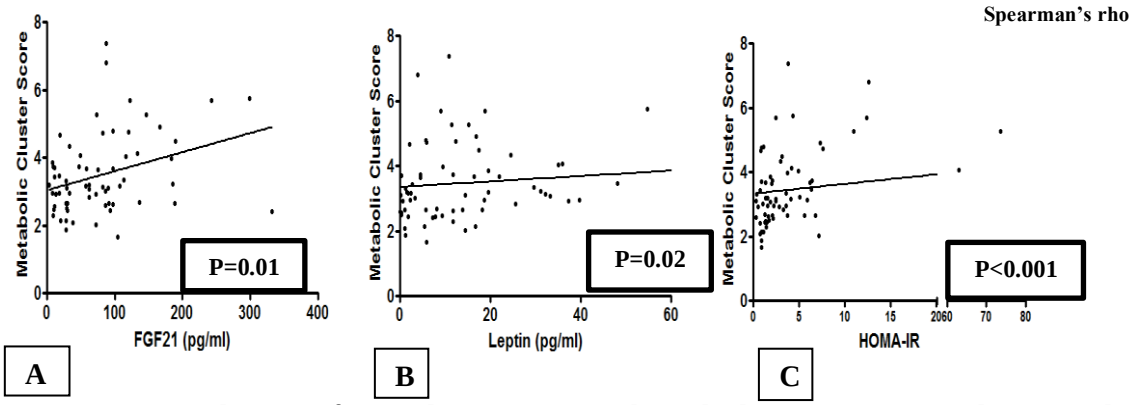

Figure 33 Correlations of MS Cluster Score with studied parameters. Panel A, B and C positive correlations of MS Cluster Score with FGF21, leptin and HOMAIR

Serum biomarkers

FGF21 correlated positively with leptin (corr. Coef. 0.3, p=0.006), with HOMAIR (corr. Coef. 0.4, p<0.001), and negatively with IGFBP1 (corr. Coef. 0.3 p=0.01) (**Figure 34)**.

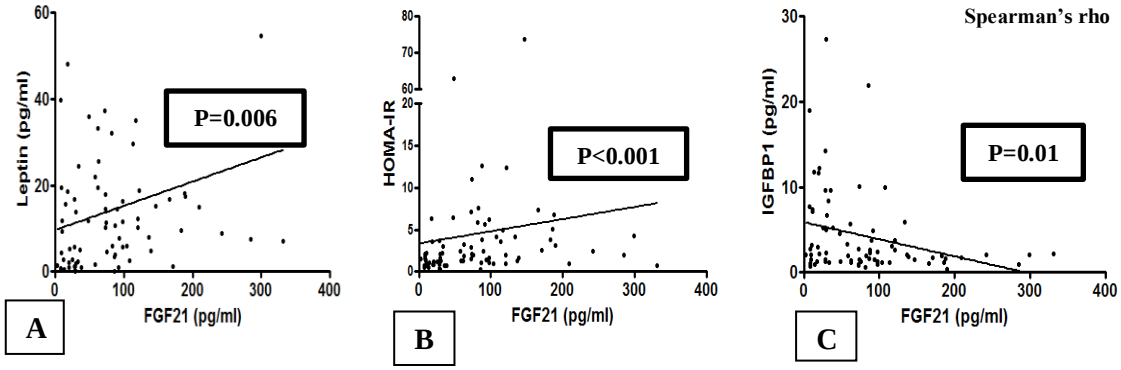

Figure 34 Correlations of FGF21 with studied parameters. Panel A and B positive correlations of FGF21 with leptin and HOMAIR respectively. Panel C negative correlation of FGF21 with IGFBP1

Leptin except for its positive correlation with FGF21 correlated also positively with HOMAIR (corr. Coef. 0.5, p<0.001) and negatively with adiponectin (corr. Coef. 0.3 p=0.01) and with IGFBP1 (corr. Coef. 0.6 p<0.001), adiponectin (corr. Coef. 0.3 p=0.01) and with normalized FMD (corr. Coef. 0.3 p=0.002) (**Figure 35)**.

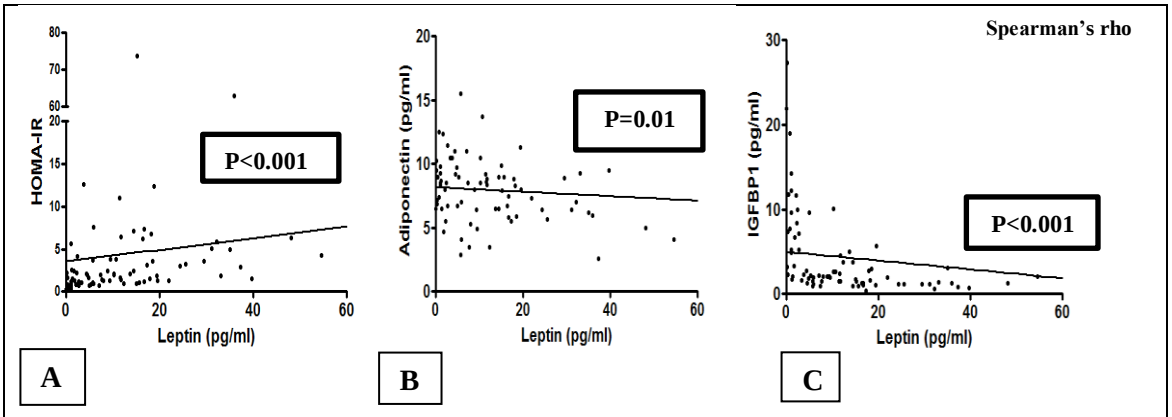

Figure 35 Correlations of leptin with studied parameters. Panel A positive correlation of leptin with HOMAIR. Panel B and C negative correlations of leptin with adiponectin and IGFBP1

IGFBP1 also negatively correlated with HOMAIR (corr. Coef. 0.4, p<0.001) (**Figure 36)**.

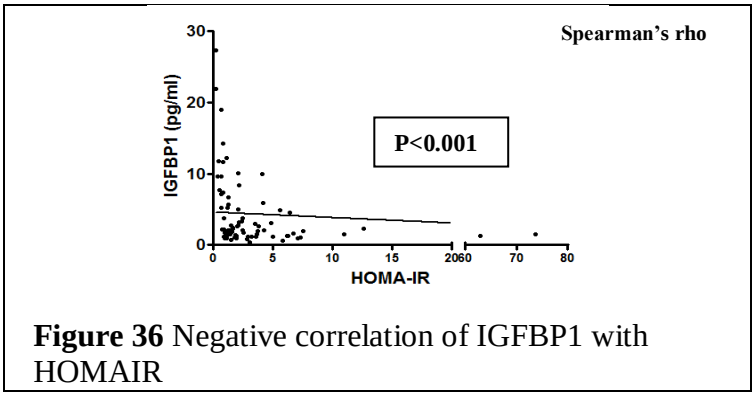

Figure 36 Negative correlation of IGFBP1 with HOMAIR

Of note, cIMT did not correlate with any of the parameters studied.

Chapter 14. Diagnostic Value of FGF21 and Leptin for Metabolic Syndrome

FGF21 and leptin both correlated with FMD and MS factors. To further explore the diagnostic value of FGF21 and leptin we applied multivariate regression analysis.

We investigated the diagnostic performance of FGF21 and of leptin. ROC analysis for FGF21 and leptin is demonstrated in Figure 37. The area under the curve (AUC) was 0.775 (p value= 0.003).

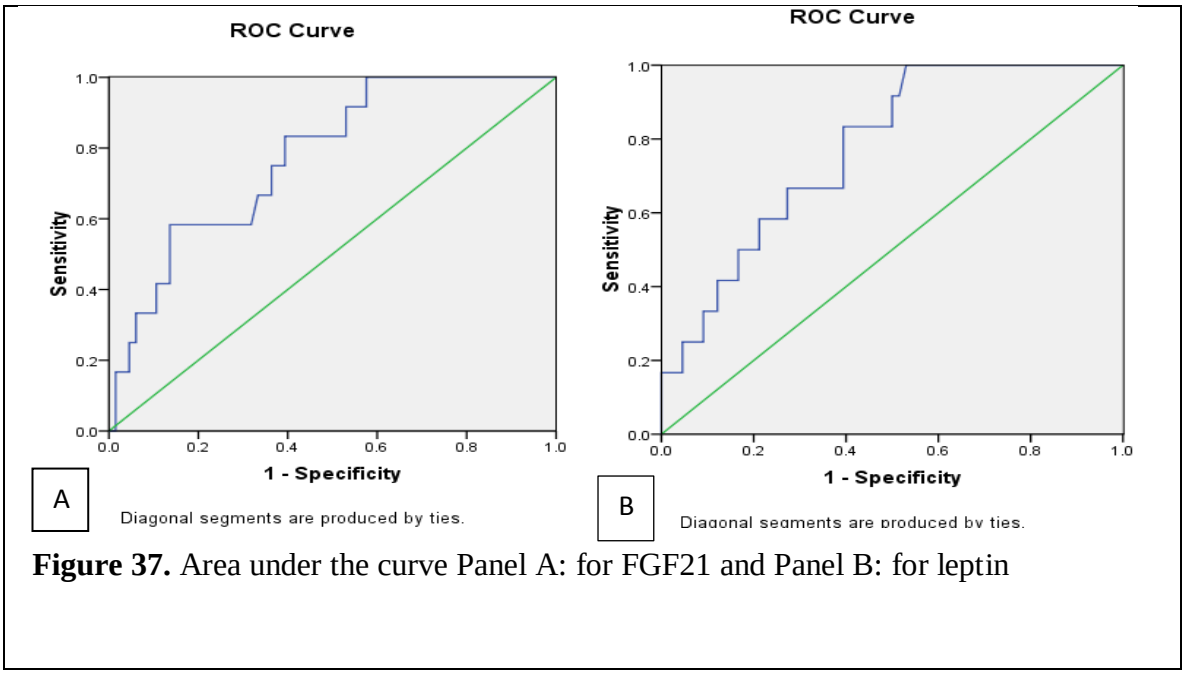

Figure 37. Area under the curve Panel A: for FGF21 and Panel B: for leptin

We identified the best cut-off value [Youden index: max (sensitivity + specificity)] of FGF21 and leptin to diagnose MS. In our population, the logistic regression analysis indicated that FGF21 levels above 121.3ng/L significantly predicted MS with sensitivity 58.3% and specificity 86.4%. Moreover, we determined that for every 10ng/L increase of FGF21 serum levels, the odds of MS increased by 12% (p= 0.006, OR 95% C.I. (1.03-1.22).

For leptin, the logistic regression analysis indicated that serum levels above 5.85ng/L significantly predicted MS with sensitivity 100% and specificity 47% and that for every 10ng/L increase of leptin serum levels, the odds of MS increased by 79% ($p< 0.001$, OR 95% C.I. (1.12-2.88).

After adjusting for BMI, age and gender, we observed that only FGF21 significantly correlated with MS while leptin was no longer correlated to MS after such adjustment. Specifically for FGF21, after adjustment for BMI, age and gender, for every 10ng/L increase in serum levels, the odds of MS increased by 10% ($p=0.04$, OR 95% C.I. 1.0-1.2).

Chapter15. Discussion

15.1 Discussion on Population Descriptives and Gender Differences

To summarize, we evaluated 78 children, 37 with normal weight and 41 overweight/obese, from northwestern Greece, aged 7 to 16 years. We further classified them into groups based on the presence or absence of MS and the state of their metabolic health.

In the whole population, we observed a possible presence of insulin resistance as the median values for HbA1c/Hb (x100)%, insulin and HOMAIR were at the highest end of normal. However, none of our children had diabetes mellitus or any other known related condition and these results could be attributed to a subtle disorder of glucose metabolism. We aimed to identify molecules that could detect even subtle metabolic disorders in healthy obese participants but also in normal weight subjects with potential underlying insulin resistance or early endothelial damage. Healthy non obese subjects may present insulin resistance associated with slight increases in glucose, insulin and triglyceride levels while increased BMI may better correlate to lipid disorders (334). The two states, insulin resistance and increased BMI, both contribute to increased risk for CVD but are not synonymous and while BMI increases are easily detectable, subtle insulin resistance states may act chronically under-detected leading thus to vascular damage.

Since the two genders were recruited in a random fashion in our population, we assessed any differences that may occur. First, the females showed a tendency for higher mean weight values and higher BMI in comparison to the boys. The female population included more obese subjects than the male population at every single year of age examined from 7 to 16, except for 12 year old males who exceeded in BMI the females. The obesity rate within gender, was higher for the females than for the males (58.3% vs. 45.0%) and the female obesity rate in the overall population was higher than the male obesity rate in the overall population (26.9 vs. 24.4%). In our population, higher rates of obesity were observed in the female gender which is the opposite of what

is usually described at a national level (31). However, evidence from regional differences in prevalence of obesity among adolescents in Greece report the boys in Epirus as having the highest rates of underweight compared to the rest of the nine geographical regions studies in 2016 (335). This is further resulting in girls exceeding boys in terms of obesity rates as well.

15.2 Discussion on Obesity Assessment

Interestingly, in our population it was observed that children with high BMI (overweight/obese) had also higher central obesity. Central obesity, as stated earlier, is known to relate to insulin resistance, lipid levels and increased blood pressure, while WC predicts CVD risk factors better than BMI (78-80). Importantly, this result indicates that obesity in childhood is linked to a pathogenic distribution of adipose tissue and constitutes a significant risk factor for metabolic and CVD.

In our obesity study, we observed a shift of glucose homeostasis towards insulin resistance in the high BMI group compared to the normal BMI group, as indicated by the increased insulin levels and the increased HOMAIR. Serum lipids turned into a more atherogenic profile with increased TRG levels and ApoB/ApoA ratio and a decrease in HDL and ApoA levels (336, 337). As expected, the inflammation marker CRP was also significantly higher in the high BMI group compared to the normal BMI group. The newer metabolic markers studied showed an interesting pattern of regulation in the high BMI group consistent to what was expected based on the literature. Our results indicate that childhood obesity is a condition in which tissues involved in major metabolic functions, liver, adipose, pancreas, are insulted. Herein we prove that in a pediatric obese population there are circulating biomarkers of tissue specific disorders involved in this condition.

Firstly, the adipose secreted hormones leptin and adiponectin were assessed. Leptin, the anorexigenic hormone, showed an up-regulation in the high BMI group in comparison to the healthy BMI group while adiponectin, the insulin sensitizing hormone, showed a trend toward lower levels as expected due to the described phenomenon of resistance

(119, 126). Secondly, IGFBP1, mainly expressed in the liver, was found to be decreased in our pediatric population with high BMI. This molecule is described to be inversely correlated to insulin resistance and to represent a possible predictor for cardiovascular damage, thus reinforcing our hypothesis that these children may have early signs of endothelial dysfunction. Furthermore, the newly discovered protein FGF21, secreted mostly by the liver, was found to be increased in the serum of the high BMI population, as expected. Interestingly, the two ratios of leptin/adiponectin and FGF21/adiponectin were significantly different in the two groups of normal BMI and high BMI. Even though adiponectin showed only a small difference between the two groups, its association with either leptin or FGF21 may provide important information on the metabolic status of the person. This confirms our hypothesis that FGF21 may be a potent biomarker and in relation to other biomarkers may constitute a diagnostic tool for the detection of early atherosclerosis.

Importantly, the obesity status in our population was related to a significant endothelial dysregulation indicating early and asymptomatic presence of cardiovascular risk factors. FMD was correlated with FGF21, leptin levels and HOMAIR and this confirms our hypothesis that FGF21, the newer metabolic regulator, accompanied by leptin and HOMAIR may represent a biomarker for early atherosclerosis in the serum of obese children.

15.3 Discussion on the Metabolic Health Assessment

In our obese population, there was a prominent presence of insulin resistance and persistent insulin resistance is known to lead to atherosclerosis. To further discriminate between obesity and insulin resistance, we adopted the metabolic health model. Indeed, in our obese population, we identified a 9% (17% of the total population studied) of children free of signs of insulin resistance and lipid imbalance. On the other hand, we also identified a 6.5% of our normal BMI population (14% of the total population studied) with signs of insulin resistance and lipid dysregulation.

15.3.1 Normal BMI Population; Metabolically Healthy and Unhealthy

Normal weight may not always represent a normal metabolism and other factors may contribute which are important to detect and evaluate. In our normal weight population, the metabolically unhealthy group showed predominant glucose homeostasis dysregulation with increased serum insulin levels and presence of insulin resistance as assessed by HOMAIR, while their lipid profile showed a trend towards imbalance with an increase in ApoB/ApoA ratio. Hormonal influence was present in this group as shown by the increase in TSH and decrease in fT4, although we were not able to further investigate it here. However, it could be part of an underlying general energy homeostasis imbalance in this group. As we have shown in mouse experiments, FGF21 and thyroid hormones show mutual regulatory dependency but have independent actions overall, thus we could hypothesize in this case that an early effect of energy imbalance through FGF21 on thyroid axis may exist (338). From the biomarkers studied in the normal BMI metabolically unhealthy children (FGF21, leptin, adiponectin and IGFBP1), only FGF21 showed a trend towards higher circulating levels. However, in this group studied, the FGF21/adiponectin ratio was significantly up-regulated and together with insulin resistance and ApoB/ApoA, these defined this specific metabolic state. Based on these results, we may speculate that the ratio FGF21/adiponectin constitutes a useful tool in detecting very early subtle metabolic dysregulations defined by imbalance in circulating biomarkers in asymptomatic normal BMI children. We may also speculate that in the absence of increased BMI, leptin, IGFBP1 and adiponectin unchanged levels may reflect the direct connection of these molecules with the state of

obesity in contrast with FGF21 that was influenced by a state of insulin resistance alone. This is further confirmed in our multivariable analysis, which showed that FGF21 is independently associated with MS (i.e. a metabolic dysregulation state), after adjusting for BMI, age and gender. In 2014 Ko et al, studied the relation of circulating levels of FGF21 to the MS in 9-year-old children. They initially showed a correlation of FGF21 with WC, triglyceride levels and HOMAIR that however was not further confirmed when data were controlled for the confounders of the MS (198). Compared to this previous study, our study enrolled different age groups and this may explain the difference in the results. This underlines the importance of appropriately evaluating the differences among age groups in childhood.

At such early stages of dysregulation we could not detect any endothelial damage as assessed by FMD and cIMT.

15.3.2 Obese Population Metabolically Healthy and Unhealthy

Obesity is a state that has been shown to have overt adverse metabolic effects and increased risk for CVD. However, not all obesity states are the same. There has been described a group of obese people who are less prone to the associated metabolic disturbances of obesity. In our study, we confirmed the existence of this differentiation in children. We observed a higher prevalence of metabolically unhealthy obesity in older children and in this group the dysregulation involved both glucose and lipid pathways. Serum biomarkers were equally dysregulated in the obese groups and did not differ between them. Only leptin showed a trend to increase in the obese metabolically unhealthy children. This result underlines firstly that different growth stages in childhood may respond differently to obesity, with older children being more prone to develop insulin resistance and lipid imbalance. Secondly, biomarkers are unable to discriminate between healthy and unhealthy obesity although to this end leptin seems to be more sensitive to TRG and HDL changes. This is further confirmed in our correlation study were leptin is shown to be positively correlated to TRG and negatively correlated to HDL levels. Based on our observations, TRGL and HDL were part of the main differences between the normal BMI and the obese condition. Specifically, in the

normal BMI unhealthy children there was a shift in the apo-lipoprotein profile, while in the obese unhealthy there was a more prominent dysregulation in TRG and HDL cholesterol. No differences in the endothelial function were observed between the two groups.

15.3.3 Normal BMI Metabolically Healthy (NMH) Compared to Obese Metabolically Unhealthy (OMU)

As expected, the comparison between the two ends of the spectrum, NMH and OMU, showed the most prominent results. In this comparison, central obesity played a pivotal role with a significant difference between the groups. This probably indicates that central obesity may reflect the most pathogenic form of obesity in a pediatric population and furthermore, that obesity in earlier ages may have a more supportive role towards the process of growing, i.e. there could be a shift of obesity from a benign to a malignant type as the growing child gradually necessitates less fat depots. Our observation regarding central obesity is consistent to what is described in the literature, where WC represents a leading feature of the MS definition in children and is also described as an independent predictor of insulin resistance, increased lipid levels and blood pressure, constituting thus a better biomarker than BMI, for CVD risk (78-80). Specifically, our OMU group of children in comparison to the NMH showed, impaired glucose homeostasis, and lipid profile imbalance with increases in TRG, ApoA, ApoB and ApoB/ApoA ratio, with concomitant decrease in HDL levels. Liver and thyroid function also showed to be weakly influenced with an increase in serum ALT and a decrease in serum fT4 that however remained within the normal range. All serum markers were strongly involved in this imbalance starting with the marker of inflammation CRP that was significantly increased although remaining within the normal range limits. FGF21 and leptin were strikingly higher in the OMU group as would be expected and IGFBP1 was down-regulated accompanied by a trend for down-regulation of adiponectin. In the comparison between these groups, there was also a trend of more impaired endothelial function in the OMU group. As we observed from our correlation study, FMD is closely linked to FGF21, leptin and HOMAIR. This leads

us to speculate that the striking effects on FGF21 and leptin metabolism in the OMU group may be closely related to the presence of endothelial insult.

15.3.4 Normal BMI Metabolically Healthy (NMH) Compared to Obese Metabolically Healthy (OMH)

Within the metabolically healthy population the obese group was also defined by higher WC confirming the presence of central obesity. The most sensitive biomarker was leptin and specifically leptin/adiponectin ratio showed a significant increase in the obese state.

15.3.5 Normal BMI Metabolically Unhealthy (NMU) Compared to Obese Metabolically Unhealthy (OMU)

The comparison within the metabolically unhealthy population, between the normal BMI and obese groups, provided helpful insight as it revealed a significantly impaired FMD in the OMU compared to the NMU. Indeed, this difference in FMD [0.06 (0.04-0.09) vs. 0.09 (0.04-0.21), p=0.05] was more prominent than the one observed between the OMU and the NMH group [0.06 (0.04-0.09) vs. 0.07 (0.04-0.12), p=0.09]. This may be related to the larger homogeneity between OMU and NMU groups than OMU and NMH groups, indicating that FMD may be more sensitive in detecting differences between strictly controlled groups. The rest of the results from this comparison indicated that the OMU children had increased central obesity, insulin and TRG levels, while of the biomarkers studied here, CRP, leptin, leptin/adiponectin and IGFBP1 significantly differed between the groups. Consistent with the literature, these findings confirm that leptin may be a sensitive marker of obesity in childhood.

15.3.6 Normal BMI Metabolically Unhealthy (NMU) Compared To Obese Metabolically Healhty (OMH)

Age was a defining difference in the NMU group when compared to the OMH group accompanied by increased serum glucose in the NMU. Regarding our earlier discussion of age as a determinant of benign obesity in children, the finding that metabolically unhealthy normal weight children were found to be older than the metabolically healthy obese children may indicate how early the protective role of age in metabolic health may be worn out. Interestingly, leptin was higher in the obese healthy group indicating that metabolic health may not influence the expression of this molecule as much as obesity does.

15.4 Discussion on the Classification Based on Metabolic Syndrome Results

In our MS group, all of the children were obese; this is also reported by Papoutsakis and associates in a Greek cohort (339). This led us speculate that the children without MS (nonMS group) may need to be further differentiated on the basis of their BMI in order to make a direct comparison between children with MS and the two different BMI categories (normal and high) in the nonMS population. This differentiation helped reveal subtle differences between MS and children who were completely free of any MS feature including obesity that was a strong cofactor in the MS group of our paediatric population.

In the MS group, there was a clear dysregulation of glucose homeostasis with increased serum insulin and presence of insulin resistance as assessed by the HOMAIR index. This was a very strong effect, consistent in all comparisons made within subgroups of our population, clearly indicating that central obesity is a key player in the dysregulation of glucose homeostasis in MS. Similar effects were observed in the lipid profile of MS children with higher circulating serum cholesterol levels in the MS compared to the nonMS group. LDL was higher in the MS group albeit without reaching statistical significance and these results may in part be explained by the fact that high TRG and low HDL levels are principal features in the definition of MS. The modified Friedewald formula indicates that LDL may decrease in the presence of low HDL but concomitant high TRG (340).

Friedewald formula

$$LDL = \text{Total Chol} - HDL - \frac{TRG}{4}$$

More accentuated effects were observed in serum lipoproteins shifting to a more atherogenic profile with an increase in ApoB and ApoB/ApoA ratio and a decrease in ApoA and Lp(a).

Liver function was also influenced with an up-regulation of ALT in the MS group versus the nonMS group. It is generally known that liver is insulted in states such as obesity or MS with the development of NASH and NAFLD (74, 167). Further assessment may be provided when necessary, by ultrasonography or MRI, to confirm the deposition of fat in the liver. A panel of biochemical markers of hepatic dysfunction in NASH/NAFLD that may diminish the need for more difficult exams, especially MRI, in small children would be extremely helpful.

Children with MS showed increased levels of FGF21 and leptin when compared to children without MS. The known effects of FGF21 in mice and humans include glucose and lipid regulation (149). Specifically, FGF21 is shown to act on white adipose tissue lipolysis, increase insulin-dependent glucose uptake in adipocytes and improve insulin resistance promoting an overall balance and supporting the metabolic homeostasis by controlling the underlying mechanisms of MS and NAFLD (150). The levels of this molecule are described to be increased in obese adults, in adults with T2DM, with NAFLD and with MS (163, 179, 341). A possible existence of resistance has been described in the obese state that in part explains how serum FGF21 levels may be increased in unfavorable conditions (161). However, in pediatric populations there is limited data on the regulation of FGF21 in the MS. In 2014, Mantzoros et al assessed this regulation in a pediatric population. The study showed no association of FGF21 in the paediatric population studied (198). This was a study on 210 children aged 9 years, thus a very specific window of age was assessed. In addition, the definition criteria for the MS used in this study were the age-modified criteria developed by the National

Cholesterol Education Program's Adult Treatment Panel III. Definition of MS based on different diagnostic criteria may be another confounding factor in studies on MS in paediatric populations. In 2012, Roth et al assessed the relation of FGF21 with obesity, MS and NAFLD in a pediatric population. Similar to the previous study, no relationship was found between FGF21 and features of the MS or NAFLD (196). This later study included 60 obese and 40 normal weight children aged 6 to 14 years and the IDF criteria for MS definition were used. However, the relation of FGF21 with features of the MS were assessed within the obese population and this may further obscure the discrimination between MS and obesity.

Leptin levels have been described to relate to FGF21 levels (196). Leptin's primary action lies in energy homeostasis and satiety perception as a key communicator that conveys signals about energy deposits in the periphery to the hypothalamus (119). The levels of this molecule are increased in obesity and this mainly reflects the role of this molecule as a messenger of energy demands from periphery to central cues of appetite (119). This increase has been previously reported to reflect a state of resistance in obesity (122, 123). There is also evidence that leptin may be involved in the process of atherosclerosis by regulating the production of different pro-inflammatory cytokines, activating immune cells and increasing oxidative stress (342, 343). The exact pathway connecting FGF21 to leptin is uncertain. However, both molecules could constitute strong markers of obesity in the paediatric population. Our study reinforces the role of leptin in MS as increased levels of leptin were found in the group of children with MS. This relationship has been little described previously; only a few studies, consistent with ours, showed that the levels of leptin were increased in children with the MS (344). In 2017, in a study in children aged 5 to 11 years in whom the IDF criteria for the diagnosis of the MS were applied, it was shown that leptin levels were significantly associated with the MS (344). In primary school children, it was also shown that leptin was the most sensitive marker able to predict the accumulation of CV risk factors and the presence of MS (345). In a Greek paediatric cohort of 1,138 healthy peri-adolescents, in whom the IDF criteria for the MS were used, leptin and adiponectin were found to independently predict the number of MS features. This finding was no longer significant after adjustment for BMI. In the framework of the Identification and

prevention of dietary and life-style-induced health effects in children and infants (IDEFICS) study, consisting of a big European cohort of 16,228 children aged 2 to 9 years, a novel definition of the MS was developed (346). In this project, higher leptin concentrations were shown to be associated with MS, even after adjusting for BMI or body fat mass confirming an early role of leptin in MS, while the association of adiponectin with MS seemed to be mediated by body fat in the specific age range studied (347).

Adiponectin is a molecule with anti-inflammatory and anti-atherosclerotic actions. In comparison to leptin, adiponectin has been described as having a protective role against vascular damage (348). On the basis of this, the leptin/adiponectin circulating levels ratio was created; this ratio expresses the levels of hyperleptinemia to hypoadiponectinemia, and has been proposed as an atherogenic index (319). In our population, adiponectin circulating levels had a trend to be reduced reflecting the overall dysregulation of serum biomarkers to a more atherogenic profile. Furthermore, in our study, both the ratio leptin/adiponectin and FGF21/leptin were found to be significantly increased in the MS compared to the nonMS group, consistent with previously reported. These findings indicate that these ratios may prove to be a useful tool in the evaluation of MS in children.

In our study, serum levels of IGFBP1 were decreased in the MS compared to the nonMS group; this finding is consistent with the known inverse correlation of IGBP1 with insulin sensitivity (141). Children with MS had decreased insulin sensitivity as assessed by HOMAIR.

Regarding arterial endothelial function that was evaluated using brachial FMD in our population, FMD showed a strong trend to decrease in children with MS in comparison to the nonMS group (0.06 (0.03-0.07) vs. 0.07 (0.04-0.1), p=0.06). Importantly, when FMD was compared between MS and nonMS-N group, the difference was more prominent [0.06 (0.03-0.07) vs. 0.09 (0.04-0.09) p=0.03]. This finding indicates the important role of FMD in detecting even subtle changes in endothelial function that become evident when both MS and obesity are taken into account to differentiate the metabolic status of our paediatric population. The evaluation of cIMT showed no

significant difference in any of the between group comparisons, indicating that this vascular index used to assess mainly structural vascular changes, may not be useful in the assessment of early vascular damage.

FMD was able to detect endothelial dysfunction in children with MS and even in some children without MS, i.e. the group free of MS but with increased BMI. These results on the vascular response combined with the results from the metabolic health assessment led us to speculate that in children, a clustering of factors may lead to endothelial damage and these are not necessarily included in the definition of the MS. Interestingly, FMD in our population correlated with the MS Cluster score, encouraging our hypotheses. Importantly, as mentioned earlier in the analysis of the studied biomarkers, only FGF21 correlated with MS independently of BMI, supporting the diagnostic value of this molecule. We showed that FGF21 predicted MS in children; in our population, for every 10ng/L increase in FGF21 serum levels, the odds of the MS increased by 10% Even though in our children, leptin was a strong marker of endothelial dysfunction correlating with MS, this correlation was lost after adjustment for BMI. In 2012, Papoutsakis et al evaluated leptin and adiponectin in a big cohort of 1,138 children, and they reported that by using the IDF criteria for MS, the correlation of these two molecules with MS was mediated by BMI, in accordance with our results for leptin (339).

Chapter 16: Limitations

The evaluation of the Tanner stage of the children who participated in our study is lacking. This information could add valuable insight and could explain better the gender differences identified in our study. In the diagram of BMI based on year of age and gender (**Figure 20**), a consistently higher BMI in the female population was found with the exception of the age of 12 for boys. The Tanner stage may have helped clarify why these boys constitute an exception to the general observation, e.g. whether this finding could be related to the growth spurt and how.

Another limitation of our study is the cohort size. A larger cohort could have provided stronger correlations and better defined differences within the various subgroups groups. Sample calculations could not be performed for the newer metabolic markers as the reference ranges are not known for the paediatric population.

Finally, this study was not designed for the longitudinal follow-up of the subjects enrolled. Indeed, it would be very interesting to follow-up these children into early adulthood and monitor their endothelial function in combination to their biomarker profile. This would allow us to directly evaluate the prognostic value of FGF21 and other metabolic markers for the development of MS and CVD in later life.

Chapter 17. Conclusions

The novelty of our study consists of the investigation of FGF21 correlation with the endothelial dysfunction. This correlation clearly shows the value of this molecule as an early marker of atherosclerosis. We have demonstrated that FGF21 is correlated to endothelial dysfunction, as assessed by normalized FMD, and thus FGF21 can be used as a biomarker of early signs of vascular damage in childhood even in subtle conditions of metabolic dysregulation. To our knowledge, this has not been previously shown in adults or children.

Moreover, we have confirmed that FGF21 is an easily measured serum metabolite with clearly different circulating levels in healthy children versus children with high BMI, chidren with MS or with imbalanced metabolic health. We propose a diagnostic cut-off value for FGF21 as we observed that 130 pg/ml corresponds to the value of serum FGF21 that has the best diagnostic accuracy for predicting the presence of the MS according to ROC curve analysis.

We were able to confirm the described homeostasis between FGF21, leptin, adiponectin and IGFBP1 by showing that FGF21 and leptin were up-regulated in the serum of children with increased BMI, with MS or metabolically unhealthy children when compared to their respective controls. Adiponectin and IGFBP1 showed the expected decrease in children with increased BMI. The FGF21/adiponectin ratio was able to discriminate the NMU group, in whom none of the two serum levels were importantly modified. Similarly, this ratio, along with the other metabolic markers studied, was proven to be helpful in defining metabolic dysregulation in clearly altered conditions such as high BMI and MS. Therefore, we propose FGF21/adiponectin ratio as a new tool that may prove to be useful in discriminating subtle metabolic dysregulation when clear dysregulation of biomarkers is not yet evident.

In conclusion, subtle metabolic dysregulation states may exist in paediatric populations, in apparently healthy subjects, even in the absence of obesity. These states of imbalance may lead to early endothelial dysfunction that can be identified with the use of serum biomarkers in correlation with FMD assessment. In our population, FGF21 was proven

to be a potent biomarker in states of metabolic dysregulation in children and to our knowledge, we are the first to report that FGF21 negatively correlates with FMD in a paediatric population. FGF21/adiponectin ratio is a useful marker in the evaluation of metabolic health in children. Finally, in children, even in absence of MS, the MS cluster score in correlation with the FMD and FGF21, may be useful to identify children at higher cardiovascular risk from their very early life. Further research and larger prospective studies are needed to show the prognostic value of these indices in childhood and adolescence as well as whether these could be used to monitor the effectiveness of targeted interventions (e.g. improved diet, regular exercise) to improve metabolic and vascular health in these sensitive populations.

ΠΕΡΙΛΗΨΗ ΔΙΑΤΡΙΒΗΣ

Νεότεροι Δείκτες Μεταβολικών και Καρδιαγγειακών Νόσων

Ελένη Μ. Δομουζόγλου

Παιδίατρος

Εισαγωγή. Η επίπτωση μεταβολικών και καρδιαγγειακών νοσημάτων όπως ο σακχαρώδης διαβήτης (ΣΔ) και η αθηροσκλήρυνση είναι ιδιαίτερα αυξημένη στις δυτικές κοινωνίες και αποτελούν σήμερα από τις συχνότερες αιτίες νοσηρότητας και θνητότητας. Η έγκαιρη διάγνωση των νόσων αυτών σε αρχικά στάδια εξέλιξής τους κατά την παιδική ηλικία αποτελεί κρίσιμο ζητούμενο για την πρόληψη αυτών των ασθενειών. Για το σκοπό αυτό αναζητούνται νέοι, μη επεμβατικοί δείκτες πρώιμης ανίχνευσης της αθηροσκλήρυνσης και των μεταβολικών νόσων. Μεταξύ αυτών μελετώνται και βιολογικοί δείκτες οι οποίοι θα μπορούν να μετρηθούν σχετικά εύκολα στον ορό των ασθενών.

Κατά τη διάρκεια της τελευταίας δεκαετίας, προέκυψε ένας νέος παράγοντας, ο *Fibroblast Growth Factor* 21 (FGF21), που φαίνεται ότι έχει πολλαπλές επιδράσεις στον μεταβολισμό. Μελετάται η μεταβολή της συγκέντρωσής του στον ορό σε διάφορες μεταβολικές ασθένειες όπως ο ΣΔ, η διαταραχή ανοχής στην γλυκόζη και το λιπώδες ήπαρ. Έχει βρεθεί ότι τα επίπεδα του FGF21 στην κυκλοφορία του αίματος είναι αυξημένα στην παχυσαρκία και συσχετίζονται με την ινσουλίνη νηστείας, το δείκτη αντίστασης στην ινσουλίνη homeostasis model assessment–insulin resistance (HOMAIR), την περιφέρεια μέσης (ΠΜ) και το δείκτη μάζας σώματος (ΔΜΣ). Πρόσφατες μελέτες έχουν δείξει ακόμη ότι ο FGF21 αυξάνεται σε ασθενείς με στεφανιαία νόσο.

Άλλες πρωτείνες όπως η λεπτίνη, η αδιπονεκτίνη ο Insulin-like growth factor binding protein 1 (IGFBP1), έχουν μελετηθεί μερικώς σε ενήλικες ως πιθανοί δείκτες μεταβολικών και καρδιαγγειακών νόσων λόγω της δράσης τους στον μεταβολισμό και

της σημαντικής μεταβολής των επιπέδων τους στο αίμα σε μεταβολικές νόσους. Ο ρόλος τους στα παιδιά παραμένει αδιευκρίνιστος.

Σκοπός της παρούσας διδακτορικής έρευνας ήταν η μελέτη νέων δεικτών όπως ο FGF21, η λεπτίνη, η αδιπονεκτίνη και ο IGFBP1 και η διερεύνηση του πιθανού ρόλου τους στη φυσιολογία και παθοφυσιολογία του μεταβολισμού και του καρδιαγγειακού συστήματος στην παιδική ηλικία. Στο ίδιο πλαίσιο διερευνήθηκε ακόμη η πιθανή συσχέτιση του FGF21 και των υπόλοιπων υποψήφιων βιοδεικτών με αγγειακούς δείκτες που θεωρούνται καθιερωμένοι δείκτες πρώιμης ανίχνευσης της αθηροσκλήρυνσης στους ενήλικες, όπως είναι η ενδοθηλιακή δυσλειτουργία, που μελετάται με την ενδοθηλιοεξαρτώμενη αγγειοδιαστολή στη βραχιόνια αρτηρία [flow mediated dilation (FMD)], και ο δείκτης πρώιμης δομικής αθηροσκλήρυνσης [πάχος έσω-μέσου χιτώνα καρωτίδων [carotid intima-media thickness (IMT)].

Μέθοδος. Μελετήθηκαν 78 υγιή παιδιά ηλικίας 7 έως 16 ετών. Καταγράφηκαν τα σωματομετρικά τους στοιχεία (βάρος, ύψος, ΠΜ, ΔΜΣ), η συστολική και διαστολική αρτηριακή τους πίεση ενώ έγινε μέτρηση βιοχημικών δεικτών στον ορό του αίματος έπειτα από ολονύκτια νηστεία. Έγιναν μετρήσεις των αγγειακών δεικτών FMD και cIMT. Καταγράφηκαν τα παιδιά που είχαν φυσιολογικό ΔΜΣ και αυτά που ήταν υπέρβαρα ή παχύσαρκα, καθώς επίσης και τα παιδιά που είχαν μεταβολικό σύνδρομο (ΜΣ) (ΜΣ, με βάση τα κριτήρια ορισμού από την International Federation of Diabetes). Επίσης διερευνήθηκε η ύπαρξη μεταβολικών διαταραχών όπως η αντίσταση στην ινσουλίνη, καθώς και η ηπατική και θυρεοειδική λειτουργία και το λιπιδαιμικό προφίλ. Σε αυτό τον πληθυσμό προσδιορίστηκαν τα επίπεδα του FGF21, της λεπτίνης, της αδιπονεκτίνης και του IGFBP1. Μελετήθηκε η πιθανή συσχέτιση των βιοδεικτών με το ΜΣ, την παχυσαρκία και με μεταβολικές διαταραχές προκειμένου να διερευνηθεί η διαγνωστική αξία των βιοδεικτών.

Αποτελέσματα. Τα επίπεδα του FGF21 βρέθηκαν στατιστικά σημαντικά αυξημένα στον ορό του αίματος των παιδιών που ήταν παχύσαρκα και των παιδιών που είχαν ΜΣ. Ο FGF21, συσχετίστηκε αρνητικά με το FMD γεγονός που υποδεικνύει ότι μπορεί να χρησιμοποιηθεί ως δείκτης ενδοθηλιακής λειτουργίας σε παιδιατρικό πληθυσμό. Ο FGF21 συσχετίστηκε επίσης με την λεπτίνη, τον IGFBP1 και τον HOMAIR καθώς και

με παράγοντες του ΜΣ όπως η ΠΜ και η HDL. Τα επίπεδα της λεπτίνης βρέθηκαν αυξημένα στον ορό του αίματος των παιδιών που ήταν παχύσαρκα και αυτών που είχαν ΜΣ. Η λεπτίνη συσχετίστηκε επίσης αρνητικά με το FMD και τον IGFBP1 και θετικά με τον FGF21 και το HOMAIR, ενώ συσχέτιση βρέθηκε και με παράγοντες του ΜΣ. Τα επίπεδα της αδιπονεκτίνης στον ορό του αίματος δεν ήταν στατιστικά σημαντικά διαφορετικά στα παχύσαρκα παιδιά και αυτά με ΜΣ σε σύγκριση με τα επίπεδα των παιδιών με φυσιολογικό ΔΜΣ και με των παιδιών χωρίς ΜΣ αντίστοιχα. Τα επίπεδα ορού του IGFBP1 βρέθηκαν μειωμένα στον ορό του αίματος των παιδιών που ήταν παχύσαρκα και των παιδιών με ΜΣ, δεν συσχετίστηκαν με το FMD αλλά συσχετίστηκαν με FGF21, λεπτίνη, HOMAIR και με παράγοντες του ΜΣ. Από τους δείκτες που μελετήθηκαν, μόνο ο FGF21 και η λεπτίνη συσχετίστηκαν με το FMD, και η σχέση αυτή παρέμεινε σημαντική έπειτα από πολυπαραγοντική μελέτη και διόρθωση για ΔΜΣ, φύλο και ηλικία, μόνο για τον FGF21. Συγκεκριμένα, για κάθε αύξηση του FGF21 κατά 10ng/L, η πιθανότητα ΜΣ αυξανόταν κατά 10%. Η διαγνωστική αξία των επιπέδων του FGF21 στον ορό του αίματος υπολογίστηκε και βρέθηκε πως τιμές μεγαλύτερες από 121.3ng/L μπορούσαν να προβλέψουν το ΜΣ. Με βάση τα παραπάνω, φαίνεται ότι ο FGF21 θα μπορούσε να χρησιμοποιηθεί ως δείκτης μεταβολικών και καρδιαγγειακών νόσων σε παιδιατρικό πληθυσμό, ενώ η λεπτίνη και ο IGFBP1 θα μπορούσαν πιθανόν να αποτελέσουν δείκτες μεταβολικών νόσων. Κανένας βιολογικός δείκτης δεν συσχετίστηκε με το cIMT, ενώ το cIMT δεν διέφερε μεταξύ των διαφόρων ομάδων παιδιών που μελετήθηκαν. Αυτό δείχνει ότι μεταξύ των δύο αγγειακών δεικτών που χρησιμοποιήθηκαν στην παρούσα μελέτη, μόνο η ενδοθηλιακή δυσλειτουργία FMD μπορεί να χρησιμοποιηθεί για τη μελέτη της αγγειακής δυσλειτουργίας καθώς επηρεάζεται πρώιμα με ήπιες διαταραχές του μεταβολισμού σε παιδιά.

Συμπεράσματα. Στον υγιή παιδιατρικό πληθυσμό ηλικίας 7-16 ετών που μελετήσαμε, τα επίπεδά του FGF21 βρέθηκαν αυξημένα στον ορό του αίματος σε παχύσαρκα παιδιά και παιδιά με ΜΣ σε σχέση τόσο με παιδιά που δεν ήταν παχύσαρκα όσο και με παιδιά που δεν είχαν ΜΣ. Στον ίδιο πληθυσμό, ο FGF21 συσχετίστηκε αρνητικά με το δείκτη ενδοθηλιακής δυσλειτουργίας FMD και η αύξησή του στο ορό μπορούσε να προβλέψει την παρουσία ΜΣ στα παιδιά.. Από τους υπόλοιπους δείκτες, η λεπτίνη και ο IGFBP1 παρουσίασαν συσχέτιση με χαρακτηριστικά του ΜΣ αλλά δεν συσχετίστηκαν

ανεξάρτητα με τον αγγειακό δείκτη και θα μπορούσαν πιθανόν να αποτελέσουν δείκτες μεταβολικών νόσων.

Περισσότερη και στοχευμένη, προοπτική έρευνα με μακροχρόνια παρακολούθηση των παιδιών απαιτείται για να δείξει αν ο FGF21 θα μπορούσε στο μέλλον να χρησιμοποιηθεί ως προγνωστικός δείκτης μεταβολικών και καρδιαγγειακών νόσων σε παιδιατρικό πληθυσμό

ABSTRACT OF THE THESIS

Newer Markers of Metabolic and Cardiovascular Diseases

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Introduction. The prevalence of metabolic and cardiovascular diseases, such as diabetes mellitus (DM) and atherosclerosis is increased especially in western societies and today these diseases, are included among the most frequent causes of morbidity and mortality. Early diagnosis of these disorders in initial stages during childhood, is essential for their prevention. For this purpose, newer noninvasive markers of early atherosclerosis and metabolic disorders are being studied. Potential candidates are biological markers that could be easily measured in the serum of the patients.

During the past decade a new molecule, Fibroblast Growth Factor 21 (FGF21), has been discovered, with multiple effects on metabolic pathways. The regulation of the serum levels of FGF21 in different metabolic disorders such as DM, insulin resistance and fatty liver, has been studied. It has been found that FGF21 serum levels are increased in obesity and that they correlate with fasting insulin, homeostasis model assessment-insulin resistance (HOMA-IR), waist circumference (WC) and body mass index (BMI). Recent studies have supported that FGF21 serum levels are found to be increased in patients with coronary artery disease.

Other proteins like leptin, adiponectin, insulin-like growth factor binding protein 1 (IGFBP1), have been partially studied in adults as potential markers of metabolic and cardiovascular disorders due to their action on metabolism and the significant regulation of their serum levels in different metabolic conditions. The role of these molecules in the children is not completely understood.

The aim of this study was to discover new markers, like FGF21, leptin adiponectin and IGFBP1 and their potential role in metabolism and cardiovascular disease in pediatric population. We also explored in a pediatric population, the possible correlation of FGF21 and the other molecules mentioned above, with markers of early vascular

dysfunction considered to be established in adults, such as brachial flow mediated dilation (FMD) and carotid intima-media thickness (cIMT).

Method. Seventy eight, healthy children aged 7 to 16 years old were included in our study. Anthropometric parameters (weight, height, WC, BMI), systolic and diastolic arterial pressure were measured and blood samples were analyzed after overnight fasting. Vascular indices FMD and cIMT were assessed. The children with normal BMI, and those who were overweight or obese as well as the children with metabolic syndrome (MS) (MS, based on the International Diabetes Federation criteria), were recorded. The presence of metabolic disorders such as insulin resistance, liver and thyroid function and the lipidemic profile have been also studied. In this population we have measured FGF21, leptin, adiponectin and IGFBP1 serum levels. We studied the potential correlation of the biomarkers with MS, obesity and with the metabolic disorders mentioned to investigate their diagnostic value.

Results. FGF21 serum levels have been found to be significantly increased in overweight/obese children and in children with MS. FGF21 has been negatively correlated with FMD indicating that it can be used as a marker of endothelial dysfunction in pediatric population. FGF21 also correlated with leptin, IGFBP1 and HOMAIR as well as with MS definition factors like WC and HDL. Leptin levels have been found to be increased in the serum of the children who were obese and those with MS. Leptin has been also found to be negatively correlated with FMD and IGFBP1 and positively with FGF21 and HOMAIR, while correlation have also been found with defining parameters of the MS. Adiponectin serum levels have not been found to be significantly different in obese children and in children with MS in comparison to the serum levels of the children with normal BMI and the children without MS, respectively. Serum levels of IGFBP1 have been found to be decreased in the serum of obese children and of children with MS, no correlation with FMD has been found but they have been correlated with FGF21, leptin and HOMAIR as well as with defining parameters of the MS. From the markers studied only FGF21 and leptin have been correlated with FMD and this correlation has remained significant after multivariable analysis and adjustment for BMI, gender and age, only for FGF21. Specifically, for

every 10ng/L increase of FGF21 serum levels, the odds for MS increased by 10%. The diagnostic value of FGF21 serum levels has been calculated and we have found that levels above 121.3ng/L significantly predicted MS. Based on these results, it has been shown that FGF21 could be used as a marker of metabolic and cardiovascular diseases in pediatric population, while leptin and IGFBP1 could constitute a marker of metabolic diseases. None of the biomarkers correlated with cIMT, while cIMT did not differ between the different groups of children studied. This indicates that between the two vascular indices used in the present study, only endothelial dysfunction assessed by FMD can be used for the evaluation of vascular dysregulation as it is influenced even in early stages, by mild metabolic disorders, in children.

Conclusions. In the healthy pediatric population aged 7 to 16 years that we studied, the levels of FGF21 were found to be significantly increased in the serum of obese children and children with MS in comparison to children who were not obese and children who did not have MS. In the same population, FGF21 negatively correlated with the index of endothelial dysfunction FMD and its increase in the serum could predict the presence of MS in children. Regarding the other biomarkers, leptin and IGFBP1 presented a correlation with features of MS but their levels did not independently correlate with the vascular index and thus these biomarkers could potentially constitute markers of metabolic diseases.

More research with larger prospective studies with long-term follow-up of children is needed to show whether FGF21 could, in the future, constitute a prognostic biomarker of metabolic and cardiovascular diseases in pediatric population.

LIST OF ABBREVIATIONS

AAP	American Academy Of Pediatrics
ALT	Alanine Aminotransferase
AMPK	Adenosine Monophsphate-Activated Protein Kinase
ApoA	Apo-Lipoprotein A
AST	Aspartate Aminotransferase
ATPIII	Adult Treatment Panel III
BAT	Brown Adipose Tisse
BMI	Body Mass Index
CAD	Coronary Artery Disease
CCA	Common Carotid Artery
CDC	Centers For Disease Control And Prevention
CHREBP	Carbohydrate Responsive Element Binding Protein
cIMT	Carotid Intima Media Thickness
CMECs	Cardiac Micruvascular Endothelial Cells
CRP	C Reactive Protein
CVD	Cardiovascular Disease
DIO	Diet Induced Obese
ERK	Extracellular Signal-Regulated Kinase
FAP	Fibroblast Activation Protein
FFA	Free Fatty Acid
FGF21	Fibroblast Growth Factor 21
FMD	Flow Mediated Dilation
fT4	Free Thyroxin
GFN	Geometric Framework For Nutrition
Glu	Serum Glucose
HbA1c	Glycosylated Hemoglobin
HDL	High Density Lipoprotein
HMGCS2	3-Hydroxy-3-Methylglutaryl-Coa Synthase 2
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance

ICAM-1	Intercellular Adesion Molecule-1
IDF	International Diabetes Federation
IGFBP1	Insulin-Like Growth Factor Binding Protein 1
Ins	Serum Insulin
IQR	Interquartile Range
ISCU	Iron-Sulfur Cluster Scaffold Homolog
KD	Ketogenic Diet
Lp(a)	Lipoprotein alpha
MeSyBePo	Metabolic Syndrome Berlin Potsdam
MHO	Metabolically Healthy Obese
MRGS	Multicentre Growth Reference Study
MS	Metabolic Syndrome
mTOR	Mammalian Target Of Rapamycin
MUO	Metabolically Unhealthy Obese
NAFLD	Non Alcoholic Fatty Liver Disease
NCEP	National Cholesterol Education Program
NCHS	National Center For Health Statistics
NEDD	Non-Endothelium-Dependent Dilation
NEFA	Non Esterified Fatty Acid
NHANESIII	National Health And Nutrition Examination Survey
NHLBI	National Heart Lung And Blood Institue
NMH	Normal weight Metabolically Healthy group
NMU	Normal Weight Metabolically Unhealthy Group
nonMS	Non Metabolic Syndrome Group
nonMS-N	Non Metabolic Syndrome Group with normal BMI
nonMS-O	Non Metabolic Syndrome Group with increased BMI
OECD	Organization For Economic Cooperation And Development
OMH	Obese Metabolically Healthy Group
OMU	Obese Metabolically Unhealthy Group
OSA	Obstructive Sleep Apnea
ox-LDL	Oxidized Low Density Lipoprotein

PCOS	Polycystic Ovary Syndrome
PCSK9	Proprotein Convertase Subtlilisin/Kexin Type 9
PDAY	Pathobiological Determinant Of Atherosclerosis In Youth
PPARα	Peroxisome Proliferator Activated Receptor Alpha
SFR	Serum Response Factor
SIRT1	Sirtuin 1
SREBP	Transcription Factor Sterol Regulatory Element-Binding Protein
T Chol	Total Cholesterole
T2DM	Type II Diabetes Mellitus
TIA	Transient Ischaemic Attack
TRG	Triglyceride
TSH	Thyroid Stimulating Hormone
VCAM-1	Vascular Cell Adhesion Molecule-1
VLDL	Very Low-Density Lipoproteins
WC	Waist Circumference
WHO	World Health Organization

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