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**POLYMORPHIC VARIATIONS IN CHILDREN WITH
OBESITY AND INSULIN RESISTANCE**

ABSTRACT

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I. INTRODUCTION

Obesity in children represents a public health issue primarily linked to dietary intake and genetic patterns. Nowadays, the young population has easy access to high-calorie and taste-appealing foods that contribute to chronic metabolic diseases when included in dietary and lifestyle choices.

The prevalence of pediatric obesity is increasing worldwide. In 2019, the World Health Organization estimated that 38 million children under 5 and 340 million children and adolescents aged between 5 and 19 were overweight or obese.

Obesity pathogenesis involves complex interactions between behavioral, environmental, and genetic factors.

This Ph.D. thesis focuses on the evaluation of the polymorphic variants in children with obesity and insulin resistance due to the impact of these two medical conditions on their health outcomes. Unfortunately the modern era facilitated sedentary lifestyle patterns and promoted accessible fast-food habits for the young generation. Also, the current SARS-CoV pandemic has contributed throughout its restrictions to the increasing prevalence of obesity in both adults and children.

The NUTRIGEN project ("Use of nutrigenomic models for personalization of dietary treatments in obesity"), developed in the Genetics Discipline of the University of Medicine and Pharmacy "Victor Babeș" Timișoara, gave us the opportunity to evaluate a large number of children with obesity. The primary purpose of the study was represented by establishing a genetic signature model involved in unsaturated omega-6/3 essential fatty acids (PUFA) metabolism and a correlation between blood/plasma concentrations of the relevant metabolites, genetic signatures and a profile for insulin resistance in obese children.

In recent years, significant studies aimed to identify the molecular mechanisms of the genes involved in the etiopathogenesis of obesity in search of high risk indicators. Adding to the fact that nutrition is one of the primary environmental factors that determinate obesity, gene-diet interactions represent a priority theme in evaluating gene variation's impact on nutrient metabolism.

The relationship between obesity and insulin resistance is proven throughout many data. Insulin is a regulator of adipocyte biology, the cell structure with the highest insulin sensitivity, promoting the storage of triglycerides in adipocytes, stimulating glucose transport, triglyceride synthesis (lipogenesis), and inhibiting lipolysis. It also increases the absorption of fatty acids derived from circulating lipoproteins by stimulating lipoprotein lipase activity in the adipose tissue.

The risk of insulin resistance and diabetes increases as body fat content measured by body mass index (BMI) is higher. Adipose tissue acts as an endocrine organ. Central (intra-abdominal) fat deposits represent a precondition for developing insulin resistance, type 2 diabetes, and cardiovascular disease.

The body weight depends on food intake, energy expenditure, and adipogenesis. Any mutation involving the genes that mediate these mechanisms has phenotypic weight-turned consequences.

Hereditary factors cover 30% to 50% in BMI variation. Although polygenic obesity is by far the most commonly observed, monogenic defects and syndromic forms of obesity are also identified.

Monogenic obesity is caused by single gene mutations. The majority of the genes responsible for monogenic obesity are involved in the leptinergic-melanocortinergic pathway. These genes encode leptin (LEP), the leptin receptor (LEPR), melanocortin 4 receptor (MC4R), proopiomelanocortin (POMC), and brain-derived neurotrophic factor (BDNF). Patients with monogenic forms generally present during early childhood with hyperphagia and severe obesity. Other genetic causes of severe obesity are Prader Willi syndrome, Alström syndrome, Bardet Biedl syndrome.

The possibility of detecting causal loci for polygenic forms of childhood obesity increases as the genome-wide association studies (GWAS) expands, providing opportunities for identifying new loci for common variants of obesity.

Genetic factors also play a crucial role in insulin resistance. Insulin receptor substrate 1 (IRS-1) is involved in the insulin signaling pathway. Fat mass and obesity-associated gene (*FTO*) variants are strongly associated with increased BMI and impaired insulin sensitivity. Transcription Factor 7 Like 2 gene (*TCF7L2*) variants show the strongest and most consistent association with the risk of developing insulin resistance and type 2 diabetes of any gene variants identified so far. N-acetyltransferase 1 (*NAT1*) and 2 (*NAT2*) mutations affect insulin sensitivity. Low concentrations of insulin-like growth factor 1 (IGF-1) are associated with decreased insulin sensitivity.

Additionally, the adipose tissue of obese individuals is infiltrated with mononuclear cells that maintain a state of chronic inflammation. These pro-inflammatory and prothrombotic adipokines (TNF α , resistin, IL-6, plasminogen-1 activator inhibitor, angiotensinogen) have a direct contribution to insulin resistance and atherogenesis.

In recent years, the modern dietary pattern of the western diet is characterized by being rich in saturated fats, refined carbohydrates and salt. In an attempt to promote healthy dietary habits, the beneficial role of n-3 PUFA intake is fully documented in literature.

The LC-PUFAs cannot be synthesized *de novo*, and their blood levels depend on nutrition and conversion pathways from the two precursors, namely n-6 linoleic acid (LA) and n-3 α -linolenic acid (ALA). The n-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are synthesized from ALA, while n-6 arachidonic acid (ARA) is from LA. Green plants, seeds, and vegetable oils represent ALA and LA intake sources, while seafood mainly provides the EPA and DHA supply. ARA is contained in a large variety of animal foods. Studies have shown the substantial health-related benefits of the n-3 LC-PUFAs intake. Maintaining the ratio between n-3 and n-6 fatty acids is extremely important. Lately, a shift has been observed in overweight children due to the western diet's decreased n-3 intake. Besides nutrition, the conversion pathway rate from ALA offers essential differences between n-3 LC-PUFAs levels. The synthesis of EPA and DHA is provided through desaturation and elongation processes, catalyzed by a group of enzymes known as desaturases. This mechanism is dependent on the expression of the fatty acid desaturase 2 gene (*FADS2*)-encoded delta-6 desaturase (D6D) enzyme.

Genetic variants of the *FADS* gene cluster have an essential role in lipid profiles and glucose homeostasis. Furthermore, gene-diet interactions emphasize the role of *FADS* polymorphisms on health.

In PUFAs' endogenous synthesis, the fatty acid desaturase (*FADS*) gene cluster (controlling the desaturation) and genes controlling the elongation (*ELOVL* genes) are involved; variations in these genes impact PUFA levels in children.

FADS2 gene (chromosome 11q12.2) plays a role in the initial desaturation step that converts n-6 LA and n-3 ALA into gamma (γ)-linolenic acid (GLA) and stearidonic acid (SDA), respectively. It also mediates the final step in n-3 PUFAs biosynthesis for DHA synthesis.

Certain polymorphic variations in the phosphatidylethanolamine N-methyltransferase (*PEMT*) and methylene tetrahydrofolate reductase (*MTHFR*) genes involved in phospholipid synthesis may also influence PUFA status.

Additionally, genetic variations in genes involved in the synthesis of phospholipids and one-carbon metabolism may further impact PUFAs' status. Phosphatidylcholine (PtdCho) is the most common phospholipid in cell membranes. PtdCho is synthesized through distinct pathways, either through the Cytidine 5'-diphosphocholine (CDP-choline) pathway or by the conversion of phosphatidylethanolamine to PtdCho by phosphatidylethanolamine N-methyltransferase (*PEMT*), which influences the abundance of certain PUFAs in phospholipids structure. The methylenetetrahydrofolate reductase gene (*MTHFR*), involved in one-carbon metabolism, may also impact omega-3 PUFA levels, probably by influencing S-Adenosyl methionine synthesis, which in its turn influences PUFAs' phospholipid composition.

PEMT gene is highly polymorphic, data indicating that some SNPs may have a protective role from nonalcoholic fatty liver (NAFLD) progression when choline intake is low.

The fatty acid composition of erythrocytes reflects the fatty acid composition of other organs. Therefore, erythrocyte membrane fatty acid levels could be potentially relevant biomarkers for assessing PUFA status in the human body and could improve the assessment of PUFA homeostasis, adding relevant information about the roles that PUFA may play in associated metabolic disorders of obesity, such as dyslipidemia, and also highlights additional links between dietary intake and various genetic variations.

II. OBJECTIVES

The thesis is structured in three studies that focus on essential scientific objectives:

Study 1. The first study's objective was to evaluate the association between *FADS2* gene variants and free plasma levels of polyunsaturated fatty acids (PUFA) in overweight and obese children, taking into account their dietary intake.

I chose to focus on this scientific topic because the imbalance between PUFA intake and its metabolism can promote metabolic imbalances that precede obesity. PUFAs are assimilated through the diet and synthesized endogenously from their precursor molecules (linoleic acid for omega-6 and alpha-linolenic acid for omega-3). Several genes can influence the status of PUFA in the body. The *FADS2* gene encodes one of the enzymes that catalyze the synthesis of PUFA, and its gene variations alter the phospholipid structure of fatty acids in the blood.

Study 2. The second study focused on developing and validating a LC-MS/MS method to fully quantitate ALA, ARA, DHA, EPA, and LA in plasma from both their free and total

forms, using a simplified sample preparation procedure without the need of chemical derivatization.

The method is intended as an improved alternative to the existing gas chromatography-based methods by offering shorter analytical runs.

Until recently, FAs were commonly quantified using gas chromatography–mass spectrometry (GS-MS) with electron impact ionization following methyl esters derivatization and gas chromatography-flame ionization (GS-FID). Liquid chromatography–mass spectrometry (LC–MS) began more recently to be used for FAs analysis. Using electrospray ionization (ESI), FAs tend to ionize in negative mode, but they were reported to exhibit low specificity. Therefore, chemical derivatization was used to improve the ESI-LC-MS detection. However, derivatization requires additional steps in the sample preparation protocol.

Study 3. The association between several gene variants and the levels of PUFA in the blood of obese children was the third objective of the thesis. Given the importance of fatty acid composition for the structure of cell membranes, the present research focused on the association between several single polymorphic variants (SNPs - single-nucleotide polymorphism) and PUFA content in erythrocytes of obese and overweight children.

This study aimed to identify potential biomarkers relevant for the evaluation of PUFA homeostasis by assessing erythrocyte membrane fatty acid (RBC) levels in relation to dietary intake and genetic variations in childhood obesity. Also, another aim was to compare the composition of phosphatidylcholine-derived fatty acids of the *PEMT* and CDP-choline pathways.

III. MATERIALS AND METHODS

1. Participants

Two hundred obese children (97 males, 103 females) were recruited at the 2nd Paediatric Clinic of Clinical Emergency County Hospital Timisoara, Romania, in the context of a bigger study. Participants were aged 7–18 years, with BMI > +2 SD as compared to the World Health Organization (WHO) reference, and abdominal circumference above the 90th percentile. Their parents or other legal guardians, as well as the children themselves, were fully informed about the aims and methods of the study, and informed consent was obtained from legal guardians. The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of the “Victor Babes” University of Medicine and Pharmacy, Timisoara, Romania, and was registered at ClinicalTrials.gov (NCT02837367).

Exclusion criteria were diagnosis of any type of cancer or medical history of cancer; any psychiatric disorder; blood coagulation disorders; endocrine-induced obesity (Cushing syndrome, hypothyroidism, growth hormone deficit); hypothalamus-induced obesity (Babinski–Fröhlich syndrome); genetic syndromes (Prader–Willi, achondroplasia, Bardet–Biedl, Fanconi, Turner, etc.), and personal history for convulsive disorders, nephrotic syndrome, or asthma with corticoid treatment. Four individuals (females) were excluded due to incomplete assessment data.

2. Anthropometric Measurements and sample collection

Anthropometric measurements were performed, following international guidelines. Weight and height were measured using an electronic scale with a stadiometer. Measurements for height and weight were recorded to the nearest 0.5 cm and 0.5 kg, respectively. BMI was calculated as kg/m².

Waist circumference was measured with an inextensible anthropometric tape, by a trained person, to the nearest 0.1 cm, in standing position, at the midpoint between the end of the rib cage and the top of the iliac crest. Hip circumference was measured around the widest portion of the hip.

Blood samples were collected after overnight fasting (at least 6 h) in EDTA sterile vacutainers.

3. Food intake

Food intake was evaluated using five-pass 24 h dietary recalls as previously described. The dietary recalls were performed four times for each participant. For younger participants, they were addressed to both a parent and the child. The declared amounts of food were converted to energy and macro- and micronutrient intakes using a web application (Nutritio, Bucharest, Romania, <https://nutritioapp.com>) and average daily intakes were computed and used in subsequent analyses.

4. Hematological and Biochemical Tests

Complete blood count was assessed using flux cytometry and cytochemistry by ISO 15189-accredited medical laboratory, acting as an external partner.

Total plasma concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), C reactive protein (CRP), total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose were performed on an Ortho Clinical Vitros 350 Chemistry System (Ortho Clinical Diagnostics Inc, Raritan, NJ, USA), using its standardized reagents, following the manufacturer's protocols. Homocysteine was measured by ELISA method on an Epoch Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). The analyzer was calibrated and maintained according to the manufacturer's instructions. A homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the following formula: fasting insulin (mIU/L) × fasting glucose (mmol/L)/22.5.

5. Quantification of fatty acids

N-3 and n-6 PUFAs—ALA, EPA, DHA, and LA and ARA, respectively—were measured in their free form in plasma samples using high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), following the method described by Serafim et al.

6. Analysis of Genetic Variants

Genotyping was performed on a MiSeq sequencer (Illumina, San Diego, CA, USA) using a custom-made hotspot sequencing panel for 55 single nucleotide polymorphisms (SNPs) within 14 genes selected as previously being associated with increased lipids, non-alcoholic fatty liver, or cardiovascular disease. The 55 genetic polymorphisms (high frequency according to the "1000 Genomes Project") metabolically relevant to obesity are: *ABCB4* (rs1149222, rs2071645, rs31672, rs4148811, rs9655950, rs1202283), *APOC3* (rs28 CH, rs28, rs2289209, rs4563403, rs4687591, rs6807783, rs7634578, rs881883), *CHKB* (rs1557502, rs1557503, rs470117, rs7238), *FADS2* (rs2526678, rs526126) *MTHFD1* (rs10135928), *MTHFR* (rs1801133, rs2066471, rs4846048, rs4846052, rs7525338, rs868014) *SCD* (rs11557927, rs11599710, rs12247426, rs2167444, rs7849), *SLC44A1* (rs10120572, rs10820799, rs193008, rs328006, rs440290, rs443094, rs7018875), *STAT3* (rs9891119), *PCYT1A* (rs1580820), *PCYT1B* (rs4898190), *EMYP* (rs1109859, rs12103822, rs16961845, rs4244593, rs4479310, rs7214988, rs7946, rs8068641, rs936108, rs13342397, rs6502603), *PNPLA3* (rs2281135, rs738409).

Sequence alignment to the reference genome and sequence quality filtering was performed using the Illumina MiSeq Reporter v2.6 platform. The sequences were aligned with Burrows-Wheeler Aligner (BWA), and variant calling was performed with Genome Analysis Toolkit (GATK) using the human reference sequence assembly hg19/GRCh37.

IV. RESULTS AND DISCUSSIONS

Study 1

The results showed that the low-frequency G allele of rs526126 was associated with higher levels of plasma DHA only for DHA and EPA intakes lower than 10.56 mg and 3.45 mg, respectively.

Our data indicated that DHA plasma levels were more closely associated with rs526126 than EPA plasma levels. This can be explained by the fact that DHA is the final product of the desaturation cascade and thus is more likely to be influenced by *FADS2* activity. Moreover, the plasma levels of free DHA were higher than the ALA and EPA, leading to the conclusion that a significant amount of DHA is synthesized endogenously, in addition to their respective intakes. Differences were even more evident when the EPA and DHA dietary intakes were low, leading to the hypothesis that *FADS2* activity is dependent on them and that the presence of rs526126 low-frequency allele (G) may result in increased desaturation activity.

Study 2

This method was designed considering the primary goal, which was to develop a method of quantification without the derivatization of fatty acids. Derivatization would have required several extra steps in an already time-consuming sample preparation protocol. Previously published methods using dimethylaminoethyl ester (DMAE) derivatives of fatty acids provided LODs in the low femtomolar range; however, it required additional incubation and extraction steps, adding more complexity to an already time-consuming protocol. Although derivatization provides unparalleled sensitivity, our method proved that FAs from human plasma can be

reliably quantified without the need for derivatization. In comparison with GS-MS and GS-FID methods, which require an approximately 20 min analytical run, our method is significantly shorter, with a duration of only 6,5 min, which results in a massive time reduction after running a large number of samples.

Study 3

Significant differences were identified between females and males regarding standardized body mass index for age (zBMI), with males presenting a higher degree of obesity.

GG and GA genotypes, when compared to the AA genotype for the *PEMT* rs1109859, were associated with higher levels of DHA and EPA in RBCs. This is in agreement with previous findings, suggesting that the PtdCho synthesized via the *PEMT* pathway contains mainly PUFAs (mainly ARA and DHA) while the CDP-choline pathway forms PtdCho containing medium-chain and saturated fatty acids. Another study also found similar evidence, indicating that DHA composition from plasma PtdCho may be a marker for *PEMT* activity.

PUFA levels in RBCs were also associated with genetic variations for the *MTHFR* gene (rs4846052). Although the mechanism still needs to be further clarified, our results suggest that the *MTHFR* rs4846052 genotype influences PUFA levels in the RBC membranes, with the TT genotype being associated with higher levels of PUFAs in the RBC membranes compared to that of TC and TT genotypes.

V. CONCLUSIONS

Study 1

Results from this study indicated that the *FADS2* gene polymorphism impacts the plasma levels of free n-3 PUFAs, specifically at lower EPA and DHA intakes. We found that the presence of rs526126 low frequency allele in the *FADS2* gene was associated with higher plasma levels of free DHA, differences being more evident when the dietary intake of n-3 PUFAs was low. However, further research is needed to confirm the extent of this association and if rs526126 minor allele has a protective role in subjects with low intakes of n-3 PUFAs.

The plasma levels of free DHA were higher than the ALA and EPA, leading to the conclusion that a significant amount of DHA is synthesized endogenously, in addition to their respective intakes. Differences were even more evident when the EPA and DHA dietary intakes were low, leading to the hypothesis that *FADS2* activity is dependent on them and that the presence of rs526126 low-frequency allele (G) may result in increased desaturation activity.

Our data indicated that DHA plasma levels were more closely associated with rs526126 than EPA plasma levels. This can be explained by the fact that DHA is the final product of the desaturation cascade and thus is more likely to be influenced by *FADS2* activity.

Study 2

The developed and validated method described offers a simplified extraction procedure without the need of derivatization, and a fast and reproducible LC-MS/MS quantification. It is suitable for quantifying n-3 and n-6 fatty acids in human plasma, in both free and total forms. Due to the fact the derivatization is not needed, the sample preparation is less time-consuming. The LC-MS/MS proved significantly faster than previously published GS-MS methods. Combining these two factors, the proposed workflow is significantly less time-consuming than existing GS-MS and LC-MS methods. The method can be used to assess changes in fatty acid metabolism, which have implications in obesity, type 2 diabetes, insulin resistance, and interrelationship with other metabolic pathways.

Study 3

Genetic variations in *PEMT* (rs1109859) and *MTHFR* (rs4846052) were associated with alterations in the content of PUFA species in RBC membranes. This finding suggests that the genetic status of *PEMT* and *MTHFR* genes may contribute to PUFA homeostasis and, therefore, could contribute to PUFA status in children with obesity. Further research is needed to establish whether these genotype-specific alterations are specific to overweight children.

GG and GA genotypes, when compared to the AA genotype for the *PEMT* rs1109859, were associated with higher levels of DHA and EPA in RBCs. Our results strengthen further the hypothesis that fatty acid composition of PtdCho synthesized via the *PEMT* pathway contains mainly PUFAs (mainly ARA and DHA) while the CDP-choline pathway forms PtdCho containing medium-chain and saturated fatty acids. This finding suggests that DHA composition from plasma PtdCho may be a marker for *PEMT* activity.

MTHFR rs4846052 genotype influences PUFA levels in the RBC membranes, with the TT genotype being associated with higher levels of PUFAs in the RBC membranes compared to that of TC and TT genotypes.

The thesis summarizes the genetic variations involved in the metabolism of polyunsaturated fatty acids (PUFA) in obese children. This research provides encouraging results with predictive role in preventing the onset of obesity and its associated comorbidities (insulin resistance, type II diabetes, non-alcoholic fatty liver, and cardiovascular disease).

Genetic variations in the metabolic pathways of methyl donors can affect lipid transport and metabolism. The interest in addressing prediction and modeling methods through obesity-associated genetic signatures has valuable scientific utility.

Keywords: pediatric obesity, insulin resistance, PUFA, *FADS2*, *PEMT*, *MTHFR*, LC-MS;