Klotho, fibroblast growth factors 19 and 21 serum concentrations in children and adolescents with normal body weight and obesity – and their associations with metabolic parameters.

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Abstract

Background: Fibroblast growth factor 19 (FGF19), Fibroblast growth factor 21 (FGF21) and Klotho are regulators of energy homeostasis. However, in the paediatric population the relationship between obesity, metabolic disorders and mentioned factors has not been clearly investigated. We analysed serum concentrations of FGF19, FGF21 and Klotho in children and adolescents with normal body weight as well as in overweight and obese subjects – and their associations with components of metabolic syndrome and insulin resistance (IR).

Methods: The cross-sectional study conducted in the group of hospitalised children and adolescents. Laboratory investigation included serum ELISA tests for FGF19, FGF21, Klotho as well as lipid profile and oral glucose tolerance test for calculation of the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index. The clinical analysis included blood pressure measurement, body fat percentage estimation and assessing the prevalence of metabolic syndrome (MS) and its components.

Results: The study was conducted on 174 children/adolescents aged 6-17 years divided into the following groups: with normal body weight (N=48), with obesity (N=92) and overweight subjects (N=34). Klotho levels were significantly higher in the group of subjects with obesity [median 168.6 pg/ml]) than those with overweight [131.3 pg/ml] and normal body weight [116.6 pg/ml] (p=0.0334). Median serum FGF21 level was elevated in the group of patients with MS in comparison to other subjects [136.2 pg/ml vs 82.6 pg/ml, p=0.0285]. Increased Klotho concentrations were noted in patients affected by IR compared with subjects with normal insulin sensitivity [185.3 pg/ml vs 132.6 pg/ml, p=0.0282]. Multivariable model for HOMA-IR showed FGF19 as an independent predictor for IR after adjusting for the pubertal stage and BMI Z-score.

Conclusions: Klotho levels were associated with body weight status in children and adolescents. Moreover, Klotho, FGF19 and FGF21 concentrations correlated with IR status and traits of MS.

Background

Overweight and obesity in children and adolescents have become a worldwide medical problem [1, 2]. Excessive body mass promotes insulin resistance (IR) in tissues, which increases the risk of type 2 diabetes, metabolic syndrome (MS) and non-alcoholic fatty liver disease (NAFLD). All these conditions contribute to future cardiovascular risk [3] and must be actively countered. One of the central agents involved in obesity is adipose tissue and its main hormone – adiponectin – which increases insulin sensitivity [4]. However, other signalling molecules, including ones not derived from adipocytes have recently drawn attention to their role in lipid and glucose metabolism [5].

Particular interest has been given to the fibroblast growth factor subfamily 19, which includes fibroblast growth factor 19 (FGF19) and fibroblast growth factor 21 (FGF21). These hormones have been reported to regulate energy homeostasis in prolonged response to nutritional status after insulin and glucagon action. FGF19 is mainly secreted from the small intestine in response to feeding and exerts insulin-like

effects: promotes glycogen synthesis and inhibits gluconeogenesis. FGF21 is released from the liver in response to starvation and exhibits glucagon-like properties: promotes lipolysis, thermogenesis and gluconeogenesis [5, 6].

It was previously shown that adult patients with obesity and metabolic diseases present reduced serum FGF19 levels with compensatory increase in FGF21 concentrations [5, 7]. However, in the group of children and adolescents the relationship between obesity, metabolic disorders and mentioned factors has not been clearly described [8, 9].

The biological action of FGF19 subfamily molecules is mediated by a transmembrane protein Klotho, which promotes their binding to specific receptors [10]. Furthermore, the soluble form of Klotho protein can itself act as hormone detectable in blood, urine and cerebrospinal fluid [11, 12]. Klotho is one of the positive regulators of adipogenesis, however, the relationship between nutritional status and Klotho serum concentration is not certain [13, 14, 15, 16].

The goal of this study was to investigate FGF19, FGF21 and Klotho serum concentrations in children and adolescents in relation to body weight status. Moreover, we aimed to evaluate the association between the factors mentioned above and occurrence of IR as well as MS and its components. Finally, we assessed the relationship between measured proteins` concentrations and IR.

Methods

Participants

This was a cross-sectional observation based of series of patients aged 6–17 years hospitalized between years 2015–2019 in the Department of Gastroenterology, Allergology and Paediatrics, Polish Mother's Memorial Hospital – Research Institute in Lodz, Poland. All patients with obesity and overweight subjects (identified by ICD code) were invited to participate in the study. Non-obese children and adolescents were included as a convenience sample (based on guardian`s consent and lack of contraindications to participate). Exclusion criteria included: admission due to acute conditions (trauma, infection, exacerbation of chronic disease), chronic inflammatory diseases, endocrine disorders (e.g., hyper and hypothyroidism, pituitary hormone deficiency, diabetes type 1, adrenal insufficiency, Cushing's syndrome), malignancy and/or current use of medications that may influence on body composition or glucose and lipid metabolism (e.g., thyroid medication, metformin, steroids). Parents and children ≥ 16 years old provided written informed consent before participation. The study was approved by local bioethical committee (PMMH-RI 39/2015).

Anthropometric measurements, blood pressure and puberty development assessment

All participants underwent measurements of body weight [kg], height [cm] (Radwag WPT 60/150 OW), waist circumference [cm], subscapular and triceps skinfold thickness [mm] (MSD Skin Fold Meter). Body

Mass Index (BMI) was calculated according to formula: weight/height^2 and transformed into Z-scores and percentiles based on national growth charts [17, 18]. Based on the 85th and 95th BMI percentile cut-off the group was divided into participants with normal body weight, overweight and obese subjects. Body fat content in % (BF%) was estimated by Slaughter`s equation [19]. However, due to the lack of modern and population-specific growth charts, BF% was not transformed into Z-scores. We also decided against standardizing BF% to body surface or other metrics to preserve it a simple and easily interpretable parameter. During the physical examination the blood pressure (systolic, diastolic) measurements were performed employing a standard procedure (auscultatory, aneroid non-mercury manometer) and interpreted using country-specific centile charts [20]. The diagnosis of arterial hypertension was based on the three measurements performed on different occasions. Physical development was assessed using the Tanner scale (from 1 to 5) [21].

Blood sampling and laboratory analyses

Venous blood samples were drawn after 12 h of fasting into standard vacuum tubes. Low-density lipoprotein cholesterol (LDL-C) was measured directly by a two-step reaction. Triglycerides (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were analysed using enzymatic colorimetric assays. Enzymatic activity of alanine aminotransferase (ALT) was measured by akinetic method. Plasma glucose was determined by oxidase method. All these assays were performed using the Vitros 5.1FS or 4600 platforms (Ortho Clinical Diagnostics, USA). Electrochemiluminescence was used to measure insulin serum levels (Cobas e 601, Roche Diagnostics, USA).

Furthermore, each child underwent standard 2-hours oral glucose tolerance test (OGTT) using 1.75 g glucose/kg (max. 75 g). After 2 hours plasma glucose between 7.8 mmol/L (140 mg/dL) and 11.1 mmol/L (200 mg/dL) was interpreted as impaired glucose tolerance.

Serum samples for FGF19, FGF21 and Klotho analysis were immediately stored at -80 °C until analysis. They were thawed at room temperature only once for the measurement. FGF19 and FGF21 concentrations were measured with Human FGF19 and FGF21 ELISA Kits (BioVendor, Brno, Czech Republic) according to the manufacturer`s instructions with an ELISA reader iMARK™ (Bio-Rad) at a wavelength of 450 nm. According to the manufacturer no cross-reactivity with human FGF19, FGF21 and FGF23 was observed. The limits of detection for FGF19 and FGF21 were 4.8 pg/ml and 7.0 pg/ml respectively.

The double-antibody sandwich ELISA Kit was used to determine Klotho concentrations in serum (ELISA Kit for Klotho SEH757Hu, Cloud-Clone Corp, Houston, TX, USA). The analysis was performed according to the manufacturer`s instructions with an ELISA reader iMARK™ (Bio-Rad) at a wavelength of 450 nm. The manufacturer reported no significant cross-reactivity or interference between Klotho and analogues. The detection range was 15.6–1,000 pg/ml.

The levels of the FGF19, FGF21 and Klotho below the detection ranges were described as 0.

Insulin resistance and metabolic syndrome diagnosis

IR was assessed by calculation of Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index according to the following formula: fasting insulinemia (μU/ml) × fasting glycemia (mmol/l)/ 22.5. Excessive IR was diagnosed when HOMA-IR exceeded 2.67 in boys and 2.22 in girls in prepubertal period and 5.22 in boys and 3.82 in girls respectively in pubertal period [22]. MS diagnosis was based on the International Diabetes Federation criteria from 2007: visceral fat obesity (waist circumference ≥ 90th percentile) plus any two of the other four factors (elevated TG ≥ 150 mg/dl), reduced HDL-C concentration (HDL-C < 40 mg/dl), elevated arterial blood pressure (≥ 95th centile or systolic ≥ 130 mmHg, diastolic ≥ 85 mmHg), elevated fasting glycemia (≥ 100 mg/dl) [23]. According to the above-mentioned criteria, in the group of children less than 10 years, MS was not recognized.

Statistical analysis

Normal weight, overweight and obese groups were compared in terms of clinical characteristics and concentrations of FGF19, FGF21 and Klotho proteins with Kruskal-Wallis ANOVA with post-hoc Dunn tests. Data were presented as medians and 25–75% ranges. The relationships between continuous variables and concentrations of FGF19, FGF21 and Klotho proteins were assessed by Spearman`s R coefficients. The frequencies of important metabolic outcomes (presence of arterial hypertension, dyslipidaemia, MS etc) were noted for each group and compared (with normal weight group used as reference) by 0dds Ratios with 95% confidence intervals (95%CI). The concentrations of investigated proteins were then compared between patients with and without specific conditions with Mann-Whitney U tests.

The relationship between measured proteins` concentrations and IR was evaluated using multivariate linear regression with HOMA-IR (log-transformed with base 10) as a continuous outcome. The initial predictors included gender, age, Tanner stage, BMI Z-score, BF% and FGF19, FGF21 and Klotho serum concentrations. After univariate assessment, BF% was discarded due to high correlation with BMI Z-score and FGF21 as well as Klotho were discarded due to nonsignificant association with HOMA-IR. Age and gender were forcefully retained in the model, despite no significant association with the outcome. For clarity sake, physical development (Tanner stage) was recoded as 1 for stage III and 0 otherwise. The final model was constructed using stepwise forward regression and its performance in predicting HOMA-IR was expressed by adjusted R^2 values. All calculations were performed with Statistica 13.1 (Statsoft) software.

Results

Group characteristics

From 5058 subjects aged 6–17 years hospitalized in the study period, 174 children/adolescents (45.4% boys) at median age 12.10 years were enrolled in the study after taking into account excluding criteria and positive participation consent. Based on the 85th and 95th BMI percentile cut-off the group was divided into participants: with normal body weight (N = 48, 35.4% boys), with obesity (N = 92, 50% boys)

and overweight subjects (N = 34, 47.1% boys). Gender structure was similar across all three subgroups (p = 0.2525).

The anthropometric and biochemical features of the studied group were presented in Table 1. Notably, the groups were similar in terms of age (p = 0.3812) and physical development stage (p = 0.8710). The groups differed significantly in terms of metabolic conditions – the components of MS as well as other abnormalities were more prevalent in overweight and obese patients than those with normal weight (Fig. 1). The frequency of impaired glucose tolerance state was similar across the groups and neither overweight [OR = 2.94 (95%CI: 0.26–33.78)] nor obesity [OR = 2.70 (95%CI: 0.31–23.8)] were associated with significantly increased risk. The groups demonstrated significant differences in IR measured by HOMA-IR (Table 1). However, only obesity significantly increased the risk of IR assessed after taking into account reference value for sex and age [exceeded in 41.3% of patients with obesity vs 6.3% of those with normal weight, OR = 10.56 (95%CI: 3.05–36.48)] (Fig. 1).

Table 1

Characteristics of the study population.

Variable	Normal weight (N = 48) median (25–75%)	Overweight (N = 34) median (25–75%)	Obesity (N = 92) median (25–75%)	p-value
Age [years]	13.6 (10.1 to 15.7)	12.0 (9.9 to 15.9)	12.0 (9.7 to 14.3)	0.3812
Tanner score	2 (2 to 4)	2 (1 to 4)	2 (2 to 4)	0.8710
BMI [kg/m^2]	18,4 (16.6 to 20.3)	24.1 (22.4 to 25.7)	27.4 (25.9 to 30.6)	N/A
BMI z-score	-0.1 (-0.6 to 0.4)	1.5 (1.3 to 1.6)	2.1 (1.8 to 2.3)	N/A
BMI percentile	45.8 (26.4 to 66.0)	92.7 (90.5 to 94.3)	98.0 (96.7 to 99.0)	N/A
Body fat [%]	23.9 (20.1 to 27.3)	37.7 (32.9 to 47.0)	45.2 (39.2 to 52.9)	< 0.0001[1]
HOMA-IR	1.8 (1.1 to 2.7)	2.5 (2.1 to 3.2)	3.2 (2.1 to 5.1)	< 0.0001[2]
TC [mg/dl]	151.5 (138.0 to 174.0)	161.5 (148.0 to 183.0)	153.0 (135.0 to 175.0)	0.176
HDL-C [mg/dl]	53.0 (49.0 to 59.5)	47.5 (42.0 to 54.0)	44.0 (38.0 to 51.0)	< 0.0001[3]
LDL-C [mg/dl]	79.0 (69.5 to 97.5)	90.5 (74.0 to 102.0)	89.0 (75.0 to 106.5)	0.1721
TG [mg/dl]	77.5 (55.5 to 95.5)	103.0 (62.0 to 135.0)	104.5 (79.5 to 128.0)	0.0007[4]
Klotho [pg/ml]	116.6 (38.5 to 163.9)	131.3 (78.0 to 313.0)	168.6 (90.2 to 375.9)	0.0334[5]

N- number of subjects, N/A-not applicable, BMI – Body Mass Index, HOMA-IR – Homeostatic Model Assessment of Insulin Resistance, TC – total cholesterol, HDL-C - high-density lipoprotein cholesterol, LDL-C - low-density lipoprotein cholesterol, TG – triglycerides, FGF19 – fibroblast growth factor 19, FGF21 - fibroblast growth factor 21.

1- Post-hoc comparisons significant between normal weight and overweight ($p < 0.0001$), normal weight and obesity ($p < 0.0001$), and overweight and obesity ($p = 0.0030$) groups

2- Post-hoc comparisons significant between normal weight and obesity ($p < 0.0001$) and normal weight and overweight ($p = 0.0435$)

3- Post-hoc comparison significant only between normal weight and obesity ($p < 0.0001$)

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5- Post-hoc comparison significant only between normal weight and obesity ($p = 0.0282$)

Variable	Normal weight (N = 48) median (25–75%)	Overweight (N = 34) median (25–75%)	Obesity (N = 92) median (25–75%)	p-value
FGF19 [pg/ml]	232.8 (126.0 to 340.5)	167.6 (118.2 to 276.6)	160.6 (87.5 to 260.2)	0.0563
FGF21 [pg/ml]	82.4 (31.6 to 128.2)	87.3 (47.1 to 181.4)	89.3 (43.3 to 193.2)	0.3783
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BMI [kg/m^2]	18,4 (16.6 to 20.3)	24.1 (22.4 to 25.7)	27.4 (25.9 to 30.6)	N/A
BMI z-score	-0.1 (-0.6 to 0.4)	1.5 (1.3 to 1.6)	2.1 (1.8 to 2.3)	N/A
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Body fat [%]	23.9 (20.1 to 27.3)	37.7 (32.9 to 47.0)	45.2 (39.2 to 52.9)	< 0.0001[1]
HOMA-IR	1.8 (1.1 to 2.7)	2.5 (2.1 to 3.2)	3.2 (2.1 to 5.1)	< 0.0001[2]
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The groups also presented significant discrepancies in the cardio-vascular profiles (Table 1, Fig. 1). Arterial hypertension was also more prevalent in both – overweight patients [38.2% vs 8.3%, OR = 6.81 (95%CI: 1.98–23.42)] and subjects with obesity [50.0% vs 8.3%, OR = 11.00 (95%CI: 3.65–33.12)].

FGF19, FGF21 and Klotho levels analysis

The proteins' concentrations were found not to correlate strongly with child's age (Klotho - R = 0.053, p = 0.4848; FGF19 - R = 0.064, p = 0.3996; FGF 21 - R = 0.159, p = 0.0360, weak association) (Table 2). Neither they were associated with the patients' sex [Klotho – males 140.6 pg/ml (88.7 to 323.1) vs 136.8 pg/ml (72.0 to 297.0), p = 0.9674; FGF19 – males 150.6 pg/ml (85.9 to 299.7) vs 197.7 pg/ml (123.5 to 279.3), p = 0.1125; FGF21 – males 85.1 pg/ml (42.2 to 160.9) vs 89.3 pg/ml (42.8 to 174.2), p = 0.5915] or stage of physical development (Klotho – p = 0.1838; FGF19 – p = 0.4569; FGF21 – p = 0.1306). The studied proteins were also independent of each other for a given patient (Fig. 2A).

Table 2

Klotho, FGF19, FGF21 concentrations in correlation with age, parameters of nutritional status, lipid profile, ALT and HOMA-IR.

	Klotho	FGF19	FGF21
Age	R = 0.053, p = 0.4848	R = 0.064, p = 0.3996	R = 0.159, p = 0.0360
BMI Z-score	R = 0.188, p = 0.0129	R=-0.206, p = 0.0062	R = 0.111, p = 0.1423
BF%	R = 0.210, p = 0.0055	R=-0.143, p = 0.0600	R = 0.202, p = 0.0020
ALT	R = 0.184, p = 0.0149	R=-0.230, p = 0.0022	R = 0.015, p = 0.8413
TC	R = 0.097, p = 0.1997	R=-0.040, p = 0.5913	R=-0.050, p = 0.5057
HDL-C	R = 0.084, p = 0.2649	R = 0.122, p = 0.1076	R=-0.212, p = 0.0047
LDL-C	R = 0.211, p = 0.0051	R=-0.030, p = 0.6852	R = 0.087, p = 0.2504
TG	R=-0.014, p = 0.8507	R=-0.201, p = 0.0077	R = 0.187, p = 0.0131
HOMA-IR	R = 0.129, p = 0.0879	R=-0.313, p < 0.0001	R = 0.098, p = 0.1978

HOMA-IR - Homeostatic Model Assessment of Insulin Resistance, BMI – Body Mass Index, BF% - body fat [%], TC - total cholesterol, LDL-C - low-density lipoprotein cholesterol, HDL-C- high density lipoprotein cholesterol, TG - triglycerides, ALT- alanine aminotransferase, FGF19 – fibroblast growth factor 19, FGF21 - fibroblast growth factor 21.

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HOMA-IR	R = 0.129, p = 0.0879	R=-0.313, p < 0.0001	R = 0.098, p = 0.1978

HOMA-IR - Homeostatic Model Assessment of Insulin Resistance, BMI – Body Mass Index, BF% - body fat [%], TC - total cholesterol, LDL-C - low-density lipoprotein cholesterol, HDL-C- high density lipoprotein cholesterol, TG - triglycerides, ALT- alanine aminotransferase, FGF19 – fibroblast growth factor 19, FGF21 - fibroblast growth factor 21.

The proteins' profiles showed weak associations with children`s body mass [Klotho – R = 0.19, p = 0.0129 (weak association), FGF19 – R=-0.21, p = 0.0062 (weak association), FGF21 – R = 0.11, p = 0.1423 (no association) and adiposity [Klotho – R = 0.21, p = 0.0055 (weak association), FGF19 – R=-0.14, p = 0.0600 (weak, not significant), FGF21 – R = 0.20, p = 0.0020 (weak association)] (Table 2). Division by body weight status (subjects with normal body weight, overweight or obese patients – Fig. 2B-D; Table 1) showed significant impact on Klotho concentration (p = 0.0334). The discrepancy was greatest among those with obesity [median concentration 168.6 pg/ml (90.2 to 375.9)] and normal body weight [median 116.6 pg/ml (38.5 to 163.9)] (post hoc p = 0.0282). The differences between overweight and obese patients (post hoc p = 1.0000) as well as overweight subjects and subjects with normal weight (post hoc p = 0.3633) were not significant. Furthermore, there were several significant associations with lipid profiles (Klotho x LDL-C – R = 0.21, p = 0.0051; FGF19 x TG – R=-0.20, p = 0.0077, FGF21 x HDL-C – R=-0.21, p = 0.0050, FGF21 x TG – R = 0.190, p = 0.0131) and ALT levels (FGF19 x ALT - R=-0.230, p = 0.0022, Klotho x ALT R = 0.184, p = 0.0149) (Table 2, Fig. 3).

Among the three proteins, only FGF19 showed significant association with HOMA-IR index [Klotho – R = 0.13, p = 0.0879, FGF19 – R=-0.31, p < 0.0001, FGF21 – R = 0.10, p = 0.1978)] (Table 2). However, dividing children by sex- and physical development-adjusted targets demonstrated that those with high IR presented higher concentrations of Klotho [185.3 pg/ml (102.1 to 398.2) vs 132.6 pg/ml (63.9 to 275.6),

p = 0.0282] and lower concentrations of FGF19 [143.0 pg/ml (81.5 to 229.3) vs 195.6 pg/ml (118.2 to 297.8), p = 0.0233] (Table 3).

Table 3

Median (IQR) serum values of Klotho, FGF19 and FGF21 in relations to the occurrence of metabolic syndrome and its components as well as insulin resistance and impaired glucose tolerance.

Subjects:	**Klotho [pg/ml]**	**FGF19 [pg/ml]**	**FGF21 [pg/ml]**
With (N = 116) vs without (N = 58) central obesity	156.4 (89.6 to 350.9) vs 118.5 (48.4 to 177.5) p = 0.0275	160.6 (92.2 to 260.2) vs 229.4 (119.5 to 345.0) p = 0.0264	93.0 (48.8 to 199.5) vs 70.1 (31.5 to 108.6) p = 0.0193
With (N = 32) low vs normal (N = 142) HDL	156.4 (92.4 to 266.7) vs 134.4 (75.5 to 335.8) p = 0.8202	147.6 (79.4 to 257.8) vs 194.2 (112.4 to 289.2) p = 0.1727	100.3 (48.8 to 165.1) vs 84.7 (42.1 to 161.0) p = 0.5064
With (N = 23) high vs normal (N = 151) TG	134.2 (90.7 to 321.1) vs 140.3 (75.5 to 313.0) p = 0.5620	124.3 (79.3 to 213.8) vs 184.9 (112.4 to 297.8) p = 0.0136	124.6 (78.6 to 363.5) vs 81.1 (39.5 to 151.3) p = 0.0035
With (N = 63) vs without (N = 111) arterial hypertension	157.5 (90.2 to 321.1) vs 136.0 (63.9 to 313.0) p = 0.1975	145.7 (93.3 to 244.5) vs 197.7 (101.1 to 299.7) p = 0.1085	124.6 (61.3 to 260.3) vs 75.2 (39.4 to 115.3) p = 0.0004
With (N = 18) vs without (N = 156) metabolic syndrome	172.7 (123.0 to 321.1) vs 135.0 (75.1 to 305.0) p = 0.2050	133.0 (62.2 to 171.2) vs 186.5 (109.2 to 284.7) p = 0.0509	136.2 (86.5 to 239.9) vs 82.6 (41.8 to 152.4) p = 0.0286
With (N = 8) vs without (N = 166) impaired glucose tolerance	159.6 (131.8 to 208.3) vs 136.4 (75.5 to 323.1) p = 0.4948	86.9 (65.4 to 149.4) vs 183.8 (106.0 to 289.2) p = 0.0416	107.2 (66.2 to 216.8) vs 86.4 (42.2 to 161.0) p = 0.4767
Insulin resistant (N = 48) vs insulin sensitive (N = 126)	185.3 (102.1 to 398.2) vs 132.6 (63.9 to 275.6) p = 0.0283	143.0 (81.5 to 229.3) vs 195.6 (118.2 to 297.8) p = 0.0233	91.9 (44.5 to 159.0) vs 82.6 (42.2 to 161.0) p = 0.6314
MS-metabolic syndrome, FGF19 – fibroblast growth factor 19, FGF21 - fibroblast growth factor 21.			
Significant differences between patients with or without each condition (in columns) were bolded.			
Comparison for insulin resistance and impaired glucose tolerance (which are not included in used metabolic syndrome diagnostic criteria) was separated from other clinical states.			

Table 3

Median (IQR) serum values of Klotho, FGF19 and FGF21 in relations to the occurrence of metabolic syndrome and its components as well as insulin resistance and impaired glucose tolerance.

Subjects:	Klotho [pg/ml]	FGF19 [pg/ml]	FGF21 [pg/ml]
With (N = 116) vs without (N = 58) central obesity	156.4 (89.6 to 350.9) vs 118.5 (48.4 to 177.5) p = 0.0275	160.6 (92.2 to 260.2) vs 229.4 (119.5 to 345.0) p = 0.0264	93.0 (48.8 to 199.5) vs 70.1 (31.5 to 108.6) p = 0.0193
With (N = 32) low vs normal (N = 142) HDL	156.4 (92.4 to 266.7) vs 134.4 (75.5 to 335.8) p = 0.8202	147.6 (79.4 to 257.8) vs 194.2 (112.4 to 289.2) p = 0.1727	100.3 (48.8 to 165.1) vs 84.7 (42.1 to 161.0) p = 0.5064
With (N = 23) high vs normal (N = 151) TG	134.2 (90.7 to 321.1) vs 140.3 (75.5 to 313.0) p = 0.5620	124.3 (79.3 to 213.8) vs 184.9 (112.4 to 297.8) p = 0.0136	124.6 (78.6 to 363.5) vs 81.1 (39.5 to 151.3) p = 0.0035
With (N = 63) vs without (N = 111) arterial hypertension	157.5 (90.2 to 321.1) vs 136.0 (63.9 to 313.0) p = 0.1975	145.7 (93.3 to 244.5) vs 197.7 (101.1 to 299.7) p = 0.1085	124.6 (61.3 to 260.3) vs 75.2 (39.4 to 115.3) p = 0.0004
With (N = 18) vs without (N = 156) metabolic syndrome	172.7 (123.0 to 321.1) vs 135.0 (75.1 to 305.0) p = 0.2050	133.0 (62.2 to 171.2) vs 186.5 (109.2 to 284.7) p = 0.0509	136.2 (86.5 to 239.9) vs 82.6 (41.8 to 152.4) p = 0.0286
With (N = 8) vs without (N = 166) impaired glucose tolerance	159.6 (131.8 to 208.3) vs 136.4 (75.5 to 323.1) p = 0.4948	86.9 (65.4 to 149.4) vs 183.8 (106.0 to 289.2) p = 0.0416	107.2 (66.2 to 216.8) vs 86.4 (42.2 to 161.0) p = 0.4767
Insulin resistant (N = 48) vs insulin sensitive (N = 126)	185.3 (102.1 to 398.2) vs 132.6 (63.9 to 275.6) p = 0.0283	143.0 (81.5 to 229.3) vs 195.6 (118.2 to 297.8) p = 0.0233	91.9 (44.5 to 159.0) vs 82.6 (42.2 to 161.0) p = 0.6314
MS-metabolic syndrome, FGF19 – fibroblast growth factor 19, FGF21 - fibroblast growth factor 21.			
Significant differences between patients with or without each condition (in columns) were bolded.			
Comparison for insulin resistance and impaired glucose tolerance (which are not included in used metabolic syndrome diagnostic criteria) was separated from other clinical states.			

The proteins concentrations were also considered in relation to the diagnosis of MS. It turned out that those with this condition presented elevated concentration of FGF21 [136.2 pg/ml (86.5 to 239.9) vs 82.6 pg/ml (41.8 to 152.4), p = 0.0286]. Moreover, the dependence of the FGF19 and FGF21 concentration on the occurrence of particular MS components was noted. We showed a markedly higher concentration of FGF21 in the subjects with arterial hypertension and elevated TG levels compared with children with normal blood pressure [124.6 pg/ml (61.3 to 260.3) vs 75.2 pg/ml (39.4 to 115.3), p = 0.0004] and normal

TG levels [124.6 (78.6 to 363.5) vs 81.1 (39.5 to 151.3), p = 0.0035]. In the group of subjects with central obesity in comparison with children without this abnormality the increase of Klotho [156.4 (89.6 to 350.9) vs 118.5 (48.4 to 177.5) p = 0.0275 and FGF21 [93.0 (48.8 to 199.5) vs 70.1 (31.5 to 108.6) p = 0.0193] as well as decrease of FGF19 [160.6 (92.2 to 260.2) vs 229.4 (119.5 to 345.0) p = 0.0264] were observed (Table 3).

The analysis of multivariate model for HOMA-IR showed that FGF19 was an independent predictor of IR in the studied subjects after adjusting for pubertal stage, sex, age and BMI Z-score (Table 4). Quantitatively, each 100 pg/ml drop in FGF19 serum concentration was associated with 8.2% increase in HOMA-IR. This effect was comparable to the impact of physical development (Eta^2 for FGF19 3.7%, for Tanner stage – 3.8%) The model, however, managed to explain only a small portion of overall HOMA-IR variation (R^2 = 30%).

Table 4

Multivariate linear regression for log10(HOMA-IR).

	Multivariate linear regression for log10(HOMA-IR) R^2 **= 0.30, adj.** R^2 **= 0.28**				
	Parameter	95%CI	p-value	Eta^2 [%]	Commentary
Intercept	0.278	(0.099 to 0.457)	0.0025	5%	
Gender - male	0.006	(-0.037 to 0.038)	0.9731	< 0.1%	Associated with 1.4% higher HOMA-IR compared with girls
Tanner - stage III	0.079	(0.018 to 0.139)	0.0112	3.8%	Associated with 19.9% higher HOMA-IR than other stages of puberty
Age [years]	0.009	(-0.003 to 0.021)	0.1509	1.2%	Associated with 2.1% higher HOMA-IR for each year
BMI Z-score [standard deviations]	0.121	(0.086 to 0.156)	< 0.0001	21.3%	Associated with 32.1% higher HOMA-IR for each unit increase in BMI Z-score
FGF19 concentrations [100 pg/ml]	-0.037	(-0.067 to -0.008)	0.0124	3.7%	Associated with 8.2% drop in HOMA-IR for each 100 pg/ml increase in FGF19
The constructed model explains a minor fraction (~ 30%) of HOMA-IR variability among the patients, which demonstrates that individual insulin resistance is highly variable and might depend on other factors than investigated in this study.					
HOMA-IR - Homeostatic Model Assessment of Insulin Resistance, BMI – Body Mass Index, FGF19 – fibroblast growth factor19					
R^2 – proportion of variance in log10(HOMA-IR) explained by the model					
Eta^2 – proportion of variance in log10(HOMA-IR) explained by each factor.					

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R^2 – proportion of variance in log10(HOMA-IR) explained by the model					
Eta^2 – proportion of variance in log10(HOMA-IR) explained by each factor.					

Discussion

In the present study, we examined concentrations of circulating FGF19, FGF21 and Klotho proteins among the normal weight, obese and overweight children and adolescents and their relationships with metabolic parameters. The results complement existing reports with thus far lacking paediatric-specific data.

We noted increased FGF21 concentrations in children and adolescents with MS compared to other subjects. Moreover, FGF21 levels correlated with clinical (adiposity, arterial hypertension) as well as biochemical (TG, HDL-C) features of MS. Despite its role in metabolism regulation, the reports on its usefulness as a biomarker for obesity and abnormalities associated with MS are conflicting [8, 24, 25, 26]. FGF21, produced during fasting mainly in the liver, promotes gluconeogenesis, lipolysis, ketogenesis, ameliorates glucose uptake and improves insulin sensitivity [5]. It was previously shown that systemic administration of FGF21 has therapeutic benefits against obesity-related medical complications in obese animals [27, 28, 29]. Despite the beneficial effects of FGF21, increased endogenous FGF21 level has been observed in adults with obesity that may result from the tissue resistance to FGF21. This paradoxical phenomenon led to the hypothesis that central obesity, strangely associated with MS, is a state of FGF21 resistance resulting from decreased of FGF coreceptor (betaKlotho) expression in white adipose tissue. This hypothesis seems to corroborate our results as well other authors previous findings [30, 31]. However, as Reinehr et al. we did not confirm the relationship between FGF21 concentrations and insulin resistance occurrence [32]. The results may be explained by new data showing that elevated FGF21 levels in obesity serve as a defence mechanism to protect against systemic IR by upregulating adiponectin in subcutaneous but not visceral fat with the following anti-inflammatory action resulting from local M2 macrophage polarization [33].

To our knowledge, this is the first study in children and adolescents to show that FGF19 beside pubertal stage and BMI Z-score, is an independent predictor for IR. The studies on animal model indicated that administration of recombinant FGF19 to obese mice led to reduction of body mass, decreased blood glucose level as well as increased insulin sensitivity by central FGF19 action [34]. However, the data focused on relationship between FGF19 levels and metabolic parameters including IR are conflicting [6, 7, 35]. Despite the fact that FGF19 actions lead to the similar metabolic processes like insulin, the differences between two hormones are still not well understood [5]. What is certain, FGF19 unlike insulin, is released from the small intestine, not pancreas, and it reaches its peak serum level at 3 h after a meal, not at 1 h [5]. Furthermore, FGF19 acts using FGFR1/betaKlotho or FGFR4/betaKlotho pathways. The activation of FGFR1/betaKlotho leads to regulation of glucose metabolism, including suppression of gluconeogenesis, stimulation of glucose catabolism and reduction of TG synthesis. On the other hand, FGFR4/betaKlotho receptor activation is connected with reduction of bile acid levels and alteration in bile acid pool composition, which potentially may promote increased TG levels [5, 6, 36]. It has been speculated that insulin and FGF19 may have an inverse effect on each other [6]. Consequently, the insulin-resistant state leading to increased levels of circulating insulin may provoke observed decreased FGF19 levels.

In our study we noted that children and adolescents affected by obesity show higher serum Klotho concentrations than those with normal body weight. This contrasts with other studies on the matter. Amitani et al. showed markedly lower plasma Klotho levels in patients with obesity and anorexia nervosa than in the control group, which suggests that Klotho may reflect normal nutritional status [14]. On the other hand, Wojcicki et al., in the group of healthy Latino neonates, found no association between either weight, length at birth or obesity in early childhood and cord blood Klotho levels [16]. However, in the

literature the data focused on the relationship between obesity and Klotho levels in children and adolescents are insufficient. Our results may be supported by the fact that Klotho is one of the regulators of adipogenesis. It was previously revealed that Klotho increases adipocyte differentiation in vitro. Mice without Klotho have less detectable adipose tissue content than wild-type animals. Moreover, mice that lack the Klotho gene are resistant to obesity induced by a high-fat diet [37].

Interestingly, we noted different Klotho levels between the groups of patients with IR and normal insulin sensitivity. This may be explained by the fact that Klotho takes part in enzymatic modification of N-glycans in insulin and IGF-1 receptors and thus inhibits the intracellular insulin/IGF-1 signalling pathway. It blocks insulin-stimulated glucose uptake that contributes to IR development [12, 38]. What is important, probably inhibition of IGF-1 signalling cascade is associated with increased resistance to the oxidative stress and lead to the extension of life, which is one of the major functions of Klotho [39].

There are potential limitations of our study. Firstly, we studied only circulating hormones without their local (i.e. liver, adipose tissue) expression, which was out of scope for this study. Secondly, we relied on BMI z-score to recognize overweight and obesity without body content assessed by Dual-energy X-ray absorptiometry (DXA), which was not available during this study. Estimated BF% could not be translated into sex- and age-independent Z-scores or percentiles due to the lack of modern paediatric charts for Polish population. Moreover, serum Klotho concentrations may depend on vitamin D and calcium-phosphate homeostasis which we did not examine in the studied subjects. However, the above-mentioned dependence was observed especially in patients with chronic kidney diseases who were excluded from this study. Our multivariate model for HOMA-IR explained only a small fraction of between-patients variability. This demonstrates that there are likely other factors which might be associated with IR in a stronger and more direct way. Moreover, the subjects were enrolled to the study in the hospital and this might be a potential limitation. On the other hand, the recruitment in inpatient condition assured the similar exposure to potential confounding factors including diet, physical activity, ambient temperature. Finally, the study was carried out in a hospital single centred with sample derived from local population, which prohibits generalisation of results onto Polish or European children.

Conclusions

Serum Klotho concentrations are associated with obesity that may be supported by the fact that Klotho is one of the regulators of adipogenesis. Increased Klotho concentrations in the group with insulin resistance suggest the existence of a mutual regulation of hormones in insulin/IGF-1 signalling pathway. The negative correlation between HOMA-IR and FGF19 concentrations implies that decreased FGF19 levels may be a compensatory effect of interaction of two hormones - insulin and FGF19. The increased FGF21 concentrations in children and adolescents with MS is probably caused by a state of FGF21 resistance observed in subjects affected by central obesity.

Abbreviations

IR - insulin resistance

MS - metabolic syndrome

NAFLD - non-alcoholic fatty liver disease

FGF19 - fibroblast growth factor 19

FGF21 - fibroblast growth factor 21

BMI - Body Mass Index

BF% - Body fat content in %

LDL-C - low-density lipoprotein cholesterol

TG - triglycerides

TC - total cholesterol

HDL-C - high-density lipoprotein cholesterol

ALT - alanine aminotransferase

OGTT- oral glucose tolerance test

HOMA-IR- Homeostatic Model Assessment of Insulin Resistance

Declarations

Ethics approval and consent to participate

The study was approved by local Bioethical Committee of the Polish Mothers Memorial Hospital-Research Institute (PMMH-RI 39/2015). Parents and children ≥16 years old provided written informed consent before participation.

Consent for publication

Not applicable.

Availability of data and materialsThe data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

ASB and EC were responsible for study design, data collection, data interpretation, literature search. KP, AS, KK and VG participated in data collection and literature research. ZG and EG performed a laboratory analysis. WF and AM were responsible for data analysis, data interpretation, generation of tables and figures. All authors were involved in writing the paper and finally, they have read and approved the manuscript.

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Not applicable.

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Tables

Table 1. Characteristics of the study population.

Variable	Normal weight (N=48) median (25-75%)	Overweight (N=34) median (25-75%)	Obesity (N=92) median (25-75%)	p-value
Age [years]	13.6 (10.1 to 15.7)	12.0 (9.9 to 15.9)	12.0 (9.7 to 14.3)	0.3812
Tanner score	2 (2 to 4)	2 (1 to 4)	2 (2 to 4)	0.8710
BMI [kg/m^2]	18,4 (16.6 to 20.3)	24.1 (22.4 to 25.7)	27.4 (25.9 to 30.6)	N/A
BMI z-score	-0.1 (-0.6 to 0.4)	1.5 (1.3 to 1.6)	2.1 (1.8 to 2.3)	N/A
BMI percentile	45.8 (26.4 to 66.0)	92.7 (90.5 to 94.3)	98.0 (96.7 to 99.0)	N/A
Body fat [%]	23.9 (20.1 to 27.3)	37.7 (32.9 to 47.0)	45.2 (39.2 to 52.9)	<0.0001[1]
HOMA-IR	1.8 (1.1 to 2.7)	2.5 (2.1 to 3.2)	3.2 (2.1 to 5.1)	<0.0001[2]
TC [mg/dl]	151.5 (138.0 to 174.0)	161.5 (148.0 to 183.0)	153.0 (135.0 to 175.0)	0.176
HDL-C [mg/dl]	53.0 (49.0 to 59.5)	47.5 (42.0 to 54.0)	44.0 (38.0 to 51.0)	<0.0001[3]
LDL-C [mg/dl]	79.0 (69.5 to 97.5)	90.5 (74.0 to 102.0)	89.0 (75.0 to 106.5)	0.1721
TG [mg/dl]	77.5 (55.5 to 95.5)	103.0 (62.0 to 135.0)	104.5 (79.5 to 128.0)	0.0007[4]
Klotho [pg/ml]	116.6 (38.5 to 163.9)	131.3 (78.0 to 313.0)	168.6 (90.2 to 375.9)	0.0334[5]
FGF19 [pg/ml]	232.8 (126.0 to 340.5)	167.6 (118.2 to 276.6)	160.6 (87.5 to 260.2)	0.0563
FGF21 [pg/ml]	82.4 (31.6 to 128.2)	87.3 (47.1 to 181.4)	89.3 (43.3 to 193.2)	0.3783

N- number of subjects, N/A-not applicable, BMI – Body Mass Index, HOMA-IR – Homeostatic Model Assessment of Insulin Resistance, TC – total cholesterol, HDL-C - high-density lipoprotein cholesterol, LDL-C - low-*density lipoprotein cholesterol, TG – triglycerides,* FGF19 – fibroblast growth factor 19, FGF21 - fibroblast growth factor 21.

1. Post-hoc comparisons significant between normal weight and overweight (p<0.0001), normal weight and obesity (p<0.0001), and overweight and obesity (p=0.0030) groups
2. Post-hoc comparisons significant between normal weight and obesity (p<0.0001) and normal weight and overweight (p=0.0435)
3. Post-hoc comparison significant only between normal weight and obesity (p<0.0001)
4. Post-hoc comparison significant only between normal weight and obesity (p=0.0005)
5. Post-hoc comparison significant only between normal weight and obesity (p=0.0282)

Table 2. Klotho, FGF19, FGF21 concentrations in correlation with age, parameters of nutritional status, lipid profile, ALT and HOMA-IR.

	Klotho	FGF19	FGF21
Age	R=0.053, p=0.4848	R=0.064, p=0.3996	R=0.159, p=0.0360
BMI Z-score	R=0.188, p=0.0129	R=-0.206, p=0.0062	R=0.111, p=0.1423
BF%	R=0.210, p=0.0055	R=-0.143, p=0.0600	R=0.202, p=0.0020
ALT	R=0.184, p=0.0149	R=-0.230, p=0.0022	R=0.015, p=0.8413
TC	R=0.097, p=0.1997	R=-0.040, p=0.5913	R=-0.050, p=0.5057
HDL-C	R=0.084, p=0.2649	R=0.122, p=0.1076	R=-0.212, p=0.0047
LDL-C	R=0.211, p=0.0051	R=-0.030, p=0.6852	R=0.087, p=0.2504
TG	R=-0.014, p=0.8507	R=-0.201, p=0.0077	R=0.187, p=0.0131
HOMA-IR	R=0.129, p=0.0879	R=-0.313, p<0.0001	R=0.098, p=0.1978

HOMA-IR - Homeostatic Model Assessment of Insulin Resistance, BMI – Body Mass Index, BF% - body fat [%], TC - total cholesterol, LDL-C - low-density lipoprotein cholesterol, HDL-C- high density lipoprotein cholesterol, TG - triglycerides, ALT- alanine aminotransferase, FGF19 – fibroblast growth factor 19, FGF21 - fibroblast growth factor 21.

Table 3. Median (IQR) serum values of Klotho, FGF19 and FGF21 in relations to the occurrence of metabolic syndrome and its components as well as insulin resistance and impaired glucose tolerance.

	Klotho [pg/ml]	FGF19 [pg/ml]	FGF21 [pg/ml]
16) vs without (N=58) central obesity	**156.4 (89.6 to 350.9) vs 118.5 (48.4 to 177.5) p=0.0275**	**160.6 (92.2 to 260.2) vs 229.4 (119.5 to 345.0) p=0.0264**	**93.0 (48.8 to 199.5) vs 70.1 (31.5 to 108.6) p=0.0193**
N=32) low vs normal (N=142) HDL	156.4 (92.4 to 266.7) vs 134.4 (75.5 to 335.8) p=0.8202	147.6 (79.4 to 257.8) vs 194.2 (112.4 to 289.2) p=0.1727	100.3 (48.8 to 165.1) vs 84.7 (42.1 to 161.0) p=0.5064
N=23) high vs normal (N=151) TG	134.2 (90.7 to 321.1) vs 140.3 (75.5 to 313.0) p=0.5620	**124.3 (79.3 to 213.8) vs 184.9 (112.4 to 297.8) p=0.0136**	**124.6 (78.6 to 363.5) vs 81.1 (39.5 to 151.3) p=0.0035**
N=63) vs without (N=111) arterial hypertension	157.5 (90.2 to 321.1) vs 136.0 (63.9 to 313.0) p=0.1975	145.7 (93.3 to 244.5) vs 197.7 (101.1 to 299.7) p=0.1085	**124.6 (61.3 to 260.3) vs 75.2 (39.4 to 115.3) p=0.0004**
=18) vs without (N=156) metabolic syndrome	172.7 (123.0 to 321.1) vs 135.0 (75.1 to 305.0) p=0.2050	133.0 (62.2 to 171.2) vs 186.5 (109.2 to 284.7) p=0.0509	**136.2 (86.5 to 239.9) vs 82.6 (41.8 to 152.4) p=0.0286**
) vs without (N=166) impaired glucose tolerance	159.6 (131.8 to 208.3) vs 136.4 (75.5 to 323.1) p=0.4948	**86.9 (65.4 to 149.4) vs 183.8 (106.0 to 289.2) p=0.0416**	107.2 (66.2 to 216.8) vs 86.4 (42.2 to 161.0) p=0.4767
Insulin resistant (N=48) vs insulin sensitive (N=126)	**185.3 (102.1 to 398.2) vs 132.6 (63.9 to 275.6) p=0.0283**	**143.0 (81.5 to 229.3) vs 195.6 (118.2 to 297.8) p=0.0233**	91.9 (44.5 to 159.0) vs 82.6 (42.2 to 161.0) p=0.6314

MS-metabolic syndrome, FGF19 – fibroblast growth factor 19, FGF21 - fibroblast growth factor 21.
Significant differences between patients with or without each condition (in columns) were bolded.
Comparison for insulin resistance and impaired glucose tolerance (which are not included in used metabolic syndrome diagnostic criteria) was separated from other clinical states.

Table 4. Multivariate linear regression for log10(HOMA-IR).

	Multivariate linear regression for log10(HOMA-IR) R^2=0.30, adj. R^2=0.28				
	Parameter	**95%CI**	**p-value**	**Eta^2 [%]**	**Commentary**
Intercept	0.278	(0.099 to 0.457)	0.0025	5%	
Gender - male	0.006	(-0.037 to 0.038)	0.9731	<0.1%	Associated with 1.4% higher HOMA-IR compared with girls
Tanner - stage III	0.079	(0.018 to 0.139)	0.0112	3.8%	Associated with 19.9% higher HOMA-IR than other stages of puberty
Age [years]	0.009	(-0.003 to 0.021)	0.1509	1.2%	Associated with 2.1% higher HOMA-IR for each year
BMI Z-score [standard deviations]	0.121	(0.086 to 0.156)	<0.0001	21.3%	Associated with 32.1% higher HOMA-IR for each unit increase in BMI Z-score
FGF19 concentrations [100 pg/ml]	-0.037	(-0.067 to -0.008)	0.0124	3.7%	Associated with 8.2% drop in HOMA-IR for each 100 pg/ml increase in FGF19

The constructed model explains a minor fraction (~30%) of HOMA-IR variability among the patients, which demonstrates that individual insulin resistance is highly variable and might depend on other factors than investigated in this study.
HOMA-IR - Homeostatic Model Assessment of Insulin Resistance, BMI – Body Mass Index, FGF19 – fibroblast growth factor19
R^2 – proportion of variance in log10(HOMA-IR) explained by the model
Eta^2 – proportion of variance in log10(HOMA-IR) explained by each factor.

Figures

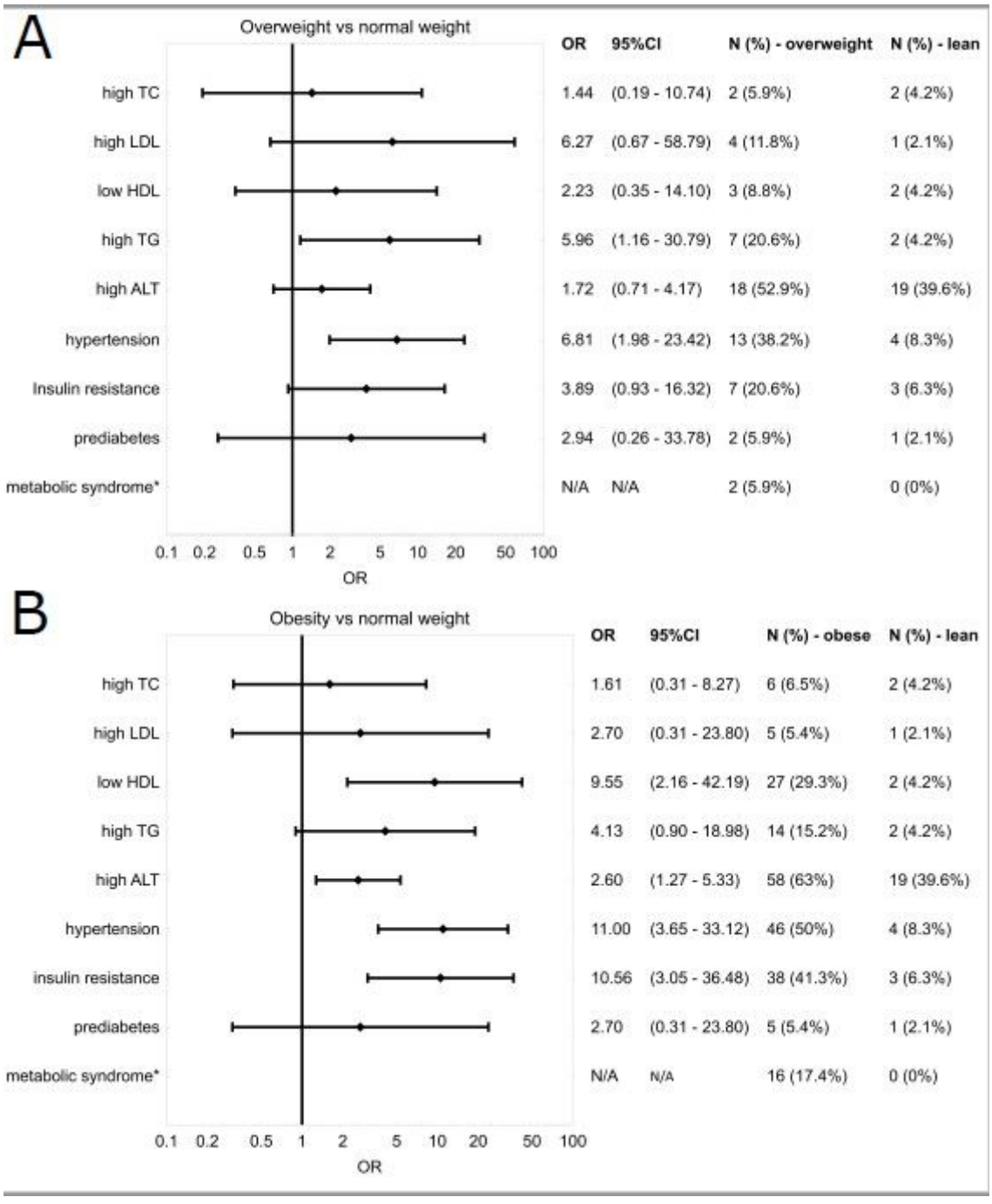

Figure 1

Relationship between overweight (A) and obesity (B) and odds of developing metabolic abnormalities, relative to children with normal body weight. The points indicate Odds Ratios (ORs) with 95% confidence intervals (95%CIs) spans. TC – total cholesterol LDL – low density lipoprotein cholesterol HDL – high density lipoprotein cholesterol TG – triglycerides ALT - alanine transaminase

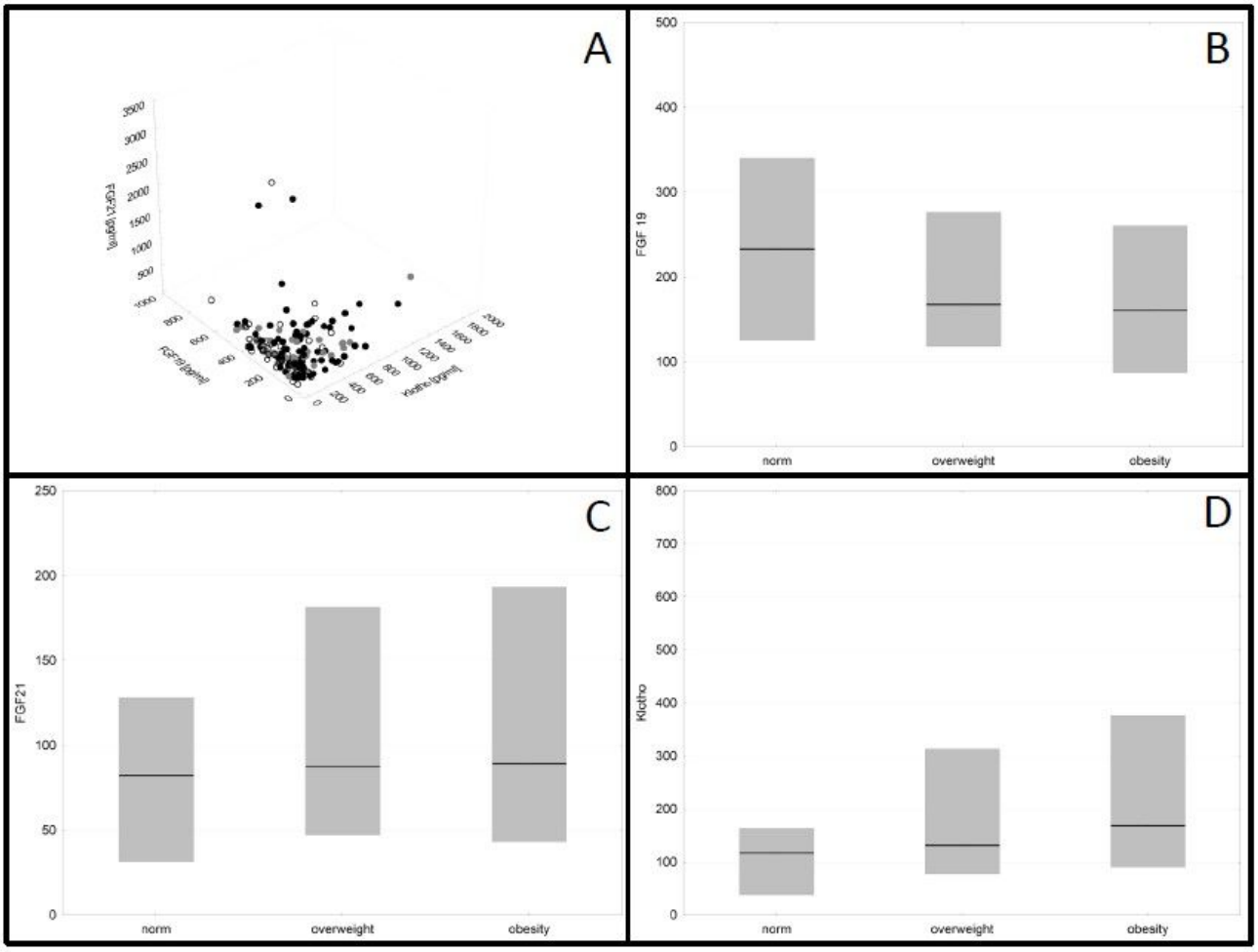

Figure 2

Klotho, FGF19 and FGF21 concentrations in the studied groups. A. Scatter plot of Klotho, FGF19 and FGF21 concentrations measured in the studied groups. Black dotes denote children with obesity, gray with overweight and white with normal body weight. There were no significant relationships between each pair of the variables - Klotho x FGF19 – R=-0.06, p=0.4552; Klotho x FGF21 – R=0.05, p=0.5334, FGF19 x FGF21 – R=<0.01, p=0.9968. B-D. The impact of body weight on FGF19 (B), FGF21 (C) and Klotho (D) concentrations. Significant relationship was noted only for Klotho concentration (p=0.0334). FGF19 showed a borderly significant association with body mass status (p=0.0563). FGF21 concentrations were similar among the groups (p=0.3783).

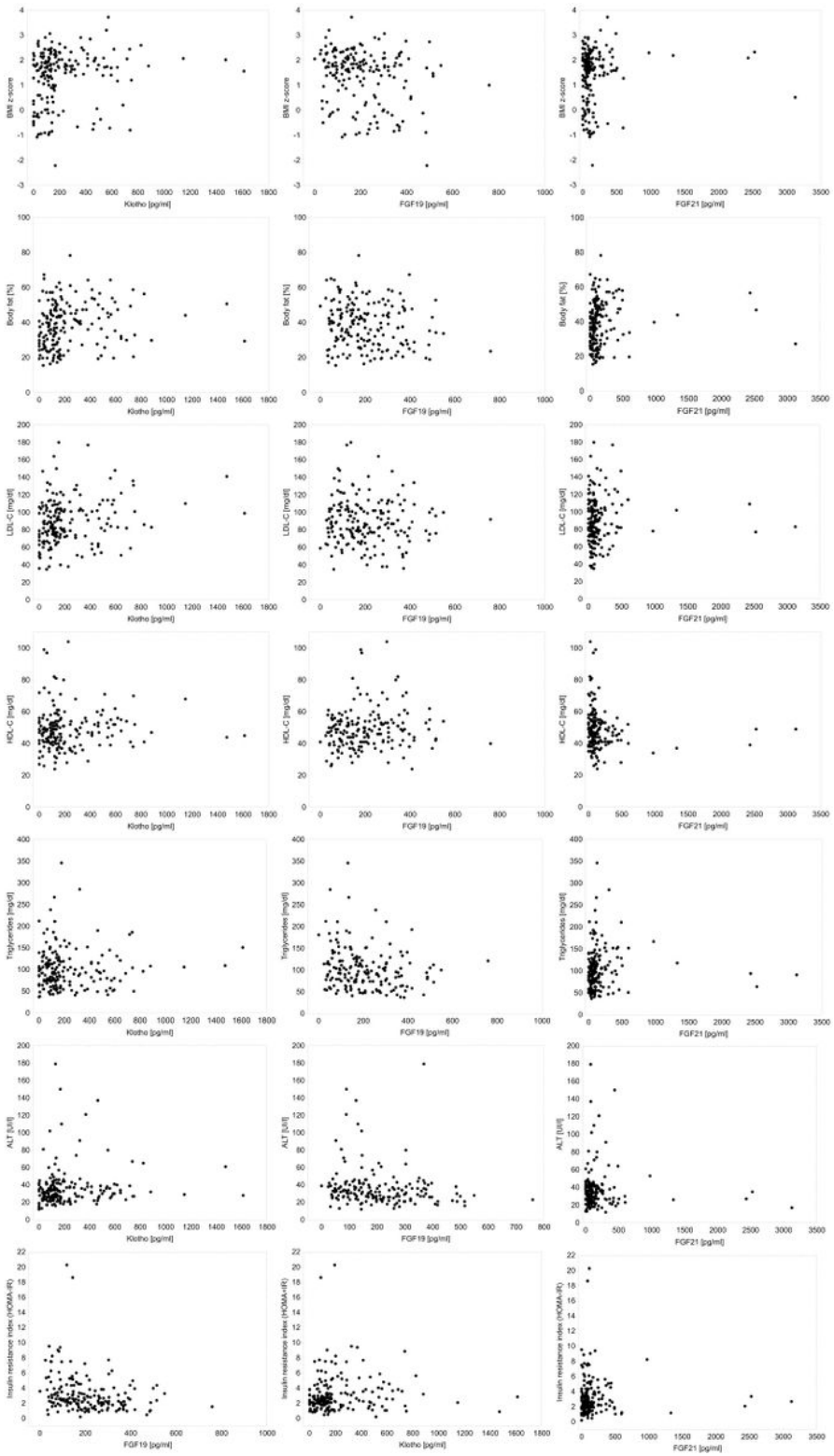

Figure 3

Correlations of Klotho, FGF19 and FGF21 concentrations with parameters of nutritional status, lipid profile, ALT and HOMA-IR.