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FASTING AND MEAL-RELATED ZONULIN SERUM LEVELS IN A LARGE COHORT OF OBESE CHILDREN AND ADOLESCENTS

Scientific Disciplinary Sector: MED/38

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Preface

Aim of this dissertation is to illustrate the main results of the research project carried out during the 3-years-PhD course, within the main research field concerning novelties in overweight and obesity in pediatric age.

The project was conceived and realized at the Pediatrics Unit of the University Hospital "G. Martino" of Messina (Italy), under the supervision of Professor Malgorzata Wasniewska and Dr. Domenico Corica.

Preliminary data of the study were selected as oral communication and presented by the candidate to the 61st Annual Meeting of the European Society for Pediatric Endocrinology (ESPE), held in September 2023 in The Hague, Netherlands.

Very recently, the present study has been accepted for publication on a scientific international journal as original research article.

Chapter 1

INTRODUCTION

1.1 Obesity in pediatric age

Nowadays, childhood obesity is acknowledged as one of the major health issues worldwide. Data of prevalence reported by WHO are of great concern, with an increasing trend registered across all age groups, in both industrialized and developing countries [1, 2]. It has been estimated that more than 340 million children and adolescents between the age of 5 and 19 years and about 41 million children under 5 years of age are overweight or obese [3]. In Europe, the prevalence of childhood obesity ranges from 10% to 40% [4]. In

Italy, the reported prevalence of overweight and obesity is about 20.4% and 9.4% respectively, with the higher percentage registered in the south of the country [5].

The pathogenesis of obesity is complex and involves the interaction of biological, developmental, behavioral, genetic, and environmental factors [6]. Furthermore, the epigenetics and the gut microbiome, as well as the intrauterine environment, breastfeeding, and nutrition in the first 1000 days, seem to play a pivotal role as significant contributors to the obesity epidemic [7-9]. What is more, adiposity rebound in early childhood is a well-known risk factor for developing obesity later in life [10].

The increasing prevalence of pediatric obesity is consequently associated with the early onset of several comorbidities, including insulin resistance (IR), impaired glucose metabolism, dyslipidemia, nonalcoholic fatty liver disease (NAFLD), cardiovascular diseases, hypertension, Obstructive Sleep Apnea (OSA), and dyslipidemia [11, 12]. Above all, having obesity in childhood increases the risk of developing type 2 diabetes mellitus (T2DM) fourfold. The severity of obesity-related complications is positively associated with the earlier onset and the degree of obesity and overweight. All the above-mentioned complications may concur to the development of the metabolic syndrome, which is characterized by a cluster of several cardio-metabolic risk factors, leading to increasing risk of future cardiovascular disease [13, 14]. In this context, IR and hyperinsulinemia - which represents the most common complication in pediatric obesity - seem to play a crucial role. Indeed, IR is

involved in the development of endothelial dysfunction in obese youths, promotes hepatic triglycerides accumulation and favors the development of NAFLD [15].

Understanding the underlying mechanisms of IR may allow an early identification of children and adolescents at risk of metabolic complications, which is essential in clinical practice to focus on targeted and early preventive interventions [16, 17].

1.2 Zonulin: from the discovery to the emerging role in overweight and obesity

Due to its recently documented role in reversible intercellular tight junction disassembly, zonulin has emerged as a valuable and promising biological marker to assess the integrity of the intestinal mucosal barrier. Human zonulin (47 KDa protein), which was discovered as an analogue of the cholera comma toxin (ZOT, zonula occludens toxin), is secreted mainly by the liver and the enterocytes, and can be isolated from multi-protein membrane complexes (claudin-occludin-guanylate kinase-like proteins ZO-1, ZO-2, and ZO-3) on the apical surface of intestinal epithelium. It represents the only measurable blood protein known to regulate the permeability of intestinal tight junctions, allowing for the paracellular transport in the gut's mucosa (figure 1) [18-21].

Dysregulation of the zonulin pathway leads to increased intestinal permeability, which may influence tolerance and immunity. High

serum levels of zonulin and the subsequent condition of "gut leakiness" have been associated with both intestinal and extraintestinal disorders, including autoimmune diseases (such as celiac disease and type 1 diabetes mellitus), cancers and diseases of the nervous system. Interestingly, genes related to these three classes of pathological states have been mapped on chromosome 16, the same where zonulin gene is located [19, 22-25].

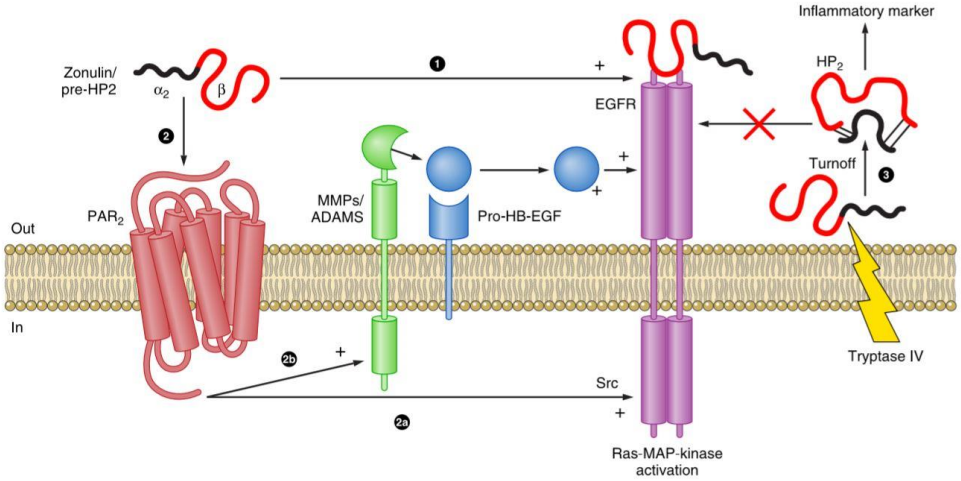


Figure 1. Zonulin is a multifunctional protein that, in its single chain form, regulates intestinal permeability, while in its two-chain form acts as a haemoglobin (HB) scavenger. To initiate tight junction disassembly, it has been proposed that zonulin activates epidermal growth factor receptor (EGFR) through direct binding (1) and/or through PAR2 transactivation (2). This second mechanism can be mediated by either Src signaling (2a) or by the release of MMPs and/or ADAMS that in turn will activate Pro-HB-EGF. Processing of zonulin via proteolytic cleavage by intestinal tryptase IV induces conformational changes in the molecule that abolish its ability to bind EGFR (3), but instead enables a different function (e.g., HB binding), and it becomes an inflammatory marker. *Adapted from "Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer" by A. Fasano, 2011, Physiological reviews, 91, p 157[19].*

Moreover, recent evidence highlighted a potential role of zonulin in the pathophysiology of obesity. Elevated circulating zonulin concentrations seem to positively correlate with body mass index

(BMI), glucose levels, dyslipidemia, systolic blood pressure, and insuline resistance [26-29]. Some authors reported significantly higher levels of zonulin in prediabetic and diabetic patients [30] and in pregnant women with gestational diabetes mellitus [31-33].

Even if the underlying mechanisms associating serum zonulin level with obesity remain unclear, experimental studies have shown a close relationship between intestinal permeability and obesity [34-36]. In this regard, it has been hypothesized that a change in the gut microbiota - caused by continuous uptake of high-fat diet - may promote chronic inflammation of the gut, and subsequently at a systemic level [37-39]. This chronic low-grade systemic inflammation, which characterizes obesity, upregulates zonulin expression and may be an important contributor to intestinal barrier dysfunction.

Zonulin has initially only been considered as an inactive precursor of haptoglobin, a protein released mainly by hepatocytes to act as a hemoglobin scavenger [40]. Haptoglobin, which has antioxidant and anti-inflammatory effects, binds to hemoglobin to determine stable complexes, preventing oxidative tissue damage [40, 41]. Therefore, the presence of zonulin could be seen not only as an indicator of intestinal permeability [20], but may also reflect a reaction secondary to inflammation [42].

In addition, elevated serum zonulin levels have been also associated with increased liver enzymes [43] and non-alcoholic fatty liver disease (NAFLD), shedding new light on the so called “gut-liver-axis” [44-49].

Despite this emerging role of zonulin, to the best of our knowledge only few studies have investigated its relationship with obesity-related clinical factors and/or laboratory biomarkers in childhood [43, 44, 50-52]. Moreover, the meal related pattern of secretion of zonulin is almost unexplored, especially in pediatric age.

A better understanding of these mechanisms may allow the identification of new prognostic markers in order to detect metabolic complications at an early stage, and therefore to provide therapeutic strategies targeted on gut microbioma, which represents the link between obesity and inflammation.

Chapter 2

Fasting and meal-related zonulin serum levels in a large cohort of obese children and adolescents

2.1 Aims and objectives

The present research project aims to explore the relationship between intestinal barrier dysfunction, assessed by serum zonulin concentration, with BMI and metabolic disorders in childhood obesity.

The primary end-point is to determine whether serum zonulin levels could be significantly influenced by anthropometric, clinical and biochemical obesity-related biomarkers, and specifically:

- to investigate the relationship between serum zonulin levels, both at baseline and postprandial, with body mass index (BMI) and biochemical markers of insulin resistance (IR), insulin sensitivity, β-cell function and

cardio-metabolic risk in obese non-diabetic children and adolescents;

- to describe, for the first time in a pediatric cohort, the meal-related pattern of secretion of serum zonulin;
- to evaluate the association between serum zonulin levels and NAFLD (ultrasonographycally assessed).

2.2 Project design and study population

This single-center and cross-sectional study was carried out from November 2020 to October 2023 at the Outpatient Clinic for Pediatric Endocrinology - Pediatrics Unit, University Hospital "G. Martino" of Messina (Italy).

The timeline of the project is described in detail in table 1.

Table 1. Gantt chart of the research study protocol.

	Start	end	0 – 6 months	6 – 18 months	18 – 30 months	30 – 36 months
Ethic Commitee, Case Report Form, purchase of laboratory equipments	0	6				
Patient recruitment	6	18				
Clinical, biochemical and ultrasonographic evaluations	18	30				
Statistical analysis, drafting scientific paper	30	36				

Children and adolescents were enrolled according to the following inclusion criteria:

- chronological age between 5 and 16 years;
- BMI ≥ +2.0 standard deviation score (SDS), according to the definition of obesity proposed by the World Health Organization (WHO) for children from the age of 5 years[53];
- caucasian ethnicity;
- born as healthy full-term infant and adequate for gestational age (AGA).

Criteria of exclusion from the study were: pre-term or post-term birth, genetic or endocrine causes of obesity, diabetes, chronic diseases or chronic pharmacological therapies, smoking.

The study design was approved by the Ethical Committee of the University Hospital AOU Policlinico "Gaetano Martino". All procedures were performed according to the Declaration of Helsinki. Written informed consent was obtained from all parents or legal tutors of the children taking part to the study protocol.

2.3 Methodology

All the patients who fulfilled the inclusion criteria reported above were enrolled for the study.

At recruitment, patients underwent a complete **medical history taking**, specifically focused on family or personal history of cardio-

metabolic risk factors). Auxological assessment was based on height measurement and BMI calculation.

Physical examination was performed by a dedicated team of pediatric endocrinologists according to standardized procedures, including measurement of height, weight, BMI, waist circumference (WC), WC-to-height ratio (WHtR), systolic and diastolic blood pressure. Standing height was measured with a Harpenden stadiometer (Holtain Ltd, Crymych, Dyfed, UK). BMI was calculated using the equation: body weight(kg)/height(m)^2. To allow the comparison between different ages and genders, height and BMI were expressed as S.D. scores (SDS). Waist circumference (WC) was measured to the nearest 0.5 cm while the subjects were standing, after gently exhaling, as the minimal circumference measurable on the horizontal plane between the lowest portion of the rib cage and the iliac crest [54]. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded at rest on the right arm in mmHg using a manual sphygmomanometer; for analysis, the average of three blood pressure values was used. Pubertal stage was determined according to the Tanner classification; patients were considered in pubertal stage from Tanner stages B2 for females and G2 for males [55].

All the patients underwent fasting biochemical assessment, including blood sampling for lipid profile, thyroid, kidney and liver function blood tests and oral glucose tolerance test (OGTT), performed at least 8 hours after the last meal. OGTT was performed according to the American Diabetes Association (ADA) guidelines [56], with

sampling at 0, + 30, +60, +90, +120 minutes for measurements of glucose and insulin. Impaired fasting glycaemia (IFG) was defined as fasting plasma glucose between 100 and 125 mg/dL. Impaired glucose tolerance (IGT) was defined as plasma glucose values between 140 and 199 mg/dL two hours after a standardized glucose load (OGTT) [56].

Homeostasis model assessment of insulin resistance (HOMA-IR), β-cell function (HOMA-B), Matsuda-index, Insulinogenic-index were calculated. Areas Under the Curves for glucose (AUCg) and insulin (AUCi) and their ratio were also evaluated. HOMA-IR was calculated according to the equation: fasting insulin (μU/ml) × fasting glucose (mg/dl)/405 [57]. The aterogenic index of plasma (AIP) was calculated according to the equation: [LOG (triglycerides/HDL cholesterol)] [58]. The following cut-offs were used to define the main indices of cardiometabolic risk as abnormal: HOMA-IR>2.5 in prepubertal children and >4 in pubertal patients [57], AIP >0.11[58], and TG/HDL ratio >1.25.

Blood samples for the serum zonulin assay were taken at fasting state, at 60-minute and 120-minute OGTT timepoint. Measurement of zonulin concentration in blood was performed by the team of UOSD Biochimica Clinica of the University Hospital G. Martino in Messina. Samples for the serum zonulin assay were collected and stored at −80°C. Concentrations of zonulin were measured by using competitive enzyme-linked immunosorbent assay (ELISA) kits (K5600, Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's instructions. The absorbance values for the

ELISA assays were determined with an Infinite 2000 Pro multimode plate reader (Tecan, Vienna, VA, USA) at 450nm.

In addition, liver ultrasonography (US) was performed in all the patients of the study. Diagnosis of liver steatosis was made by conventional liver US according to at least two of the following criteria: 1) diffusely increased echogenicity of the liver compared with kidney or spleen; 2) US beam attenuation; 3) poor visualization of intrahepatic structures [59].

2.4 Statistical analysis

Numerical data were expressed as mean, standard deviation (S.D.), median and interquartile range (Q1-Q3); categorical variables were expressed as absolute frequencies and percentage. Non-parametric approach was used since the numerical variables were not normally distributed, such as verified by the Kolmogorov-Smirnov test.

The Mann Whitney test was applied with reference to numerical parameters in order to identify possible significant differences between the different groups of patients listed below: male and female subjects, pubertal and pre-pubertal youths, patients with impaired vs normal fasting glycaemia, patients with impaired glucose tolerance vs normal glucose tolerance, patients with high or normal HbA1c, patients with or without liver steatosis, and patients with or without insulin-resistance. The Chi Square test was applied to compare the same above-mentioned groups with reference to categorical variables.

The Spearman correlation test was applied to assess the correlation between zonulin level at 0', 60' and 120' minutes with all the numerical variables.

A scatterplot was used to show the interdependence between Zonulin serum levels at 120' and BMI.

Friedman test was applied to evaluate the trend of zonulin serum levels in the three analyzed OGTT timepoints (0', 60' and 120' minutes). In addition, all two-by-two comparison were performed using Dunn test; for this analysis the Bonferroni's correction was applied by dividing the significance level (0.050) into the total number of the possible two-by-two comparisons; as a result, the adjusted significance level was 0.017. The Mann-Kendall statistical test for trend was used to assess whether our set of data values was increasing over time or decreasing over time, and whether the trend in either direction was statistically significant.

A stepwise multiple regression models was estimated in order to identify significant predictors of zonulin serum level at baseline: in particular, we tested the influence of the following covariates: gender, age, BMI, waist circumference, glutamic oxaloacetic transaminase (GOT), aspartate transaminase (AST), total cholesterol, triglycerides, liver steatosis, HbA1c, C-reactive protein (CRP), insulin resistance, impaired fasting glycaemia (IFG), impaired glucose tolerance (IGT). Results were expressed as regression coefficient (b), 95% confidence interval (C.I.) and p-

value. A boxplot and a scatterplot were built to allow the visualization of the results of the regression analysis.

Statistical analyses were performed using IBM SPSS for Windows, Version 22 (Armonk, NY, IBM Corp.). A *p*-value lower than 0.05 was considered statistically significant.

2.5 Results

2.5.1 Main clinical and biochemical data

The study involved 104 overweight and obese children and adolescents (48 males and 56 females), mean age 11.43 ±2.66 years, 68.3% of them were pubertal and 31.7% prepubertal.

The main clinical and biochemical characteristics of the study population are detailed in *Table 2*.

Overall, the mean BMI of the study population was 29.29 ± 4.3. Impaired fasting glycaemia was documented in 29/104 patients (27.9%). After OGTT load, 12/104 (11.5%) of patients reported a condition of impaired glucose tolerance. 72/104 (69.2%) patients had insulin resistance. Liver steatosis was diagnosed in 41 patients (39.4%). The degree of the steatosis was described ultrasonographycally as *mild* in 29 patients, *moderate* in 9 patients

and *severe* in 3 patients. An increase in liver volume was reported in 24 patients (23.1%).

A comparison analysis was performed in the entire cohort for sex and for pubertal status (pubertal/prepubertal) without evidence of significant difference in zonulin levels, both at baseline and postprandial.

Likewise, we did not find any statistically significant difference when comparing subjects with impaired vs normal fasting glycaemia, impaired vs normal glucose tolerance, high vs normal HbA1c and presence/absence of liver steatosis.

Table 2. Clinical and biochemical features of the study population.

	Mean	SDS
Age (years)	11.43	2.66
Height (SDS)	0.52	1.09
Weight (SDS)	2.20	0.55
BMI	29.29	4.30
BMI (SDS)	3.06	0.71
WC (cm)	86.30	15.67
HC (cm)	96.01	18.14
WC/HC	0.90	0.06
WHtR	0.59	0.05
SBP (mmHg)	114.34	8.79
DBP (mmHg)	69.89	8.69
GOT (U/L)	21.02	8.41
GPT (U/L)	23.66	20.62
GGT (U/L)	16.05	16.55
Total cholesterol (mg/dl)	169.92	25.89
LDL-cholesterol (mg/dl)	89.71	27.19
HDL-cholesterol (mg/dl)	52.69	15.67
Triglycerides (mg/dl)	84.58	36.76
Triglycerides/HDL-ratio	1.80	1.15
Total cholesterol/HDL-ratio	3.47	1.19
Uric acid (mg/dl)	26.53	5.81
CRP (mg/dl)	0.99	6.21
HbA1c (%)	5.33	0.44
Fasting glucose (mg/dl)	95.83	8.12

1h-postprandial glucose (mg/dl)	129.58	21.86
2h-postprandial glucose (mg/dl)	114.92	16.85
Fasting insulin (mUI/ml)	21.16	12,06
1h-postprandial insulin (mUI/ml)	131.70	108.25
2h-postprandial insulin (mUI/ml)	121.41	89.23
HOMA-IR	5.05	3.10
HOMA-B	237.82	131.48
IGI	2.89	2.77
Matsuda-index	2.50	1.28
AUCg	260.18	152.05
AUCi	245.29	217.30
AUCi/AUCg ratio	0.93	0.61
FT4 (pmol/L)	15.29	2.52
TSH (uUI/ml)	2.57	2.60
Fasting Zonulin (pg/ml)	19.2693	12.53050
1h-postprandial Zonulin(pg/ml)	21.0482	15.38648
2h-postprandial Zonulin(pg/ml)	21.7583	16.02727

Numerical data are expressed by mean ± SDS.

Body mass index (BMI), standard deviation score (SDS), waist circumference (WC), hip circumference (HC), WC-to-height ratio (WHtR), systolic blood pressure (SBP), diastolic blood pressure (DBP), glutamic-oxaloacetic transaminase (GOT), Glutamate Pyruvate Transaminase (GPT), gamma-glutamyl transpeptidase (GGT), C-reactive protein (CRP), glycated haemoglobin (HbA1c), homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment for β-cell function (HOMA-B), insulinogenic index (IGI), Area under the curve for glucose (AUC_g) and insulin (AUC_i). thyroid stimulating hormone (TSH), free thyroxine (FT4).

When obese patients were categorized in IR and non-IR subjects according to HOMA-IR, no significant difference in zonulin levels were recorded, both at baseline and postprandial (*Table 3*).

Overall, we found a higher rate of impaired fasting glycaemia in pubertal patients (p= 0.048).

Liver steatosis was significantly more frequent in boys than in girls (p=0.037) and in IR subjects than in non-IR ones (p=0.054).

Table 3. Comparison analysis between insulin resistant (IR) and non-insulin resistant subjects.

	IR subjects (n=72)	Non-IR subjects (n=32)	p-value
Age (years)	11.44±2.54	11.39±2.95	
Sex (M/F)	32/40	16/16	0.600
Height (SDS)	0.65±1.04	0.25±1.18	**0.035**
Weight (SDS)	2.28±0.56	2.05±0.52	**0.025**
BMI	29.72±4.38	28.33±4.03	0.220
BMI (SDS)	2.24±0.49	2.11±0.45	0.214
WC (cm)	87.70±14.41	83.20±18.04	0.173
HC (cm)	97.84±16.83	91.97±20.48	0.076
WC/HC	0.90±0.06	0.92±0.08	0.144
WHtR	0.59±0.05	0.58±0.05	0.648
SBP (mmHg)	114.98±9.16	112.96±7.94	0.253
DBP (mmHg)	70.44±9.28	68.74±7.31	0.283
GOT (U/L)	21.31±9.69	20.40 ±4.50	0.504
GPT (U/L)	25.82±23.41	18.87±11.33	0.080
GGT (U/L)	17.82±19.38	11.82±3.02	**0.015**
Total cholesterol (mg/dl)	169.73±27.56	170.34±22.14	0.542
LDL-cholesterol (mg/dl)	92.80±27.55	83.16±25.60	0.137
HDL-cholesterol (mg/dl)	50.60±13.85	57.34±18.52	**0.032**
Triglycerides (mg/dl)	88.42±39.40	76.06±28.87	0.163
Triglycerides/HDL-ratio	1.90±1.11	1.59 ±1,24	0.055
Total cholesterol/HDL-ratio	3.53±0.92	3.35± 1,66	0.101
Uric acid (mg/dl)	4.95±1.19	4.87±1.30	0.795
CRP (mg/dl)	1.36±7.41	0.14± 0.11	**0.003**
HbA1c (%)	5.37±0.33	5.25±0.62	**0.025**
Fasting glucose (mg/dl)	97.04±7.70	93.12±8.53	**0.012**
1h-postprandial glucose (mg/dl)	130.79±20.21	126.90 ±25.28	0.410
2h-postprandial glucose (mg/dl)	117.61±16.62	108.97 ±16,06	**0.012**
Fasting insulin (mUI/ml)	25.65±11.76	11.08±3.97	0.000
1h-postprandial insulin (mUI/ml)	146.19±123.01	99.56±53.30	**0.012**
2h-postprandial insulin (mUI/ml)	141.05±98.82	79.06±39.48	**0.000**
HOMA-IR	6.19±3.07	2.52±0.89	**0.000**
HOMA-B	278.53±130.12	146.24±78.48	**0.000**
IGI	3.19±3.21	2.2523±1.159	0.097

Matsuda-index	1.98±0.86	3.68±1.30	**0.000**
AUCg	269.55±181.16	239.13±31.62	**0.047**
AUCi	282.08±249.55	162.54±63.63	**0.000**
AUCi/AUCg ratio	1.04±0.69	0.67±0.24	**0.000**
FT4 (pmol/L)	15.10±2.62	15.73±2.21	0.310
TSH (uUI/ml)	2.78±3.09	2.15±0.79	0.770
Fasting Zonulin (pg/ml)	20.33±13.93	16.88±8.29	0.526
1h-postprandial Zonulin(pg/ml)	22.86±17.45	17.48±9.43	0.185
2h-postprandial Zonulin(pg/ml)	23.68±18.39	17.92±8.81	0.259
Liver steatosis (n)	32/64	9/30	**0.054**

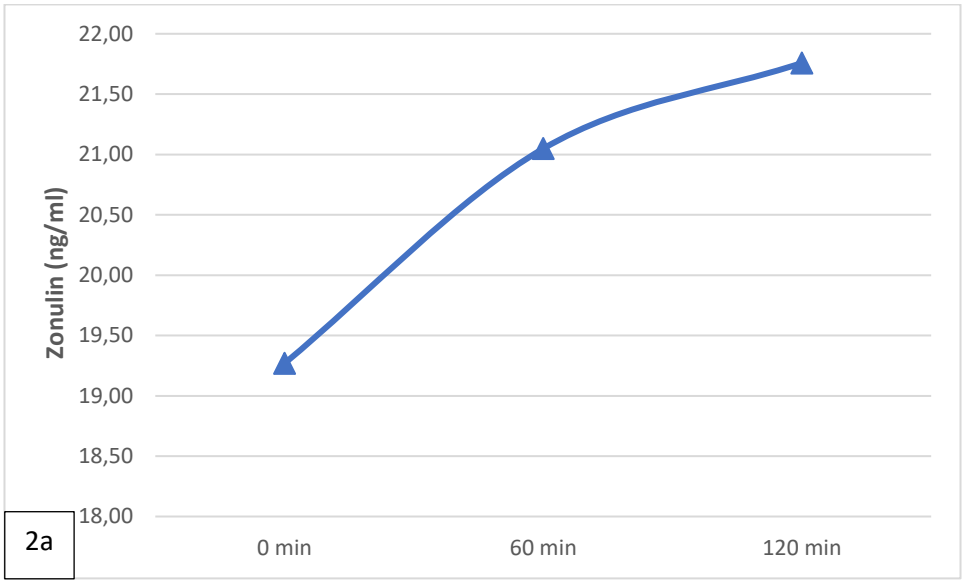
Numerical data are expressed by mean ± SDS.

Body mass index (BMI), standard deviation score (SDS), waist circumference (WC), hip circumference (HC), WC-to-height ratio (WHtR), systolic blood pressure (SBP), diastolic blood pressure (DBP), glutamic-oxaloacetic transaminase (GOT), Glutamate Pyruvate Transaminase (GPT), gamma-glutamyl transpeptidase (GGT), C-reactive protein (CRP), glycated haemoglobin (HbA1c), homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment for β-cell function (HOMA-B), insulinogenic index (IGI), Area under the curve for glucose (AUC_g) and insulin (AUC_i). thyroid stimulating hormone (TSH), free thyroxine (FT4).

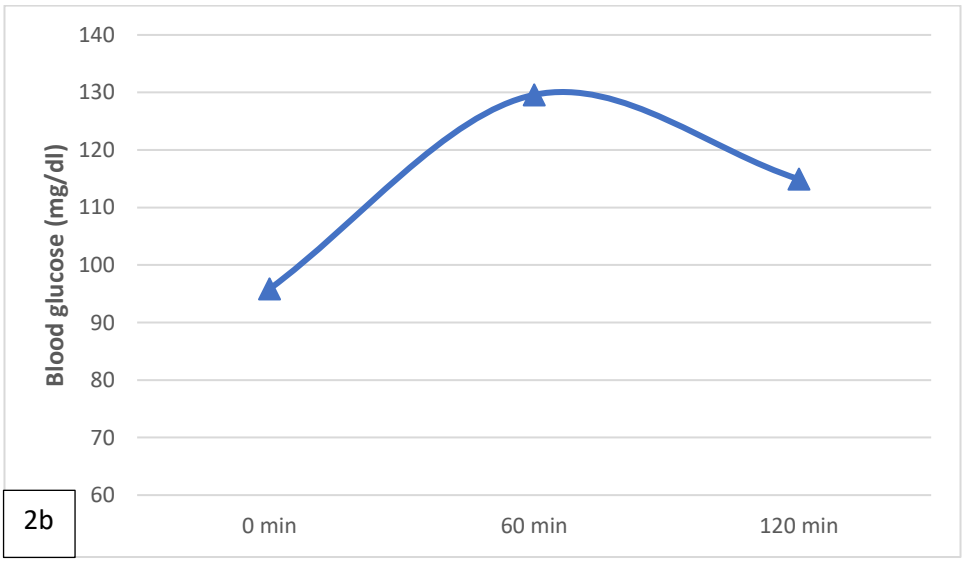
2.5.2 The meal-related pattern of serum zonulin and its relationship with obesity-related biomarkers

Zonulin serum levels significantly increased from baseline to 60-minute and 120-minute OGTT timepoint ($p<0.001$) in the entire study population (*figure 2a*). Such statistically significant increasing trend of zonulin secretion over time was confirmed by the Mann-Kendall test for trend ($p=0.000$). Conversely, blood glucose and insulin curves did not show a statistically significant trend ($p=0.184$ and $p=0.168$ respectively) in the entire cohort. Indeed - as expected - blood glucose and insulin levels reached a peak after 60 minutes after

OGTT load, followed by a postprandial decrease from 60 to 120 minutes, showed in figure 2b and 2c.



2a



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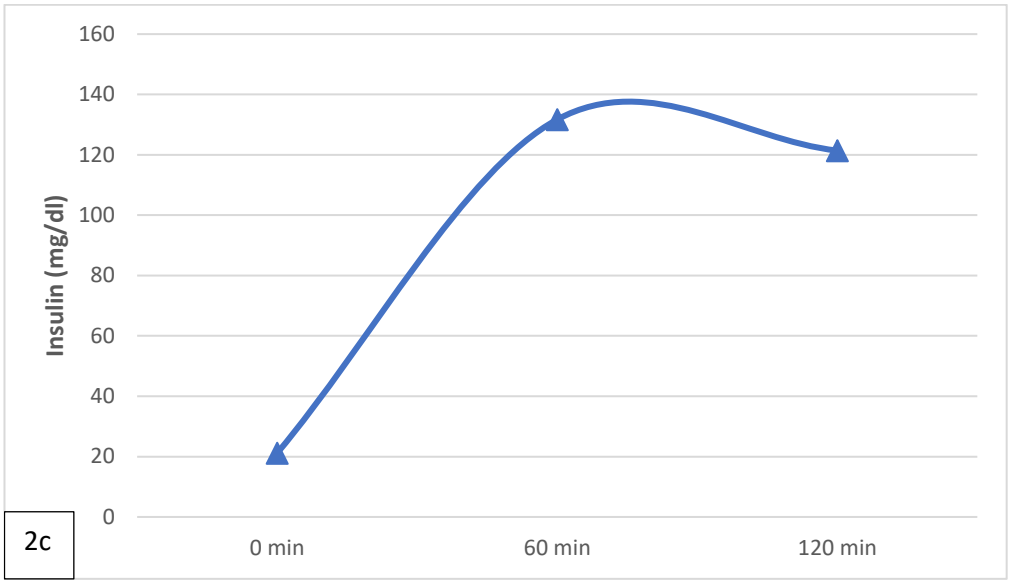

Figure 2. The meal related pattern of secretion of zonulin (2a), blood glucose (2b) and insulin (2c), evaluated from baseline to 60 minutes and 120 minutes after oral glucose tolerance test (OGTT).

A positive correlation was demonstrated between **BMI SDS** and serum zonulin levels measured at 120-minute after OGTT load (r=+0.208, p=0.046), as showed in *figure 3*.

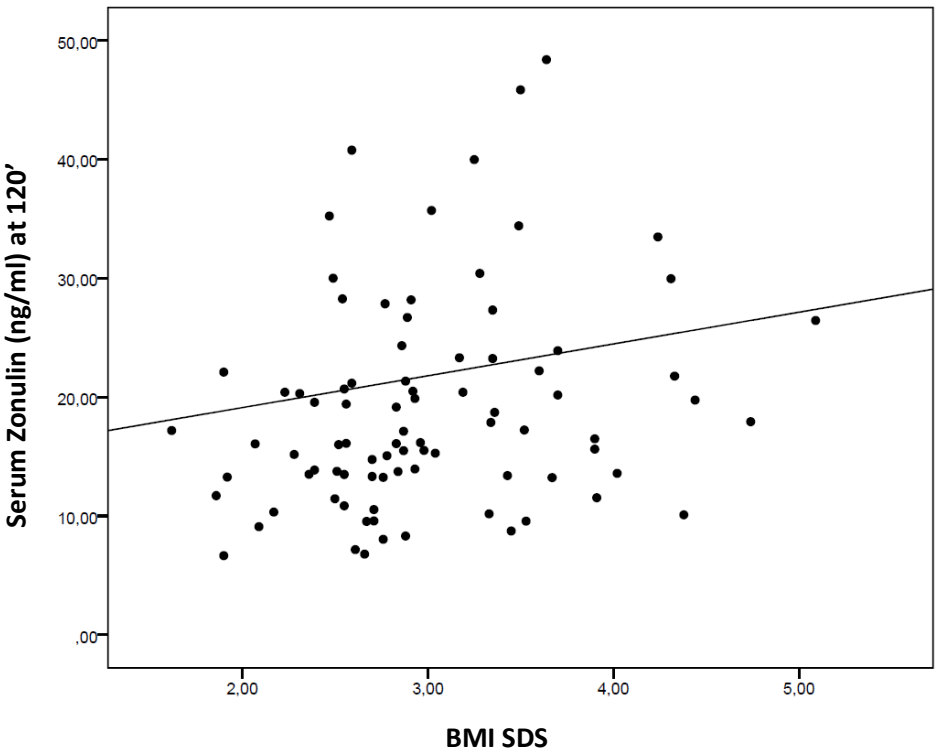

Figure 3. The scatter plot shows the interdependence between body mass index (BMI) SDS and serum zonulin levels measured at 120 minutes after oral glucose tolerance test (OGTT).

Multiple linear regression model highlighted a significant positive association of zonulin fasting levels respectively with **IR** ($p = 0.039$) and **glutamic-oxalacetic transaminase (GOT)** levels ($p = 0.038$), as showed in *figures 4* and *5*.

No significant differences in serum zonulin levels were demonstrated for age, sex, pubertal status, glucose, lipid profile and the other studied clinical and biochemical variables.

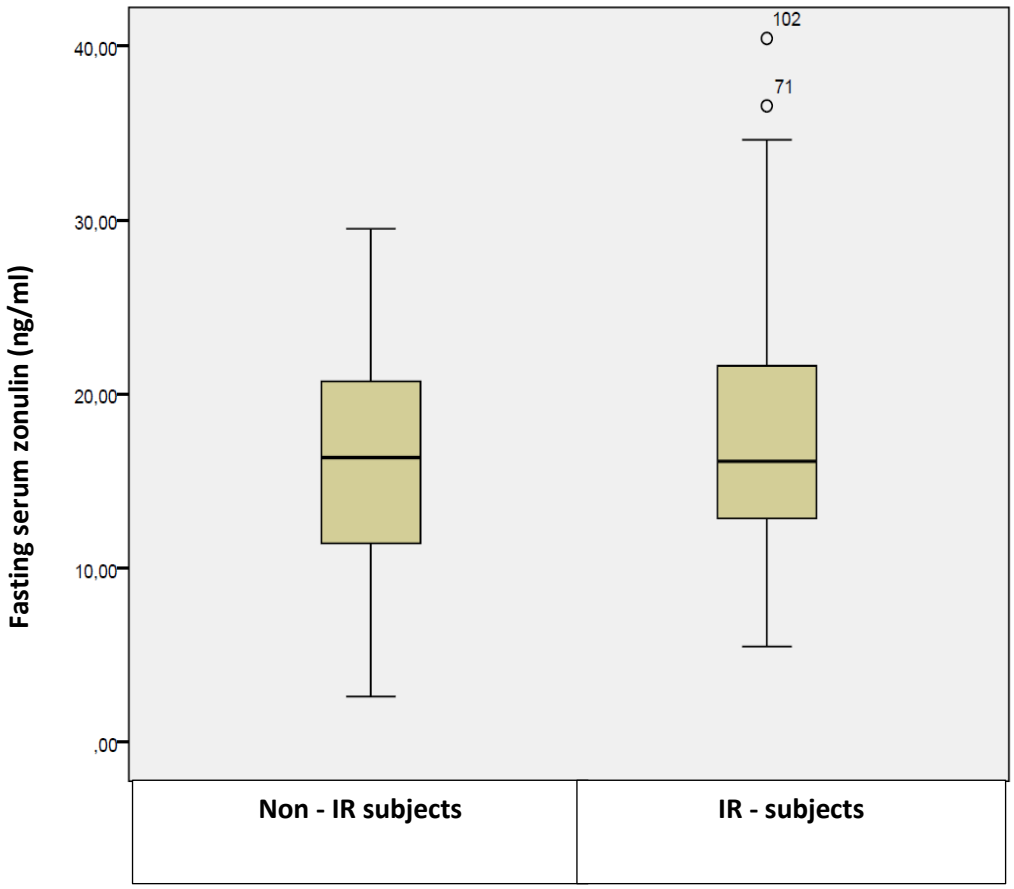

Figure 4. Box plot of fasting serum zonulin levels in insulin-resistant (IR) and non-insulin resistant patients.

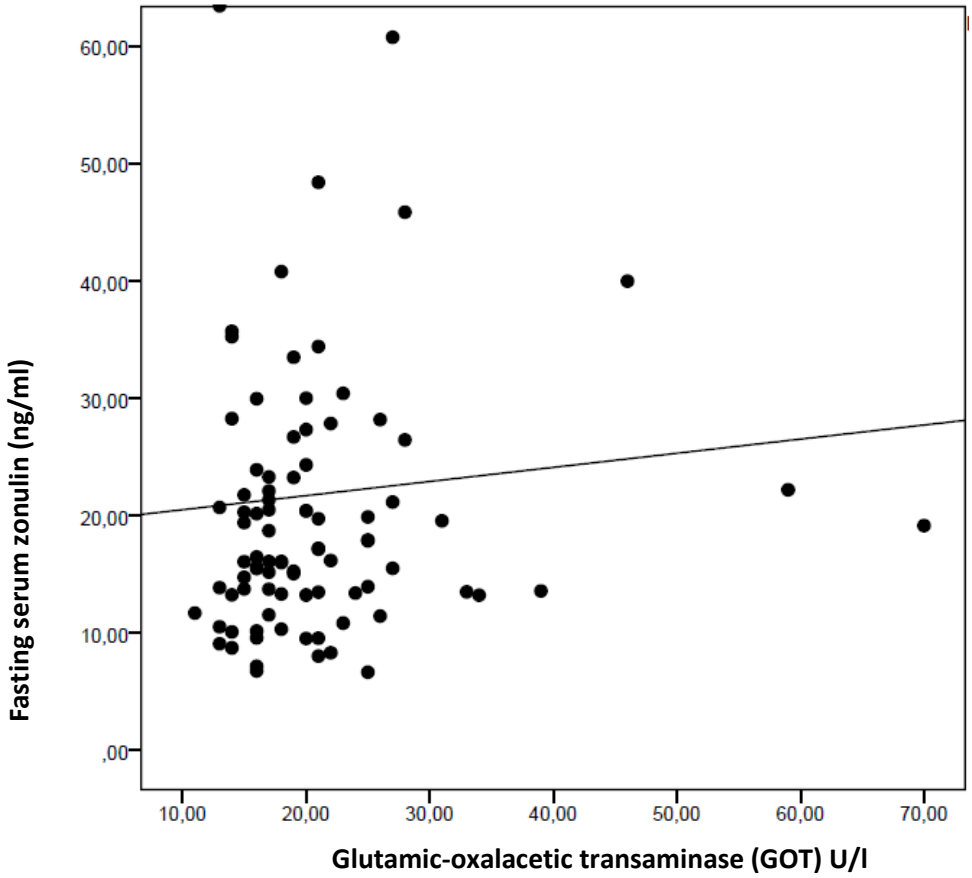

Figure 5. The scatterplot highlights a positive association between fasting serum zonulin levels and glutamic-oxalacetic transaminase (GOT) in the entire study population.

2.6 Discussion

To the best of our knowledge, this is the first study to investigate the meal-related pattern of secretion of serum zonulin in a pediatric large cohort of obese non-diabetic children and adolescents.

According to the available literature reports, serum zonulin could be regarded as a novel biomarker of intestinal permeability and is suggested to play a role in the development of metabolic syndrome found in obese patients.

Recently, higher serum zonulin levels were reported in obese subjects compared to healthy controls. These findings are well-demonstrated in adulthood [26-29]. Conversely, only few studies investigated the relationship between zonulin secretion and obesity-related biomarkers in pediatric age.

The results of the present study confirm a close relationship between serum zonulin concentration and the degree of obesity and overweight in pediatric age. Indeed, we documented a positive correlation between zonulin levels at 120' OGTT timepoint and BMI SDS in the entire study population. This finding is consistent with previous pediatric reports [43, 51, 52]. In addition, we found a positive association of zonulin fasting levels with insulin-resistance (IR) at multiple linear regression analysis. Studies investigating the relationship between zonulin concentration and IR have yielded different results [26-28]. Moreno-Navarrete et al. explored for the first time this relationship in adulthood, describing increased circulating zonulin levels in patients with glucose intolerance and

obesity-associated IR versus controls, and enhancing the role of subclinical inflammatory cytokine IL-6 in this pathway [27]. Likewise, another adult study by Zhang et al. reported higher serum zonulin levels in patients with diabetes mellitus type 2 (T2DM) than in impaired or normal glucose tolerant subjects [28]. In pediatric population, Kim et al. confirmed a positive association between zonulin concentration and BMI, fasting insulin, and HOMA-IR [43]. Conversely, other authors did not find that IR was associated with serum zonulin levels [28, 50, 52].

Moreover, in our cohort we found a positive association between fasting zonulin and glutamic-oxalacetic transaminase (GOT) levels. The association between zonulin and liver enzymes in childhood obesity was reported also by Kim et al., who found a positive association with alanine aminotransferase (ALT) [43]. In addition, other studies reported that circulating zonulin is increased in children and adolescents with non-alcoholic fatty liver disease (NAFLD) and correlates with the severity of liver steatosis [44], which we did not find in our study. Likewise, zonulin seems to correlate also with the risk of progression from liver steatosis to fibrosis. In this regard, Parkhomenko et al. reported a significant positive correlation between serum zonulin and PNFI (pediatric non-alcoholic fatty liver disease fibrosis index) in obese adolescents. Recently, an increasing number of studies demonstrated a close connection between the gut microbiota and the liver, shedding new insights on the so called “gut-liver axis” [44-47, 49]. In particular, the results of intestinal permeability studies confirmed that impairment of intestinal wall

integrity may play a major role in the development and progression not only of obesity itself, but also of obesity-related metabolic disorders, such as liver steatosis and NAFLD [44, 47, 49, 51]. Impairment of intestinal permeability is not easy to assess. Nowadays, serum zonulin assay seems to become a reliable and easy way to reflect dysfunctional intestinal barrier, since zonulin is acknowledged as the only measurable blood protein to reversibly regulate intestinal permeability through modulation of intercellular tight junctions [21]. It has been hypothesized that intestinal dysbiosis, secondary to high-fat and low-fibre diet, is a trigger for the increased synthesis of zonulin by the lamina propria of the intestinal epithelium. Zonulin secretion leads to an increase in gut permeability secondary to the disassembly of the zonula occludens-1 from the tight junctional complex [60]. Such an increased intestinal permeability may allow bacterial translocation, as well as promoting chronic inflammation of the gut, and subsequently at a systemic level. This chronic low-grade systemic inflammation, which characterizes obesity, is an important contributor to intestinal barrier dysfunction and might in turn upregulate zonulin expression [34, 38, 39, 61].

Finally, our results highlighted, for the first time in a pediatric cohort, the meal-related pattern of secretion of serum zonulin, which tends to significantly increase during and at 2-hours postprandial assessment. The increasing trend exhibited by zonulin did not reflect the dynamics of the glycemic and insulinemic curves, which instead are characterized by a postprandial peak followed by decreasing trend. These data show the effect of acute hyperglycemic stress

induced by oral glucose tolerance testing (OGTT) on zonulin levels in our cohort of obese youths. Previously, one study investigated zonulin levels before and after OGTT in adulthood. Saitoguillari et al. reported a positive correlation between fasting and after 2-hours zonulin in prediabetic patients versus controls [30]. Moving from our results, we can argue that, during acute hyperglycemia induced by OGTT, up-regulation of zonulin is long-lasting and may affect intestinal function. In the light of the above-mentioned relationship between intestinal permeability, chronic low-grade inflammation, and obesity, increased zonulin seems to reflect not only intestinal permeability, but may also reflect a reaction, secondary to inflammation and IR, establishing a persistent and self-maintaining vicious circle.

Chapter 3

CONCLUSIONS

Overall, our study provided new evidence about the relationship between serum zonulin and obesity-related parameters in pediatric age, shedding new light on the emerging role of zonulin as a promising and valuable biomarker also in childhood obesity. Therefore, we can argue that zonulin might be proposed not just as a marker of impaired intestinal permeability in children and adolescents with overweight and obesity, but also as a possible indicator of early metabolic disorders, helping to identify patients at increased risk of developing obesity-related complications due to insulin-resistance.

We acknowledge that our study has some limitations:

i) the lack of a sex and age-matched control group of normal weight subjects;

ii) the cross-sectional design which does not allow to understand the cause-and-effect relationship and to explore variation of zonulin levels over time, possibly after weight modifications;

iii) the uncertainty on the intestinal origin of zonulin, since the protein is secreted mainly from the liver and the enterocytes, but

also from adipose tissue, brain, heart, immune cells, lungs, kidney, and skin.

Additional studies, especially in pediatric age, are needed to clarify the exact role of zonulin in the pathogenesis of obesity, and to confirm whether increased zonulin levels may have negative effects on intestinal permeability.

Future perspectives of this research field may involve longitudinal studies to investigate the possible variation of serum zonulin levels over time, after dietary regimen and lifestyle intervention outcome (weight loss or persistence/worsening of overweight).

Advanced understanding of this relationship would not only allow to identify children and adolescents at risk for metabolic disorders at an early stage, but may also provide new therapeutic choices focused on microbiome modifications (e.g., via dietary pattern changes or probiotics administration).

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