Obesity and related metabolic disorders: influence of genetic variability and nutritional factors

PhD Thesis

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Frequent abbreviations used in the thesis

BMI: body mass index
BF: body fat
MS: metabolic syndrome
SNP: single nucleotide polymorphisms
UCP2: uncoupling protein 2
GIIS: glucose induced insulin secretion

LCPUFA: long chain polyunsaturated fatty acid

n-6 LCPUFAs
linoleic acid (C18:2n-6, LA)
gamma-linolenic acid (C18:3n-6, GLA)
eicosadienoic acid (C20:2n-6)
dihomo-gamma-linolenic acid (C20:3n-6, DHGLA)
arachidonic acid (C20:4n-6, AA)

n-3 LCPUFAs
alpha-linolenic acid (C18:3n-3, ALA)
eicosapentaenoic acid (C20:5n-3, EPA)
docosapentaenoic acid (C22:5n-3)
docosahexaenoic acid (C22:6n-3, DHA)

Introduction

Epidemiology of obesity

Obesity has become an epidemic in many parts of the world, according to numerous studies conducted in adults and in the much limited data collected from young people (1-3). From the 1970 to the end of the 1990s, the prevalence of overweight or obesity in school-age children doubled or tripled in several large countries (4). In Hungary, the prevalence of obesity increased from 12% to 16% between 1980' and 1990's among schoolchildren (5). Although the increasing trend seems to be uniform in all countries, North America, Europe and parts of the Western Pacific have the highest prevalence of overweight among children (approximately 20-30%) (4).

By 2010, about one in every ten children in the European region is predicted to be obese (4).

Definition of obesity

In recent years, body mass index (BMI) has been increasingly accepted as a valid indirect measure of adipose tissue in both children and adolescents for survey purposes (3). Currently a group of researchers developed an internationally acceptable definition of child overweight and obesity, specifying the measurement, the reference population, and the age and sex specific cut offs (6). In this method the percentile levels corresponding to a BMI of 25 kg/m^2 (overweight) and 30 kg/m^2 (obese) at age 18 were identified and projected backwards into childhood using a large (n=97 876) sample of youth from 6 countries (Britain, US, Holland, Singapore, Hong Kong, Brazil).

Etiology of obesity

Obesity results from the interaction of environmental factors (inappropriate eating behaviours and/or reduction in physical activity) and hereditary factors. This has been shown by numerous epidemiological studies carried out in large and different populations (7). Obesity has a very heterogeneous phenotypic expression and the molecular mechanisms involved in its development are more than diverse.

a.) Role of genetic factors in the etiology of obesity

According to several studies, 30 to 80% of weight variation might be determined by genetic factors. Today, the contribution of genetic factors to obesity can be summarized as below:

• **Monogenic obesity**: single mutations contribute to the development of obesity. These forms of obesity are rare, very severe and generally start in childhood (8). To date, nearly 200 cases of human obesity have been associated with a single gene mutation (9;10). These cases, which obey Mendelian genetics, are characterized by extremely severe phenotypes that present themselves in childhood and are often associated with additional behavioural, developmental, and endocrine disorders (11).

• **Syndromic obesity**: There are between 20 and 30 Mendelian disorders in which patients are clinically obese, yet are additionally distinguished by mental retardation, dysmorphic features, and organ-specific developmental abnormalities (10;11). Such cases are referred to as syndromic obesity. These syndromes arise from discrete genetic defects or chromosomal abnormalities, and can be either autosomal or X-linked disorders. The most common disorders known are Prader-Willi syndrome (PWS), Bardet-Biedl syndrome (BBS), and Alström syndrome.

• **Polygenic obesity**: several genetic variants interact with an 'at-risk' environment in common obesities. Here each susceptibility gene, taken individually, would only have a slight effect on weight. The cumulative contribution of these genes would become significant when there is an interaction with environmental factors predisposing to their phenotypic expression (overeating, reduction in physical activity, hormonal changes, and socio-economic factors).

There are two main approaches to identify DNA-based markers associated with quantitative traits.

The **genome-wide association study** is a hypothesis-generating approach that examines the whole genome to identify the approximate location of new genes for a disease or trait of interest. Although this approach has proven to be successful for mendelian disorders and for rare diseases with large genetic effects, its success in

common diseases and continuous traits such as obesity and BMI has been limited (12).

In the **candidate gene approach** genes that are thought to be involved in the pathogenesis of different traits, based on data derived from animal models, cellular systems, are identified. Genetic variants at these loci are then tested for association at the population level.

The number of genetic association studies has grown exponentially over the past 15 years, which was paralleled by a vast increase in the number of candidate genes.

The ***CD36* gene** is one of the promising candidate gene for obesity. CD36 is an 88 kDa membrane protein expressed at the surface of a wide variety of cell types including adipocytes, skeletal muscle cells, and monocytes/macrophages (13). It belongs to the class of B scavenger receptor and shows high affinity toward lipid-based ligands such as modified low density lipoprotein and long chain polyunsaturated fatty acids (LCPUFA) and apoptic cell membranes (13;14).

Gene invalidation and overexpression experiments indicate that CD36 plays a role in energy metabolism, fat storage and adipocyte differentiation in mice (15;16). Muscle-specific *CD36* overexpression enhances fatty acid oxidation, decreases plasma fatty acid (FFA), glucose and insulin levels and lowers body weight (15). In contrast, *CD36* invalidation is associated with high plasma FFA and triglycerides, low fasting glucose levels and less weight gain on a high fat diet (17). In humans, CD36 is also related to metabolic disorders. Firstly, CD36 deficiency (type 1) is associated with features of metabolic syndrome (18;19). Secondly, CD36 expression in adipocytes is positively correlated to body fat (20) and its expression is reduced after a period of weight loss (21). Finally, several single nucleotide polymorphisms (SNPs) in *CD36* gene have been associated with metabolic disorders related to excess fat depots (22-26), and a (TG)-repeat in intron 3 has been linked to elevated BMI in Korean patients with coronary heart disease (CHD) (27).

These studies have assessed the association between *CD36* SNPs and metabolic disorders in adults (22;24;26). However, whether *CD36* SNPs influence body fat in adolescents has not been reported in population studies. Genetic association studies

in young people are important in that the influence of behavioural and exogenous factors are less marked than in adults, leaving a larger share to the SNPs to affect the phenotype. Therefore, the aim of the present study was to assess the relationship between *CD36* genetic variability and obesity and body fat accumulation in adolescents.

An other well known and promising candidate gene for obesity is the **uncoupling protein 2 (*UCP2*) gene**. The human *UCP2* gene is mapped to chromosome 11q13, it contains 8 exons and spans 8 kb (**Figure 1**).

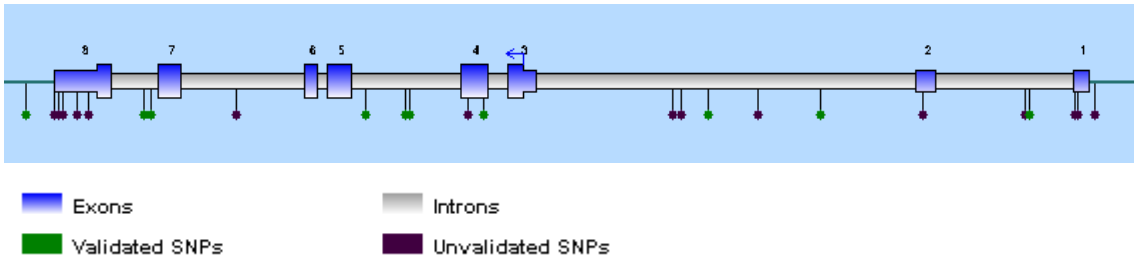

Figure 1. The human *UCP2* gene.

UCP2 gene is widely expressed in human tissues, including white adipose tissue, endocrine pancreas, skeletal muscle and liver (28). The effect of UCP2 on obesity can be due to its suspected function in energy metabolism. UCP2 might enhance the proton leak, induce respiratory uncoupling, thereby releasing the energy stored within the proton motive force as heat, and resulting in a decrease in ATP synthesis (29). Furthermore, it has been shown to effect carbohydrate and lipid metabolism. In pancreatic β-cells, increased expression or activity of UCP2 may contribute to impairing insulin secretion by reducing the ATP-ADP ratio. The mechanism(s) by which UCPs may enhance lipid catabolism is still unknown, but could rely on their ability to transport fatty acid anions outside of the mitochondrial matrix. (30).

The variant A allele of the common -866G/A polymorphism in the promoter region of the human *UCP2* gene enhances its transcriptional activity. The enhancement results in increased UCP2 mRNA levels in human fat cells; consequently, this polymorphism is associated with a reduced risk of obesity but an increased risk of type 2 diabetes (31;32). Recently, an association between obesity, disorders of lipid and carbohydrate metabolism and the G-allele of the -866G/A polymorphism in the promoter region of *UCP2* gene was reported (33).

In the present study, we investigated the fatty acid composition of plasma lipids and the association of n-6 LCPUFAs to glucose induced insulin secretion (GIIS) evaluated by standard oral glucose tolerance test (OGTT) in obese children stratified according to the -866 G/A polymorphism of UCP2.

b.) Role of long chain polyunsaturated fatty acids in the etiology of obesity

Several environmental factors affect the obesity phenomenon significantly. Evidence has accumulated showing that early nutrition programs later obesity risk. The mechanisms involved are poorly understood, beside the role of several nutrients, LCPUFAs may also play a significant role.

LCPUFAs are fatty acids with a minimum chain length of 18 carbons containing at least 2 double bonds. LCPUFAs are classified into 1 of 2 families: n-3 and n-6 (**Figure 2**).

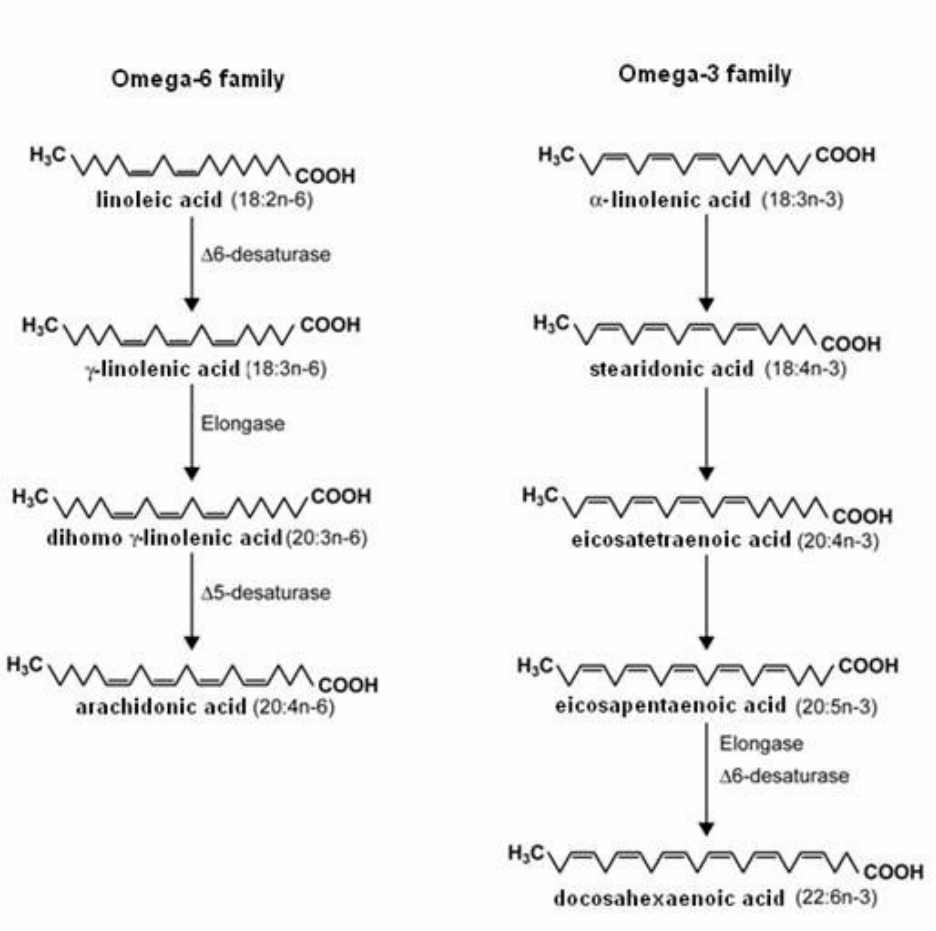

Figure 2. The metabolism of n-3 and n-6 fatty acids.

The n-3 and n-6 nomenclature refers to the location of the first unsaturated carbon from the methyl ('n') terminus of the fatty acid. The first double bond is located at carbon 3 for n-3 fatty acids and at carbon 6 for n-6 fatty acids. Alpha-linolenic acid (ALA, C18:3n-3) and linoleic acid (LA, C18:2n-6) are the parent compounds for the n-3 and n-6 series of LCPUFAs, respectively. Because humans lack the ability to insert a double bond prior to carbon 9 in the fatty acid chain, ALA and LA cannot be synthesized endogenously and are therefore dietary essential fatty acids.

The role of LCPUFA in obesity development is supported by several facts. First, both in rodents and humans, LCPUFA (especially arachidonic acid [AA, C20:4n-6]) enhances the formation of adipocytes. When compared to control adipogenic conditions, a brief exposure of preadipocytes to LCPUFAs appears sufficient to trigger in vitro both hyperplasia and hypertrophy (34). Secondly, LCPUFA is known to influence gene expression of lipolytic and glycolytic enzymes (35) and proteins regulating energy metabolism and thermogenesis (for example *PPARγ* and *UCP2*), leading to changes in metabolism, growth, and cell differentiation. Third, a recent study showed that overweight and symptoms of metabolic syndrome can be programmed in the adult animal by modulating essential LCPUFA in the perinatal period (36). Fourth, human studies also provide evidence that obesity per se is associated with a disturbed n-6/n-3 LCPUFA balance in the maternal diet during pregnancy, lactation, and early childhood (37). Finally, different LCPUFA composition of obese patients compared to normal weight controls were reported in studies (38;39). Previously we found significantly higher percentage contributions of the n-6 long-chain polyunsaturated fatty acids, gamma-linolenic acid (GLA, C18:3n-6), dihomo-gamma-linolenic acid (DHGLA, C20:3n-6), and AA, to the fatty acid composition of plasma lipid classes in obese children than in nonobese controls (38).

Beside the potential role in obesity development, LCPUFAs have several other important functions as well. The availability of LCPUFAs and especially the supply of docosahexaenoic acid (DHA, C22:6n-3) appears to modify the development of visual and cognitive functions during early life (40-44). The LCPUFA content of the diet influences membrane structure and function, and also effects the production of second messengers, such as eicosanoids that influence many cell-associated

functions (45). During gestation and infancy, the supply of preformed LCPUFA can influence neural functions (45).

Essential fatty acids can not be synthesised by humans and must be supplied through placental transport to the fetus and with human milk or milk substitute formulae to the infant. Human milk supplies not only the essential fatty acids linoleic and alpha-linolenic acids, but also their LCPUFA metabolites (40;46). It provides considerable amounts of LCPUFAs, and is considered to provide optimal form of nutrition for young infants (46-49). During gestation, major amounts of DHA and other LCPUFAs are transferred to the developing fetus for optimal growth and development by an active and selective transport (50;51). Tissue values of AA and DHA increases steadily with increasing gestational age (52); LCPUFAs accumulate in the fetus, especially during the last trimester of pregnancy (40;47;53).

Lipid content and fatty acid composition of human milk is reportedly influenced by a variety of variables such as genetic background of the lactating woman, maternal nutritional status, maternal dietary intakes immediately before and during gestation, the number of previously breastfed infants and the stage of lactation (40;47;52). It has also been debated whether fatty acid composition of human milk differs after preterm as compared to full-term delivery (54-58). In our previous study percentage contributions of arachidonic and docosahexaenoic acids as well as those of the intermediary metabolites of essential fatty acid metabolism were all significantly higher in early human milk samples of mothers giving birth to very low birth weight preterm as compared with full-term infants (58).

Preterm infants represent a small (about 5% to 10%, depending on several genetic and socioeconomical factors), but highly vulnerable subgroup of infants. Optimal nutrition of preterm infants, including the prevention of obesity, is of obvious importance from the point of view of the health of the community. However, solid information of the composition of human milk of mothers of preterm infants is a prerequiste of the optimisation of the composition of formulae to be used in the nutrition of premature babies. In this study we systematically reviewed the published information on fatty acid composition of human milk in mothers of preterm as compared to those of full-term infants.

Metabolic consequences of obesity

As the prevalence of childhood obesity increases, its health implications are becoming more evident. Obesity is associated with significant health problems in the pediatric age group and is an important early risk factor for much of adult morbidity and mortality. Many of the metabolic and cardiovascular complications of obesity are already present during childhood. In fact, there are few organ systems that obesity does not affect (59).

According to the latest estimates of Lobstein et al. over 27 000 obese children in the European Union have type 2 diabetes (most of it unrecognised and hence untreated), and over 400 000 have impared glucose tolerance (60).

Over a million obese children are likely to have a range of cardiovascular disease indicators, with an estimated 1.1 million suffering hypertension (61). Nawrot and colleagues (62) report that systolic blood pressure increases by 0.8 mmHg per 1 kg/m^2 increase in BMI in 15 to 19 year old males and by and 1.2 mmHg per 1 kg/m^2 increase in BMI in 15 to 19 year old females. In the Bogalusa Heart Study youth with BMI values >75th percentile were 8.5 times more likely to become hypertensive as adults than were their lean counterparts (63).

Obese children and adolescents have consistently been observed to have a more unfavourable lipid and lipoprotein profile than children and adolescents with a normal body weight (61). 52% of obese children 8 to 12 years old were found to have elevated total cholesterol concentrations compared with a prevalence of 16% in non-obese children (64). There is increasing evidence that atherosclerosis begins in childhood (65;66); however, the rate of progression is directly related to plasma lipoprotein concentrations (67). In the Bogalusa Heart Study, autopsy studies of children showed a clear relationship between the number and severity of risk factors, principally obesity, with atherosclerosis in both the aorta and coronary arteries (68).

Overweight and obesity in youth plays a central role in the metabolic syndrome (MS) - defined as a clustering of insulin resistance/hyperinsulinemia, dyslipidemia and hypertension. A recent publication estimated the prevalence of MS in Europe on the basis of data obtained from a literature search and extrapolated them to the 25 member states (60). Most of the studies used for the calculation of weighted average prevalence were performed on USA children (n=6) and only one was European (69).

Concerning the definition of MS in children and adolescents no consensus has been reached yet, although the clustering of metabolic risk factors was described in children long before the term "metabolic syndrome" emerged (70-72). The adult definitions of MS (World Health Organization [WHO] (73), European Group for the Study of Insulin Resistance [EGIR] (74), National Cholesterol Education Program Adult Treatment Panel III [NCEP] (75), International Diabetes Federation [IDF] (76), American Heart Association (77)) varying in terms of the criteria and cut off points and do not apply to children because of the age- and sex- dependent changes in several of the components of the MS. In the majority of the studies on MS performed in children and adolescents, the authors adapted the criteria from the adult definitions with widely varying risk factor cut-offs. The main differences concerning the definitions are the techniques used to estimate adiposity BMI or waist circumference) and the variables for evaluating glucose metabolism (fasting glucose, fasting insulin, hyperinsulinemia/insulin resistance, IGT or known type 2 diabetes). Recently, the IDF proposed its own pediatric definition of MS (78), which follows the adult IDF definition, and the cut-off points are identical to those used for adults, despite the more favorable distribution of lipid profiles and blood pressure normally found in childhood (79). The aim of the present study was to review the data concerning the prevalence of metabolic syndrome in European children and adolescents, and to determine and compare the prevalence of MS among overweight and obese children and adolescents in five European countries using four MS definitions.

The presence of multiple metabolic disorders persists from childhood into adulthood 25%–60% of the time (70;80). The alarming increases in obesity in developing countries has led the World Health Organization to estimate that cardiovascular disease CVD will rise from number 5 to number 1, the leading killer in the entire world after another decade (2;81).

For all these reasons, CVD is and will remain the leading killer in the most developed countries. Thus, the long-term consequences of childhood obesity could cause our current generation of children to become the first in the history to have a decreased life expectancy than their parents (82;83).

Aims of the study

1. **Association among single nucleotide polymorphisms at CD36 locus and obesity in European adolescents**

a.) To explore the relationship between polymorphisms in the CD36 gene and obesity in a case-control study of adolescents.

b.) To validate our findings on anthropometric markers of obesity in an independent cross sectional study of European adolescents.

2. **Association of n-6 long-chain polyunsaturated fatty acids to -866G/A genotypes of the human uncoupling protein 2 gene in obese children**

a.) To examine the effect of −866 G/A polymorphism of UCP2 on the fatty acid composition of plasma lipids in obese children.

b.) To investigate the association of n-6 long-chain polyunsaturated fatty acids (LCPUFAs) with glucose-induced insulin secretion (GIIS) in obese children stratified according to the −866 G/A polymorphism of UCP2.

3. **Systematic review of fatty acid composition of human milk from mothers of preterm compared to full-term infants**

To systematically review the published information on fatty acid composition of human milk in mothers of preterm as compared to those of full-term infants.

4. **Prevalence of metabolic syndrome in European obese children**

a.) To review the data concerning the prevalence of metabolic syndrome in European children and adolescents.

b.) To determine and compare the prevalence of MS among overweight and obese children and adolescents in five European countries using four MS definitions.

1. Association among single nucleotide polymorphisms at CD36 locus and obesity in European adolescents European adolescents

Patients and methods

The Pécs Case-control study of adolescent obesity. The study population consist of 307 obese adolescents referred to the outpatient clinic of the Department of Pediatrics University of Pécs (Pécs, Hungary) for obesity and 339 healthy normal weight adolescents recruited via general schools of Pécs aged between 14-17 years. Obesity was defined as a BMI over the value given by Cole *et al.* (84), corresponding to 30 kg/m^2 at the age of 18 years. None of the subjects had chronic diseases, was taking drugs or was dieting. Blood samples for DNA extraction was collected in EDTA K3 tubes. Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures.

HELENA cross-sectional study (HELENA-CSS) (validation setting). Recruitment and phenotyping of the participating adolescents in the HELENA study ("Healthy Lifestyle in Europe by Nutrition in Adolescence", www.helenastudy.com) have been described previously (85). Briefly, a total of 3865 adolescents were recruited between 2006 and 2007. Data were collected in 10 centres from 9 European countries (86). Subjects were randomly selected according to a proportional cluster sampling methodology taking into account geographical repartition in each city, private/public school ratio, and number of classes by school. One third of the classes were randomly selected for blood collection, resulting in a total of 1155 blood samples for the subsequent clinical biochemistry assays and genetic analyses.

Anthropometry. Skinfold thicknessess were measured at 6 sites: bicipital, tricipital, sub-scapular, suprailiac, thigh and calf using a Holtain Caliper. Reliability of skinfolds thickness measurements were adequate for epidemiological surveys (87). The percentage of body fat (BF%) was estimated from skinfold measurements according to the equations of Parizkova et al (88). The BMI was calculated.

Blood analysis. Blood for DNA extraction was collected in EDTA K3 tubes, stored at the Analytical Laboratory at the University of Bonn and then sent to the Genomic Analysis Laboratory at the Institut Pasteur de Lille (IPL) in France (89). DNA was extracted from white blood cells with the Puregene kit (QIAGEN, Courtaboeuf, France) and stored at -20°C.

In both the ***Pécs Case-control study*** and the ***HELENA-CSS*** data were collected on a detailed case report form according to standardised procedures. In each centre, trained nurses and physicians carried out complete physical examinations including weight, height and blood pressure.
For both studies, the protocol was approved by the ethics committee of each recruiting centre (90). A written, informed consent was obtained from each adolescent and both of his/her parents or legal representatives. Participation in the studies was voluntary.

Gene selection and genotyping. With the criterion used in our SNP selection procedure (a minor allele frequency (MAF) above 0.1 and tag SNPs with an r^2 value above 0.8), the HapMap database (2007 release) describes 5 haplotype blocks and 2 independent SNPs that span the whole gene. In the present study, we selected 1 SNP from each of the five haplotype blocks (block 1: rs1527479, block 2: rs3211816, block 3: rs3211867, block 4: rs3211883 and block 5: rs3211931) and the two independent SNPs (rs3211908 and rs1527483). We also selected 3 other SNPs (rs1984112, rs1761667 and rs1049673) from the literature (24) in order to cover the whole range of gene variability (**Figure 3**). Altogether, subjects were genotyped on an Illumina system, 1 SNP (rs1761667) using the VeraCode technology and the 9 other SNPs using GoldenGate technology.

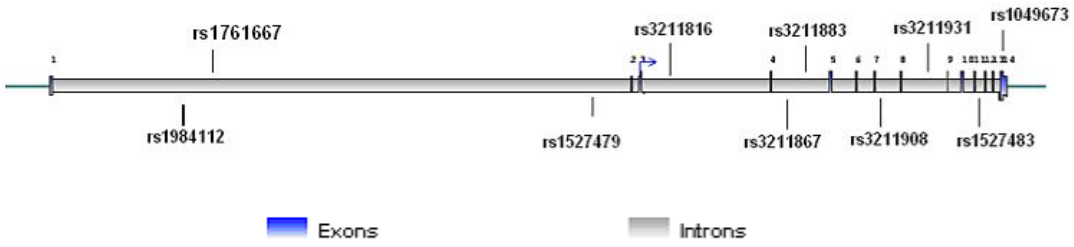

Figure 3. The CD36 gene and the selected single nucleotide polymorphisms.

Statistical methods. Statistical analyses were performed with SAS software (SAS Institute Inc., Cary, NC, USA). Departure from the Hardy–Weinberg equilibrium within the study groups was tested using a χ^2 test. Inter-locus linkage disequilibrium was tested using a log-likelihood-ratio test (91) and expressed in terms of a normalised difference D'=D/Dmax or D/Dmin (92). Allele frequencies were estimated by gene-counting. In the Pecs case-control study, multivariate logistic regression was used to calculate the odds ratio of obesity for different allele exposures, using different genetic models. Adjustment variables were age and gender. The General Linear Model (GLM) was used to compare mean values of anthropometric markers among genotypes in the cross sectional study. Adjustment variables were age, gender and centre. Dominant and recessive models were tested. For the case-control study the statistical significance threshold was set to $p \leq 0.007$ after Bonferroni correction and to $p \leq 0.05$ for the HELENA-CSS. Haplotype analyses were based on a maximum likelihood model (93) linked to the SEM algorithm (94) and performed using Thesias software developed by INSERM unit U525, Paris, France (http://ecgene.net/genecanvas).

Results

Table 1 shows the clinical characteristics of the subjects from the case-control and the cross-sectional studies. The mean age years (SD) of the obese and normal weight adolescents of the case-control study was 15.0 (1.1) and 14.6 (1.1) respectively, and 14.8 (1.4) years for the adolescents of the cross-sectional study. There were more girls among the normal weight controls and in the cross-sectional study than in the group of obese adolescents. As expected obese adolescents had significantly higher

BMI and BF% compared to normal weight adolescents of the case-control study (all $p<0.001$).

Table 1. Characteristics of the subjects

	Obesity case-control study		HELENA cross-sectional study
	Normal weight	Obese	
n	339	307	1155
Boys/girls	164/175	165/142	552/603
Age (years)	15.0 (1.1)	14.6 (1.1)	14.8 (1.4)
Weight (kg)	56.4 (8.9)	91.4 (18.6)	58.3 (13.0)
Height (cm)	168.4 (8.7)	166.8 (8.8)	165.0 (9.6)
BMI (kg/m^2)	20.1 (2.5)	32.7 (5.5)	21.3 (3.7)
BF (%)	25.1 (6.4)	39.4 (6.0)	26.5 (6.9)
Systolic BP (mmHg)	118.6 (13.5)	127.6 (11.0)	118.2 (13.8)
Diastolic BP (mmHg)	66.6 (8.6)	72.4 (8.4)	67.3 (9.8)

BMI: body mass index, BF: body fat, BP: blood pressure

DNA samples were genotyped for the 10 selected *CD36* SNPs (**Figure 3**). The genotyping success rate varied between 97.1 and 100%. In each study, all the observed SNP frequencies obeyed the Hardy–Weinberg equilibrium. The linkage disequilibrium pattern for the SNPs was assessed in the HELENA-CSS using both the D' and r^2 values (**Figure 4**). The 3 SNPs selected from literature data (rs1984112, rs1761667 and rs1049673) were in strong linkage disequilibrium with three haplotype blocks described by the HapMap database (rs1761667 with rs1527479 (block 1) [D' = 0.98, r^2 = 0.93], rs1984112 with rs3211816 (block 2) [D' = 0.97, r^2 = 0.89], and rs1049673 with rs3211931 (block 5) [D' = 1, r^2 = 0.98]). Thus, further analyses were performed with the 7 following SNPs: rs1527479, rs3211816, rs3211867, rs3211883, rs3211908, rs3211931 and rs1527483.

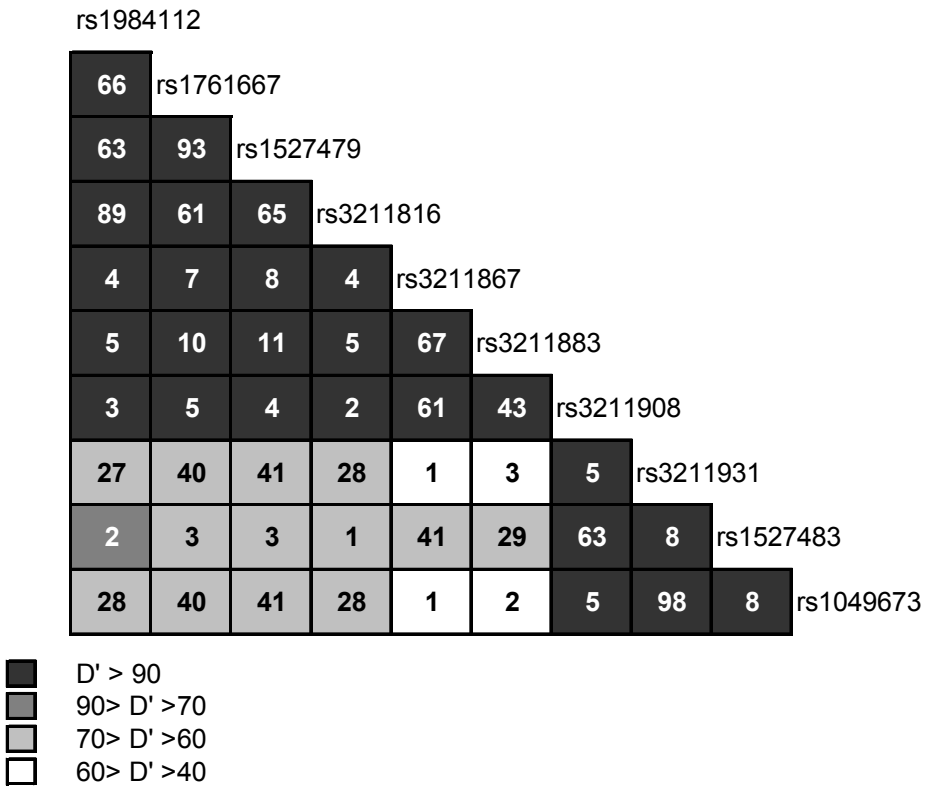

Figure 4. The linkage disequilibrium pattern (D' and r^2) across the investigated SNPs of the CD36 gene. Color is D', values are r^2.

Table 2 shows the genotype distribution of the 7 SNPs in obese and normal weight subjects, the age- and gender- adjusted odds ratio (OR) and the 95% confidence interval (95%CI) for obesity from the case-control study. In a dominant model, 4 SNPs were associated with a higher risk of obesity (rs3211867: OR [95%CI]= 1.96 [1.26-3.04], p=0.003; rs3211883: OR=1.73 [1.16-2.59], p=0.007; rs3211908: OR=2.42 [1.47-4.01], p=0.0005 and rs1527483: OR=1.95 [1.25-3.05], p=0.003). After Bonferroni correction (significance: $p \leq 0.007$), the rs3211867, rs3211883, rs3211908 and rs1527483 SNPs were still significantly associated with a greater risk of obesity.

Table 2. Genotype distribution of the investigated SNPs in obese and control subjects of the Pécs case-control study

	Normal weight			Obese			*p**	OR	[95% CI]	*p***
	11	12	22	11	12	22				
rs1527479	98 (0.29)	174 (0.52)	66 (0.19)	64 (0.21)	155 (0.51)	86 (0.28)	*0,01*	1,42	[1.06-2.10]	*0,09*
rs3211816	134 (0.40)	162 (0.48)	40 (0.12)	111 (0.36)	152 (0.50)	43 (0.14)	*0,55*	1,13	[0.82-1.57]	*0,44*
rs3211867	299 (0.882)	40 (0.118)	0	244 (0.797)	60 (0.196)	2 (0.007)	*0,004*	**1,96**	[1.26-3.04]	*0,003*
rs3211883	284 (0.840)	53 (0.157)	1 (0.003)	233 (0.764)	68 (0.223)	4 (0.013)	*0,03*	**1,73**	[1.16-2.59]	*0,007*
rs3211908	311 (0.917)	28 (0.083)	0	255 (0.831)	49 (0.160)	3 (0.009)	*0,0009*	**2,42**	[1.47-4.01]	*0,0005*
rs3211931	108 (0.32)	163 (0.48)	66 (0.20)	84 (0.27)	149 (0.49)	74 (0.24)	*0,26*	1,26	[0.89-1.78]	*0,19*
rs1527483	299 (0.885)	39 (0.115)	0	248 (0.808)	55 (0.179)	4 (0.013)	*0,004*	**1,95**	[1.25-3.05]	*0,003*

Data are n (freq). Frequent allele :1 ; Minor allele: 2. p* value is for chisquare test. ORs and p** values are for dominant model. p values are adjusted for age and gender

Table 3 shows the BF% and BMI as a function of the CD36 genotypes in the cross-sectional study. Multivariate analyses (adjusted for age, gender and centre) revealed that the mean BMI and BF% were significantly higher in carriers of at least one minor allele with rs3211867 (BMI: p=0.03, BF%: p=0.02), rs3211883 (BMI: p=0.03, BF%: p=0.05), rs3211908 (BMI: p=0.04, BF%: p=0.02) and rs1527483 (BMI: p=0.05, BF%: p=0.04) compared with individuals who were homozygous for the frequent allele. These associations were not modified by further adjustment for pubertal status (data not shown).

Haplotype analyses using the 7 SNPs (order: rs1527479, rs3211816, rs3211867, rs3211883, rs3211908, rs3211931 and rs1527483) were performed to assess the relationship with obesity in the case-control study and the association with BMI and BF% in the HELENA-CSS. Nine haplotypes had an estimated frequency of over 1% (**Table 4/a, 4/b**). Compared with the most common haplotype (GGCTGGG; estimated frequency: 0.44), 1 haplotype: AGAAAAA (estimated frequency: 0.05, minor alleles underlined) was significantly associated with a higher risk of obesity (OR: 2.28 for obesity; p=0.0008). This haplotype was also associated with a higher BF% (p=0.03) and BMI (p=0.04) in the cross-sectional study.

Table 3. Genotype distribution and mean values of body mass index and body fat percentage according to the SNPs in the HELENA-CSS

	11	12	22	*p dominant*
rs1527479	**GG (n=326)**	**GA (n=557)**	**AA (n=261)**	
BMI	21.0 (3.5)	21.3 (3.9)	21.6 (3.7)	*0,30*
BF%	26.2 (6.8)	26.4 (7.1)	26.9 (6.6)	*0,28*
rs3211816	**GG (n=449)**	**GA (n=503)**	**AA (n=161)**	
BMI	21.2 (3.6)	21.4 (3.8)	21.4 (3.7)	*0,72*
BF%	26.5 (6.6)	26.3 (7.2)	26.8 (6.6)	*0,38*
rs3211867	**CC (n=999)**	**CA (n=139) + AA (n=6)**		
BMI	21.2 (3.7)	21.8 (3.8)		*0,03*
BF%	26.4 (6.9)	27.3 (6.8)		*0,02*
rs3211883	**TT (n=945)**	**TA (n=189) + AA (n=10)**		
BMI	21.2 (3.7)	21.7 (3.6)		*0,03*
BF%	26.4 (6.9)	26.8 (6.8)		*0,05*
rs3211908	**GG (n=1041)**	**GA (n=101) + AA (n=2)**		
BMI	21.3 (3.7)	21.7 (3.8)		*0,04*
BF%	26.4 (6.9)	27.4 (6.8)		*0,02*
rs3211931	**GG (n=339)**	**GA (n=575)**	**AA (n=230)**	
BMI	21.0 (3.4)	21.4 (3.7)	21.6 (4.2)	*0,19*
BF%	26.2 (6.7)	26.5 (7.2)	26.9 (6.9)	*0,58*
rs1527483	**GG (n=1002)**	**GA (n=138) + AA (n=4)**		
BMI	21.3 (3.7)	21.7 (4.0)		*0,05*
BF%	26.4 (6.9)	27.1 (6.9)		*0,04*

Data are means (SD). Frequent allele :1 ; Minor allele: 2. BMI: body mass index, BF: body fat. P values are adjusted on age, center and gender.

Table 4/a. Haplotype frequencies, OR and 95% CI in the case-control study

Haplotype	Frequency	OR	95% CI	p
GGCTGGG	0,44	reference		
AACTGAG	0,29	0,98	0.77-1.26	0,52
AACTGGG	0,07	1,58	1.01-2.48	0,13
GGCTGAG	0,05	0,93	0.55-1.58	0,82
AGAAAAA	0,05	2,28	1.33-3.92	0,0008
AGCAGAG	0,02	1,18	0.50-2.77	0,37
AGAAGGG	0,01	1,66	0.56-4.87	0,34
GGCTGAA	0,01	0,69	0.22-2.15	0,69
AGCTGAG	0,01	0,75	0.23-2.41	0,58

Table 4/b. Haplotype frequencies, delta values of mean BF% and delta values of mean BMI in the HELENA-CSS

Haplotype	Frequency	delta BF %	p	delta BMI	p
GGCTGGG	0,45	reference		reference	
AACTGAG	0,28	0,25	0,44	0,08	0,62
AACTGGG	0,07	0,22	0,70	0,12	0,69
GGCTGAG	0,06	0,38	0,54	0,22	0,49
AGAAAAA	0,04	1,5	0,03	0,76	0,04
AGCAGAG	0,02	0,08	0,92	0,02	0,93
AGAAGGG	0,02	-0,14	0,92	0,09	0,87
GGCTGAA	0,01	0,11	0,96	-0,15	0,82
AGCTGAG	0,01	1,42	0,33	0,5	0,51

Only haplotypes with a frequency above 1% are presented. Order of the SNPs used for the analysis: rs1527479, rs3211816, rs3211867, rs3211883, rs3211908, rs3211931, rs1527483. Minor alleles for each SNP are underlined. Delta BF refers to the mean percentage of BF difference between the common haplotype (GGCTGGG) and other haplotypes.

Discussion

The results of the present study showed that rs3211908 was consistently associated to a higher risk of obesity in the case-control study and excess adiposity in the cross-sectional study. Further analyses identified a haplotype carrying the minor allele of rs3211908 that was linked with obesity and a higher body fat percentage in adolescents. These findings suggest that *CD36* gene variability is associated to the risk of fat accumulation in adolescents.

The rs3211867, rs3211883 and rs1527483 SNPs were also related to the risk of obesity and the BF% although the associations were weaker. The later SNPs were in partial linkage disequilibrium with rs3211908 ($0.43< r^2 <0.64$), suggesting that these associations reflect a single signal. This hypothesis was further supported by the haplotype analysis which showed that only one haplotype carrying the rs3211908 variant was associated with obesity and BF%. All other haplotypes were not consistently linked to excess body fat suggesting that the culprit variant is the minor allele of rs3211908. Finally, the remaining SNPs (rs1527479, rs3211816 and rs3211931) were not linked to an excess fat deposition phenotype.

Rs3211908 is located 100 bp down-stream the donor site of exon 7, that codes the amino acids corresponding to the interaction region with the LCPUFAs (95). The Genomatix MatInspector software (www.genomatix.de), a functional analysis tool, indicated that the T allele of rs3211908 abolishes a predicted glucocorticoid receptor binding site (responsive element) which may affect *CD36* gene expression. Alternatively, rs3211908 SNP could be in linkage disequilibrium with a yet undetected functional SNP located elsewhere in the *CD36* gene.

Several hypotheses might explain the link between *CD36* genetic variability and body fat depot. Firstly, CD36 is a cell membrane transporter of LCPUFA in a wide variety of metabolically active tissues, including heart, muscle and adipocytes (96). A defect in CD36 function could decrease the rate of fatty acids oxidation in muscles and thus increase the availability of fatty acids for storage in adipocytes (97;98). Secondly, cell co-culture experiments have shown that activated macrophages secrete various factors that inhibit the formation of mature adipocytes (99). A dysregulation of CD36 in macrophages embedded in the adipose tissue could alter the signalling pathway that retrocontrol adipocyte expansion (99). Thirdly, trough the provision of LCPUFA and oxidised low density lipoprotein cholesterol,

CD36 plays a key role in the activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) (100;101), a nuclear receptor responsible of adipocyte differentiation and adipogenesis (102). Thus an alteration of CD36 may impact PPAR-γ mediated adipocyte differentiation (103).

This study has several strengths and limitations. The analyses were carried out in 2 independent samples of European adolescents reducing the likelihood of spurious findings. The study was performed in adolescents, thus providing an advantage to discover unknown associations. One concerns is the limited number of SNPs used to tag *CD36* gene due to the selection of tag SNPs with a minor allele frequency >0.10, which does not allow the identification of rare alleles with possible stronger influences.

2. Association of n-6 long-chain polyunsaturated fatty acids to -866G/A genotypes of the human uncoupling protein 2 gene in obese children

Patients and methods

The investigations were carried out in 80 obese children (age: 13.0 [2.7] (8.1 – 17.0) years, body mass index: 41.7 [4.4] (27.3 – 49.7) kg/m^2, body fat: 39.1 [4.2] (31.6 – 56.1) %, mean [SD] (min. – max.)) referred to the Outpatient Clinic for Obesity of the Department of Paediatrics, University of Pécs, (Pécs, Hungary) because of their overweight. None of the subjects had any chronic disease, they did not receive any drug treatment, and none of them were dieting. We considered children as obese if their body weight exceeded the normal weight for height by more than 20%, and if body fat content was higher than 25% in boys and 30% in girls.

The study was carried out in accordance with the Declaration of Helsinki II and with approval of the ethics committee of the University of Pécs. Informed parental consent was obtained for each child before enrolment into the study. All participants remained anonymous throughout the survey.

Anthropometric measurements were carried out by the same investigator. Body height and weight were measured in the survey unit with approved medical care instruments, and body mass index was computed as weight (kilograms) divided by squared height (meters squared). The waist and hip circumferences were measured with the subject in the supine position. Body fat was estimated according to Parizkova and Roth (104) from five skinfold thicknesses measured on the left side of the body three consecutive times with the help of a Holtain caliper. Lean body mass was calculated by subtracting body fat from actual body weight. Relative body weight was calculated as the ratio between the actual body weight and the ideal body weight for age, gender and height (105).

Blood samples were taken after an overnight fast. Plasma glucose concentrations were determined by the glucose oxidase method. Plasma insulin concentrations were measured with commercially available radioimmunoassay kits (Isotope Institute of the Hungarian Academy of Sciences, Budapest, Hungary). With this method, the cutoff value for insulin was 18.7 μU/ml (95th percentile value of 100

healthy children). The OGTT consisted of oral administration of glucose (1.75 g/kg body weight, maximum 75 g) followed by plasma glucose and insulin determinations at 30, 60, 90, 120, and 180 min. The test results were evaluated according to the recommendation of the American Diabetes Association (106). Triacylglycerol and cholesterol were determined with an enzymatic kit (Boehringer Mannheim, Mannheim, Germany). High-density lipoprotein cholesterol was measured by the precipitation method of Steele et al (107).

Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures. The -866 G/A polymorphism in the promoter region of the human UCP2 gene was investigated with polymerase chain reaction (PCR) and subsequent diagnostic restriction fragment length polymorphism analyses (RFLP) with the restriction enzyme Bsh1236I which either cut (-866 G-allele) or did not cut (-866 A-allele) the 201 basepair (bp) PCR amplicon. The primers used were: forward primer 5'-TGACTGAACGTCTTTGGGACT-3' and reverse-primer 5'-GATGAGAAAAGCCGTCAGGA-3'. PCR was performed by initial denaturation at 94°C for 3 min, 36 cycles at 94°C for 30 s, 57°C for 30s, 72°C for 30s, and a final extension at 72°C for 4 min. The samples were then run on a 3.0% agarose gel, stained with ethidium bromide and analysed under ultraviolet light.

Venous blood samples for lipid analysis were taken from the antecubital vein into tubes containing 2 mg/ml EDTA as anticoagulant. The plasma was removed within 30 min and stored at –80 °C until analysis. All samples were thawed only once.

Fatty acids were analysed by high-resolution capillary gas-liquid chromatography using a Finnigan 9001 gas chromatograph (Finnigan/Tremetrics Inc., Austin, TX, USA) with split injection (ratio: 1 to 25), automatic sampler (A200SE, CTC Analytic, Switzerland) and flame-ionisation detector with a DB-23 cyanopropyl column of 40m length (J &W Scientific, Folsom, CA, USA). The analytical procedure is described in detail elsewhere (108). Peak identification was verified by comparison with authentic standards. Fatty acid results were expressed as percentages (weight by weight) of fatty acids detected with a chain length between 12 and 24 carbon atoms.

Statistical analysis Results were evaluated with SPSS for Windows, Release 11.5 (SPSS Inc., Chicago, IL, USA). All data except fatty acids are presented as mean

(SD) and were evaluated by analysis of variance followed by the least significant difference comparison test to compare mean values of subgroups. The fatty acid data are presented as median and range from the first to the third quartile values, because skewed distributions were found in several parameters, especially in fatty acids present at low concentrations. Fatty acids were analysed by the Kruskal-Wallis nonparametric analysis of variance followed by Mann-Whitney's twosided rank test to compare median values of subgroups. Results were regarded as statistically significant at $p<0.05$. Nonparametric Spearman's correlation analysis was performed in order to determine the relationship between plasma fatty acids and plasma insulin concentrations during OGTT. Because more than one correlation was calculated, we performed Bonferroni corrections to have a more conservative estimate of the p value.

Results

The clinical features of the study subjects are shown in **Table 5**. There was no significant difference among the groups in anthropometric indices of obesity, in the concentrations of plasma lipid classes, or in fasting plasma glucose and insulin concentrations (**Table 5**).

Table 5. Main clinical characteristics of 80 obese children stratified according to the -866 G/A polymorphism in the promoter region of the uncoupling protein 2 (UCP2)

UCP2 genotype:	-866G/G (n = 34)	-866G/A (n = 34)	-866A/A (n = 12)
Sex (male:female)	22:12	16:18	5:07
Age (yr)	12.8 (2.7)	12.6 (2.9)	13.8 (2.7)
Weight (kg)	79.2 (21.7)	75.6 (23.0)	73.8 (21.4)
Height (cm)	159 (16)	157 (12)	154 (9)
BMI (kg/m^2)	30.7 (4.2)	29.8 (4.9)	30.6 (6.2)
LBM (kg)	48.0 (12.4)	45.7 (13.1)	43.7 (8.6)
RBW (%)	167.7 (20.0)	164.8 (21.7)	173.2 (26.3)
BF (%)	38.9 (4.5)	39.1 (3.5)	39.3 (5.7)
Waist/hip ratio	0.85 (0.05)	0.85 (0.09)	0.86 (0.06)
HDL-cholesterol (mmol/L)	1.16 (0.35)	1.33 (0.46)	1.28 (0.50)
Total cholesterol (mmol/L)	4.19 (0.74)	4.23 (0.61)	4.42 (0.93)
Triglyceride (mmol/L)	1.39 (0.56)	1.32 (0.42)	1.78 (1.29)
Fasting plasma glucose (mmol/L)	4.3 (0.77)	4.1 (1.78)	3.96 (0.71)
Fasting plasma insulin (µU/mL)	26.86 (13.61)	24.77 (10.84)	23.08 (12.28)

Data are mean (SD). BMI, body mass index; LBM, lean body mass; RBW, relative body weight; BF, body fat content; HDL-cholesterol, high-density lipoprotein cholesterol

In plasma phospholipids (**Table 6**) and sterol esters (**Table 7**), there were no significant differences in the values of 33 individual fatty acids including 8 saturated, 10 cis-monounsaturated, 3 trans-isomeric, 8 n-6 polyunsaturated and 4 n-3 polyunsaturated fatty acids. In contrast, values of eicosadienoic acid (C20:2n-6) were significantly lower in plasma phospholipids in children with the -866A/A genotype

than in the other two groups. Values of dihomo-γ-linolenic acid (DHGLA, C20:3n-6) were significantly lower in children with the -866A/A than in those with the -866G/A genotype in plasma phospholipids (**Table 6**) and were significantly lower in children with the -866 A/A genotype than in the other two groups in plasma sterol esters (**Table 7**).

In the phospholipid fraction, C20:3n-6 values in children with the -866G/G genotype showed significant positive correlations with plasma insulin concentrations at 60 minutes of the OGTT (**Table 8**). No such correlation was seen in children with -866A/A and -866G/A genotype. In plasma sterol esters, C20:3n-6 values showed significant positive correlation with plasma insulin concentrations at 60 minutes of the OGTT in children with the -866G/G and -866G/A, but not in those with the -866A/A genotype (**Table 8**). Significant inverse correlations were seen at 0, 30 and 60 minutes of the OGTT between C20:4n-6 and insulin values in children with the -866A/A genotype (**Table 8**). No such negative correlation was seen in the other two groups.

Figure 5 shows the correlation between insulin area under the curve during OGTT and the values of C20:4n-6 in sterol ester lipids. Significant inverse correlation was seen in children with the -866A/A genotype between C20:4n-6 and the insulin area under the curve, whereas no such correlation was observed in the other groups.

Table 6. Fatty acid composition of plasma **phospholipids** in 80 obese children stratified according to genotype in the promoter of the uncoupling protein 2 (UCP2)

UCP2 genotype:	-866G/G (n = 34)	-866G/A (n = 34)	-866A/A (n = 12)
Saturated fatty acids			
$C_{14:0}$	0.37 (0.14)	0.37 (0.12)	0.32 (0.08)
$C_{16:0}$	28.22 (1.86)	27.88 (2.29)	27.20 (2.38)
$C_{18:0}$	16.13 (1.77)	17.24 (1.76)	16.72 (2.14)
Total	48.24 (3.34)	49.07 (1.69)	48.18 (2.17)
Trans fatty acids			
$C_{16:1n-7}$	0.12 (0.03)	0.13 (0.03)	0.14 (0.30)
$C_{18:1n-9/7}$	0.19 (0.11)	0.19 (0.13)	0.17 (0.17)
Total	0.40 (0.12)	0.38 (0.16)	0.38 (0.24)
Monounsaturated fatty acids			
$C_{16:1n-7}$	0.49 (0.19)	0.47 (0.15)	0.44 (0.11)
$C_{18:1n-7}$	1.17 (0.26)	1.22 (0.24)	1.21 (0.11)
$C_{18:1n-9}$	7.23 (1.65)	7.08 (1.07)	6.76 (1.55)
Total	12.19 (2.21)	12.51 (1.62)	11.75 (1.71)
N-6 Polyunsaturated fatty acids			
$C_{18:2n-6}$	18.50 (3.47)	18.26 (2.62)	19.10 (2.89)
$C_{18:3n-6}$	0.07 (0.05)	0.06 (0.04)	0.07 (0.04)
$C_{20:2n-6}$	0.51 (0.17)[a]	0.50 (0.10)[b]	0.41 (0.11)[ab]
$C_{20:3n-6}$	3.53 (0.84)	3.56 (1.01)[a]	3.01 (0.42)[a]
$C_{20:4n-6}$	11.34 (2.54)	10.79 (2.74)	11.40 (1.49)
$C_{22:4n-6}$	0.56 (0.15)	0.54 (0.09)	0.55 (0.17)
$C_{22:5n-6}$	0.45 (0.15)	0.46 (0.18)	0.49 (0.19)
Σ n-6 LCPUFA[c]	16.44 (2.60)	16.04 (2.88)	16.50 (2.77)
Σ n-6 PUFA	34.98 (4.17)	34.55 (2.46)	35.53 (2.30)
N-3 Polyunsaturated fatty acids			
$C_{18:3n3}$	0.07 (0.07)	0.06 (0.03)	0.07 (0.07)
$C_{20:3n3}$	0.03 (0.02)	0.03 (0.01)	0.02 (0.02)
$C_{20:5n-3}$	0.24 (0.27)	0.23 (0.11)	0.24 (0.08)
$C_{22:5n-3}$	0.42 (0.27)	0.40 (0.10)	0.39 (0.14)
$C_{22:6n-3}$	2.56 (0.77)	2.53 (0.93)	2.67 (0.80)
Σ n-3 LCPUFA[d]	3.37 (1.02)	2.97 (0.95)	3.42 (1.20)
Σ n-3 PUFA	3.46 (1.03)	3.03 (1.00)	3.49 (1.24)

Data are percentage of weight per weight expressed as median (interqartile ranges); [a, b] $p<0.05$ between groups. [c]Total N-6 long-chain polyunsaturated fatty acids (LCPUFA) denotes ($C_{20:2n-6}$ + $C_{20:4n-6}$ + $C_{22:4n-6}$ + $C_{22:5n-6}$). [d]Total N-3 LCPUFA denotes ($C_{20:5n-3}$ + $C_{22:5n-3}$ + $C_{22:6n-3}$).

Table 7. Fatty acid composition of plasma **sterol esters** in 80 obese children stratified according to genotype in the promoter of the uncoupling protein 2 (UCP2)

UCP2 genotype:	-866G/G (n = 34)	-866G/A (n = 34)	-866A/A (n = 12)
Saturated fatty acids			
$C_{14:0}$	0.67 (0.28)	0.71 (0.25)	0.80 (0.44)
$C_{16:0}$	10.85 (1.60)	11.00 (1.19)	11.32 (1.46)
$C_{18:0}$	1.19 (0.30)	1.28 (0.43)	1.37 (0.22)
Total	13.33 (1.63)	13.55 (1.79)	14.42 (1.88)
Trans fatty acids			
$C_{16:1n-7}$	0.53 (0.10)	0.54 (0.19)	0.59 (0.38)
$C_{18:1n-9/7}$	0.05 (0.06)	0.08 (0.09)	0.06 (0.03)
Total	0.66 (0.22)	0.66 (0.24)	0.71 (0.44)
Monounsaturated fatty acids			
$C_{16:1n-7}$	2.90 (1.53)	2.98 (1.12)	3.00 (1.57)
$C_{18:1n-7}$	1.08 (0.31)	1.12 (0.20)	1.07 (0.13)
$C_{18:1n-9}$	16.58 (2.63)	15.69 (4.03)	13.99 (1.88)
Total	20.68 (3.84)	20.31 (3.84)	18.40 (3.39)
N-6 Polyunsaturated fatty acids			
$C_{18:2n-6}$	53.75 (5.93)	52.83 (6.38)	53.85 (4.73)
$C_{18:3n-6}$	0.87 (0.51)	0.81 (0.38)	0.86 (0.44)
$C_{20:2n-6}$	0.12 (0.05)	0.14 (0.05)	0.11 (0.02)
$C_{20:3n-6}$	0.94 (0.25) [a]	0.92 (0.23) [b]	0.73 (0.22) [ab]
$C_{20:4n-6}$	8.28 (3.12)	8.14 (2.90)	7.78 (1.38)
$C_{22:4n-6}$	0.06 (0.07)	0.04 (0.04)	0.05 (0.12)
$C_{22:5n-6}$	0.05 (0.05)	0.05 (0.03)	0.04 (0.04)
Σ n-6 LCPUFA[c]	9.62 (2.88)	9.34 (2.99)	8.87 (1.83)
Σ n-6 PUFA	63.90 (4.49)	64.18 (6.30)	64.90 (3.72)
N-3 Polyunsaturated fatty acids			
$C_{18:3n3}$	0.28 (0.11)	0.25 (0.10)	0.27 (0.17)
$C_{20:3n3}$	0.90 (0.02)	0.92 (0.23)	0.73 (0.22)
$C_{20:5n-3}$	0.23 (0.15)	0.21 (0.10)	0.24 (0.16)
$C_{22:5n-3}$	0.01 (0.01)	0.01 (0.02)	0.01 (0.01)
$C_{22:6n-3}$	0.46 (0.13)	0.45 (0.26)	0.44 (0.11)
Σ n-3 LCPUFA[d]	0.72 (0.23)	0.70 (0.30)	0.66 (0.28)
Σ n-3 PUFA	1.06 (0.39)	0.96 (0.32)	0.99 (0.36)

Data are percentage of weight per weight expressed as median (interqartile ranges); [a, b] $p<0.05$ between groups. [c]Total N-6 long-chain polyunsaturated fatty acids (LCPUFA) denotes ($C_{20:2n-6}$ + $C_{20:4n-6}$ + $C_{22:4n-6}$ + $C_{22:5n-6}$). [d]Total N-3 LCPUFA denotes ($C_{20:5n-3}$ + $C_{22:5n-3}$ + $C_{22:6n-3}$).

Table 8. Spearman rank correlation coefficients between arachidonic (C20:4n-6) and dihomo-γ-linolenic (C20:3n-6) acid values in plasma phospholipids and sterol esters, on the one hand, and plasma insulin concentrations measured during standard oral glucose tolerance test, on the other hand, in 80 obese childrenstratified according to the −866 G/A polymorphism in the promoter of the uncoupling protein 2 (UCP2)

	C20:3n-6			C20:4n-6		
UCP2 genotype:	-866G/G	-866A/G	-866A/A	-866G/G	-866A/G	-866A/A
Phospholopids						
Insulin 0'	0.19	0.39	0.52	0.17	0.06	-0.12
Insulin 30'	0.43	0.07	0.04	-0.06	0.28	0.07
Insulin 60'	0.53*	0.47	0.32	0.17	0.40	-0.11
Insulin 90'	0.39	0.40	-0.1	-0.08	0.28	0.18
Insulin 120'	0.44	0.23	-0.12	0.09	0.15	0.1
Insulin 180'	0.15	0.1	-0.07	0.16	0.01	-0.07
Sterol esters						
Insulin 0'	-0.06	0.39	0.2	0.01	-0.06	-0.80*
Insulin 30'	0.31	0.03	0.32	-0.27	0.37	-0.75*
Insulin 60'	0.51*	0.48*	0.42	-0.08	0.43	-0.75*
Insulin 90'	0.22	0.26	0.28	-0.33	0.17	-0.68
Insulin 120'	0.34	0.29	0.15	-0.07	0.19	-0.44
Insulin 180'	0.09	0.12	-0.35	-0.03	0.07	-0.59

*$p<0.008$; value calculated by using Bonferroni correction.

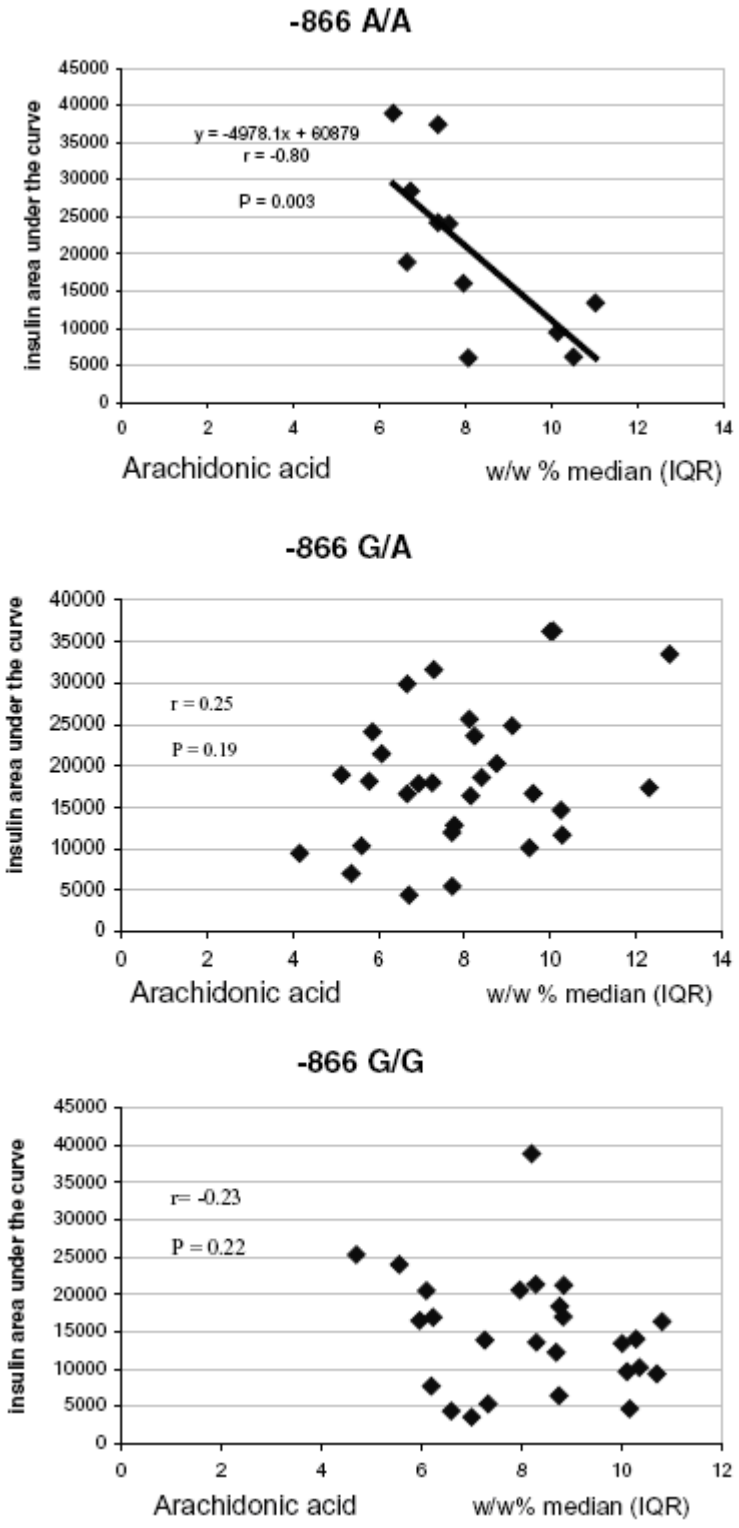

Figure 5. Linear correlation between insulin area under the curve during oral glucose tolerance test and the values of arachidonic acid in sterol ester lipids in 80 obese children stratified according to the UCP2 -866 G/A genotype.

Discussion

In the present study we observed significant differences in the values of important n-6 LCPUFAs according to -866G/A genotypes of the human uncoupling protein 2 gene in obese children. Furthermore, by correlating the fatty acid levels with the insulin response and the values calculated for insulin area under the curve during OGTT we observed considerable differences in relations to -866 polymorphism of the UCP2.

These differences cannot be explained by different degree of obesity or age. The negative correlation between 20:4n-6 values and insulin concentrations in the -866 A/A genotype group might be of special interest, because this group showed significant reduction in the values of the intermediate precursors of 20:4n-6 synthesis, 20:2n-6 and 20:3n-6. The A allele variant of the -866 G/A polymorphism is reportedly related to enhanced risk to develop diabetes mellitus (31;32). One of the potential mechanisms of the relation of altered availability of 20:4n-6 to insulin sensitivity might be the role of 20:4n-6 as a precursor of various eicosanoids.

Data obtained in this observational study can not answer the question whether altered availability of C20:4n-6 leads to changes of GIIS, or vice verse. While decreased UCP2 message might result in diminished energy expenditure and development of obesity, this very same mechanism would increase insulin secretion and thus, protect against type 2 diabetes mellitus (109). The association between the availability of n-6 polyunsaturated fatty acids and GIIS may open further opportunity to the dietary amelioration of metabolic consequences of obesity. The data obtained in the present study may provide some general messages for research on fatty acids in obesity as well. Controversial results seen in previous investigations on LCPUFAs in apparently similar groups of obese subjects may, at least in part, originate from the different genetic background including *UCP2* polymorphism (38;39).

3. Systematic review of fatty acid composition of human milk from mothers of preterm compared to full-term infants

Context

Fatty acid composition of human milk serves as guidance for the composition of infant formulae. The aim of the study was to systematically review data on the fatty acid composition of human milk of mothers of preterm compared to full-term infants.

Methods

We performed an electronic literature search in English (Medline (www.pubmed.com) and Medscape (www.medscape.com)) and German (SpringerLink (www.springerlink.com)) databases from their start dates to November 2005 onwards. The searching expressions were as follows: human milk or breast milk, combined with essential fatty acid or long-chain polyunsaturated fatty acid or arachidonic acid or docosahexanoic acid. We also checked the list of references in the publications identified. We attempted to reveal unpublished evidence by consulting abstract books of relevant scientific meetings and by contacting experts working in this field.

Fatty acid composition of preterm or full-term human milk was reported in several studies, whereas data obtained in direct comparison of the fatty acid composition of preterm and full-term human milk within the same study were published only in five reports. Each of these studies were longitudinal studies and investigated human milk from apparently healthy mothers who had delivered preterm or full-term infants. Both pregnancies and deliveries were normal in all these studies. The gestational ages of preterm infants were between 25 and 36 weeks, whereas those of full-term infants were over 37 weeks. There was no history of any disease of the offspring. All the women in the study of Genzel-Borovicezény et al., Luukkainen et al. and Kovács et al. were apparently on omnivorous diet. No information was given about the dietary background of mothers in the study of Bitman et al. and Rueda et al.

There were some methodological differences in the studies reviewed here. The sample collections were at different time points of lactation (for details see results). Storage temperature of the milk samples was either -20°C or -70°C or -80°C. For

fatty acid analysis, lipids were extracted either with chloroform-methanol or using a mixture of benzene-methanol-acetylchloride. Fatty acid methylesters were determined by capillary gas liquid chromatography in all studies except the classical study of Bitman et al (54), who used packed column gas liquid chromatography being the predominant method at the time. Results of the fatty acid composition were expressed as weight percentages of all fatty acids and were presented as means and SD (57), means and SE (54), median and IQR (56;58) or means and 95% confidence intervals (55). For a better comparability, we calculated all data into the form of means and SD from the published data or the original data bases of the two studies where medians and interquartile ranges were published (56;58). For arachidonic acid (AA) and docosahexanoic acid (DHA) values, i.e. the principal LCPUFAs of human milk we also calculated the difference of means between preterm and full-term milk and the 95% confidence intervals (CIs) of the difference of means by using the following formulae:

$$PSD = \sqrt{[(N_1 - 1)s_{1x}s_1 + (N_2 - 1)s_{2x}s_2 / (N_1 + N_2 - 2)}$$

$$SED = PSD \times \sqrt{[(1/N_1) + (1/N_2)]}$$

$$95\%\ CI = (X_1 - X_2) \pm t^* \times SED$$

(Abbrevations: PSD = Pooled SD, N_1 = number of subjects in group one (in our study: preterm), s_1 = standard deviation of group one, N_2 = number of subjects in group two (in our study: full-term), s_2 = standard deviation of group two; SED = Standard Error of the Difference, X_1 = mean of group one, X_2 = mean of group two, t* = value of t-distribution at the given confidence level with degree of freedom = $N_1 + N_2 - 2$.)

Results

Fatty acid compositions of preterm and full-term human milk investigated in 18 comparisons published in 5 studies are shown in **Tables 9, 10** and **11**.

Preterm breast milk showed higher contents of the saturated fatty acids myristic acid (C14:0) at two different time points in the study of Kovács et al (58) and in the study of Genzel-Boroviczény et al (56), palmitic acid (C16:0) values at one time point in the study of Kovács et al (58) and stearic acid (C18:0) values at one time point in the study of Luukkainen et al (55) (**Table 9**). The cis monounsaturated fatty

acid oleic acid (C18:1n-9) was higher in preterm milk at two different time points in the study of Kovács et al (58) and at three different times in the study of Rueda et al (57) (**Table 9**). Values of the principal n-6 polyunsaturated fatty acids, linoleic acid (C18:2n-6, LA), GLA and AA values were reported in all studies, whereas DHGLA values were reported only in four studies. Data on the principal n-3 polyunsaturated fatty acids, docosapentaenoic acid (C22:5n-3) and docosahexaenoic acid (C22:6n-3, DHA) were reported in all studies, whereas eicosapentaenoic acid (C20:5n-3, EPA) values were reported only in four and alpha-linolenic acid (C18:3n-3, ALA) values in 3 studies.

Values of LA, ALA and EPA did not differ between preterm and full-term human milk in any of the five studies. In contrast, GLA values were significantly higher in colostrum of mothers giving birth to preterm infants than in those delivering at term in the study of Kovács et al (58). Values of the principal n-3 LCPUFA DHA were significantly higher in preterm breast milk at 1, 4, 7, 14 and 21 days, and of the n-6 LCPUFA AA at all time points except day 14 in the study of Kovács et al (58), whereas both fatty acids were found at higher levels only at days 28-35 by Luukkainen et al (55) (**Tables 10** and **11**). Values of the major intermediary metabolite of the synthesis of AA, DHGLA were found to be significantly higher in milk samples obtained from mothers of preterm than in those collected from mothers of full-term infants in the study of Kovács et al (58), and values of C22:5n-3 (intermediary metabolite of the synthesis of DHA) were significantly higher in preterm breast milk in the study of Kovács et al (58) at 7 and 21 days, and in the study of Luukkainen et al (55) at 12 and 26 weeks (**Table 10** and **11**).

The differences of the means and the 95% confidence intervals (CIs) of the difference for DHA and AA values measured in milk samples obtained from mothers of preterm and full-term infants in the five studies are shown in **Figure 6** and **Figure 7**, respectively. The difference of mean DHA values (preterm vs term) were significantly different from zero in 3 studies (55;56;58), covering 3 different time points. In the study of Kovács et al (58) AA value difference were positive at 3 different time points of lactation; whereas the data of Rueda et al (57) showed negative 95% CIs for AA values at 2 different time points of lactation.

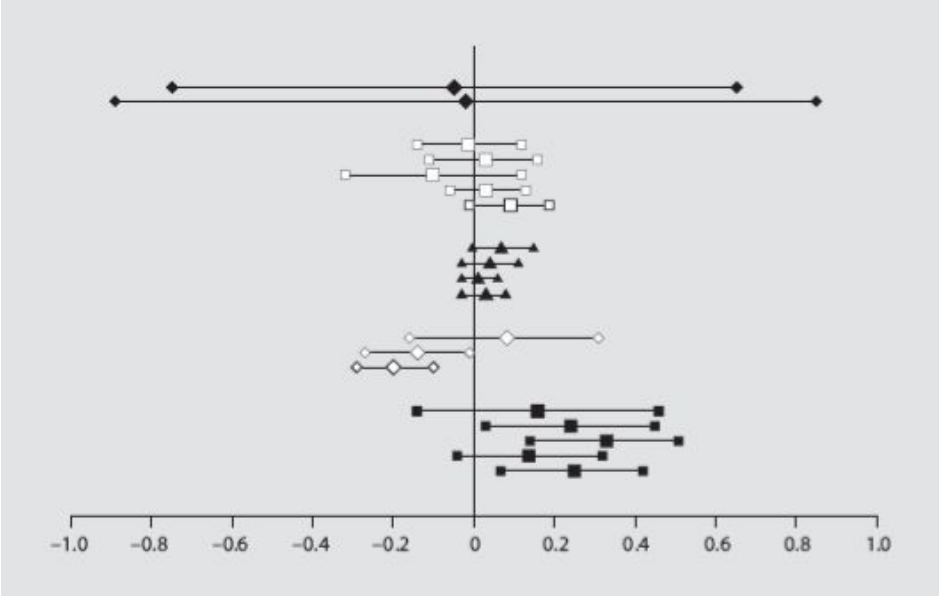

Figure 6. The differences of the means and the 95% confidence intervals (CIs) of the difference for arachidonic acid values measured in milk samples obtained from mothers of preterm and full-term infants in five studies. Each horizontal line represents one statistical comparison. The middle symbol on the horizontal line depicts mean value, whereas the lateral symbols show 95% CIs. Symbols: ◆: Bitman et al (Bitman): preterm (upper line) and very preterm (lower line) at day 42 ■: Luukainen et al (Luukainen): at days: 1-7, 8-14, 15-21, 22-27, 28-35 (top down) ▲: Genzel-Boroviczeny et al (Genzel-Boroviczeny): at days: 5, 10, 20 and 30 (top down) ◆: Rueda et al (16) at days: 1-5, 6-15 and 16-35 (top down) ■: Kovács et al (Kovacs) at days: 1, 4, 7, 14 and 21 (top down)

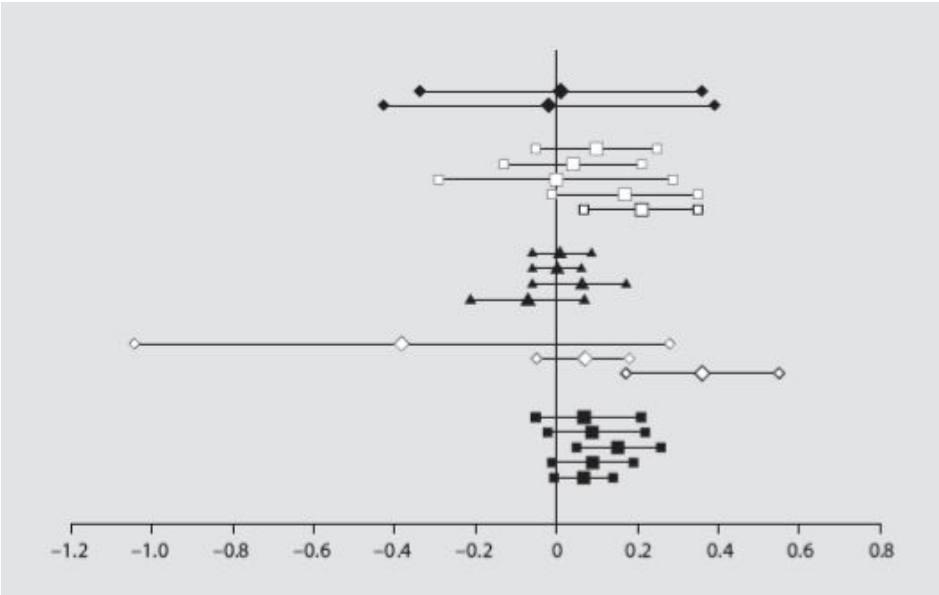

Figure 7. The differences of the means and the 95% confidence intervals of the difference for docosahexaenoic acid values measured in milk samples obtained from mothers of preterm and full-term infants in five studies. For further details of legend see **Figure 6.**

Table 9. Major saturated and cis monounsaturated fatty acids in preterm and full-term human milk

Day of lactation	Number of samples		C14:0		C16:0		C18:0		C18:1n–9	
	pre-term	full-term	preterm	full-term	preterm	full-term	preterm	full-term	preterm	full-term
Bitman et al. [13] (USA)										
42 (VPT)	18	6	8.41 (3.32)	5.68 (3.31)	20.13 (5.6)	22.2 (5.58)	7.24 (4.52)	7.68 (4.53)	33.41 (6.69)	35.51 (6.69)
42 (PT)	28	6	7.55 (4.71)	5.68 (3.31)	23.16 (7.59)	22.2 (5.58)	7.25 (6.17)	7.68 (4.53)	33.74 (9.13)	35.51 (6.69)
Luukkainen et al. [14] (Finland)										
1–7	12	8	7.47 (5.10)	6.64 (4.67)	28.27 (4.70)	29.04 (3.63)	9.8 (1.47)	9.95 (3.16)	39.17 (6.85)	38.92 (6.88)
8–14	11	10	9.88 (6.14)	8.12 (5.12)	27.89 (2.20)	28.39 (5.37)	9.62 (1.35)**	11.38 (2.16)	37.65 (4.45)	38.00 (5.88)
15–21	9	7	7.96 (4.11)	6.36 (6.65)	26.00 (4.36)	27.66 (6.88)	9.48 (2.47)	10.43 (2.55)	38.83 (4.45)	39.71 (7.47)
22–27	16	10	8.77 (3.40)	7.86 (5.77)	26.20 (3.25)	27.53 (5.08)	10.29 (1.61)	10.82 (3.31)	38.47 (2.42)	38.03 (7.33)
28–35	14	5	7.7 (2.57)	8.7 (5.85)	26.12 (4.26)	28.02 (7.35)	10.27 (1.66)	10.00 (7.43)	38.61 (2.64)	36.95 (6.49)
Genzel-Boroviczény et al. [15] (Germany)										
5	19	32	7.65 (1.64)	6.93 (2.49)	23.84 (3.16)	24.56 (5.14)	6.66 (0.92)	7.23 (1.10)	33.49 (6.67)	32.16 (4.22)
10	19	38	8.7 (2.26)*	7.07 (2.5)	22.74 (3.02)	22.29 (6.25)	7.61 (1.48)	7.60 (1.22)	29.73 (9.58)	31.59 (4.26)
20	17	38	9.15 (2.73)*	7.29 (1.81)	22.42 (1.88)	23.16 (3.07)	8.03 (1.50)	7.69 (1.21)	31.18 (3.95)	31.74 (3.04)
30	13	38	8.98 (3.08)	7.39 (2.51)	22.74 (2.64)	22.76 (1.62)	7.73 (1.22)	7.47 (1.22)	31.52 (4.28)	30.74 (5.81)
Rueda et al. [16] (Spain)										
1–5	6	16	6.70 (2.17)	6.60 (1.79)	21.05 (0.79)	20.81 (2.19)	5.69 (0.53)	7.36 (4.13)	30.70 (3.49)*	34.93 (2.67)
6–15	6	16	7.57 (2.04)	8.21 (2.25)	19.56 (2.19)	19.59 (2.19)	6.09 (1.05)	6.28 (1.01)	39.86 (5.47)**	33.61 (3.26)
16–35	6	16	6.27 (1.60)	6.42 (3.00)	19.03 (4.68)	18.85 (2.57)	6.50 (1.97)	6.70 (0.89)	38.14 (5.25)*	47.37 (6.42)
Kovács et al. [17] (Hungary)										
1	8	10	6.18 (2.25)	5.19 (1.51)	24.21 (3.05)*	27.78 (2.21)	4.99 (1.17)	5.33 (0.99)	35.82 (4.42)	35.73 (3.18)
4	8	10	9.36 (5.13)	6.10 (1.66)	23.97 (2.46)	25.47 (2.72)	4.97 (0.85)	5.29 (1.33)	33.24 (5.47)	36.02 (2.14)
7	8	10	9.68 (4.64)**	5.69 (1.63)	23.02 (2.46)	25.16 (3.25)	5.35 (1.01)	5.44 (1.07)	32.3 (4.25)	36.37 (2.8)
14	8	10	9.87 (4.59)*	5.55 (1.82)	22.83 (3.38)	23.49 (1.69)	5.42 (0.43)	5.32 (0.93)	32.38 (3.92)**	35.94 (2.54)
21	8	10	8.36 (2.69)	5.68 (2.34)	22.41 (4.87)	23.78 (3.38)	5.41 (0.57)	5.78 (1.45)	31.21 (2.84)*	36.01 (4.24)

Data are in weight percent presented as means (SD). * $p < 0.05$, ** $p < 0.001$, VPT denotes very preterm and PT denotes preterm.

Table 10. Major n-3 polyunsaturated fatty acids in preterm and full-term human milk

Day of lactation	C18:3n–3		C20:5n–3		C22:5n–3		C22:6n–3 (DHA)	
	preterm	full-term	preterm	full-term	preterm	full-term	preterm	full-term
Bitman et al. [13]								
42 (VPT)	0.76 (0.52)	1.03 (0.51)	0.55 (0.72)	n.d.	0.42 (0.36)	0.11 (0.36)	0.24 (0.36)	0.23 (0.34)
42 (PT)	0.76 (0.71)	1.03 (0.51)	n.d.	n.d.	0.12 (0.51)	0.11 (0.36)	0.21 (0.46)	0.23 (0.34)
Luukkainen et al. [14]								
1–7	0.85 (0.34)	1.01 (0.27)	0.1 (0.05)	0.08 (0.04)	0.32 (0.78)	0.24 (0.49)	0.54 (1.32)	0.44 (0.89)
8–14	1.01 (0.66)	1.06 (0.67)	0.09 (0.15)	0.08 (0.08)	0.22 (0.54)	0.16 (0.33)	0.42 (1.03)	0.38 (0.77)
15–21	1.33 (1.05)	1.22 (0.78)	0.08 (0.07)	0.09 (0.08)	0.19 (0.46)	0.24 (0.49)	0.3 (0.73)	0.3 (0.61)
22–27	1.21 (0.49)	1.3 (0.49)	0.11 (0.09)	0.1 (0.08)	0.21 (0.51)**	0.1 (0.20)	0.35 (0.86)	0.18 (0.37)
28–35	1.24 (0.44)	1.45 (1.16)	0.09 (0.02)	0.07 (0.1)	0.22 (0.53)**	0.08 (0.16)	0.39 (0.95)**	0.18 (0.37)
Genzel-Boroviczény et al. [15]								
5	0.73 (0.49)	0.66 (0.19)	0.04 (0.06)	0.11 (0.21)	0.23 (0.05)	0.22 (0.08)	0.45 (0.11)	0.45 (0.11)
10	0.74 (0.26)	0.84 (0.27)	0.04 (0.06)	0.03 (0.08)	0.17 (0.04)	0.17 (0.05)	0.37 (0.14)	0.39 (0.09)
20	0.78 (0.21)	0.82 (0.27)	0.08 (0.15)	0.06 (0.09)	0.17 (0.11)	0.17 (0.05)	0.29 (0.26)	0.30 (0.13)
30	0.96 (0.38)	0.74 (0.17)	0.05 (0.07)	0.12 (0.18)	0.15 (0.06)	0.17 (0.08)	0.25 (0.05)	0.32 (0.25)
Rueda et al. [16]								
1–5	n.d.	n.d.	0.15 (0.04)	0.20 (0.19)	0.42 (0.2)	0.4 (0.2)	0.48 (0.11)	0.86 (0.96)
6–15	n.d.	n.d.	0.19 (0.01)	0.10 (0.05)	0.31 (0.07)	0.26 (0.29)	0.48 (0.2)	0.41 (0.12)
16–35	n.d.	n.d.	n.d.	0.07 (0.03)	n.d.	0.17 (0.04)	0.69 (0.42)	0.33 (0.12)
Kovács et al. [17]								
1	0.42 (0.27)	0.28 (0.14)	n.d.	n.d.	0.19 (0.11)	0.10 (0.09)	0.32 (0.12)*	0.25 (0.14)
4	0.39 (0.20)	0.34 (0.23)	n.d.	n.d.	0.14 (0.05)	0.08 (0.07)	0.30 (0.13)*	0.20 (0.11)
7	0.49 (0.21)	0.41 (0.25)	n.d.	n.d.	0.12 (0.05)*	0.06 (0.05)	0.32 (0.13)**	0.16 (0.09)
14	0.49 (0.25)	0.43 (0.19)	n.d.	n.d.	0.11 (0.06)	0.05 (0.03)	0.24 (0.12)*	0.15 (0.09)
21	0.46 (0.20)	0.39 (0.14)	n.d.	n.d.	0.12 (0.07)*	0.05 (0.03)	0.19 (0.07)*	0.13 (0.07)

n.d. denotes not determined. For symbols, abbrevations and number of samples analysed see Table 1.

Table 11. Major n-6 polyunsaturated fatty acids in preterm and full-term human milk

Day of lactation	C18:2n–6		C18:3n–6		C20:3n–6 (DHGLA)		C20:4n–6 (AA)	
	preterm	full-term	preterm	full-term	preterm	full-term	preterm	full-term
Bitman et al. [13]								
42 (VPT)	15.75 (4.88)	15.58 (4.87)	n.d.	n.d.	0.51 (0.52)	0.53 (0.51)	0.55 (0.72)	0.6 (0.71)
42 (PT)	13.83 (6.63)	15.58 (4.87)	n.d.	n.d.	0.43 (0.51)	0.53 (0.51)	0.58 (0.97)	0.6 (0.71)
Luukkainen et al. [14]								
1–7	8.8 (3.08)	9.92 (3.69)	0.48 (1.17)	0.43 (0.88)	n.d.	n.d.	0.59 (1.44)	0.6 (1.22)
8–14	8.79 (2.72)	8.69 (3.96)	0.34 (0.83)	0.33 (0.67)	n.d.	n.d.	0.47 (1.15)	0.44 (0.89)
15–21	11.75 (7.32)	9.85 (5.75)	0.31 (0.76)	0.37 (0.75)	n.d.	n.d.	0.39 (0.95)	0.49 (1.0)
22–27	10.32 (3.91)	10.46 (2.38)	0.28 (0.68)	0.28 (0.57)	n.d.	n.d.	0.36 (0.88)	0.33 (0.67)
28–35	11.32 (3.69)	11.52 (8.71)	0.27 (0.66)	0.18 (0.37)	n.d.	n.d.	0.37 (0.91)*	0.28 (0.57)
Genzel-Boroviczény et al. [15]								
5	9.86 (2.17)	9.81 (3.26)	0.31 (0.64)	0.11 (0.08)	0.59 (0.29)	0.55 (0.23)	0.72 (0.12)	0.76 (0.14)
10	10.34 (2.79)	10.99 (3.51)	0.11 (0.08)	0.18 (0.12)	0.46 (0.12)	0.45 (0.19)	0.62 (0.11)	0.60 (0.14)
20	9.89 (2.18)	11.11 (4.13)	0.15 (0.11)	0.32 (0.57)	0.40 (0.12)	0.39 (0.09)	0.50 (0.05)	0.50 (0.09)
30	10.46 (2.36)	11.81 (3.44)	0.16 (0.05)	0.29 (0.46)	0.40 (0.12)	0.41 (0.15)	0.48 (0.05)	0.46 (0.05)
Rueda et al. [16]								
1–5	12.59 (2.7)	12.42 (1.83)	0.14 (0.06)	0.22 (0.2)	0.73 (0.15)	0.69 (0.25)	0.91 (0.29)	0.83 (0.3)
6–15	13.07 (2.75)	14.22 (3.66)	0.18 (0.04)	0.09 (0.04)	0.55 (0.07)	0.62 (0.2)	0.71 (0.12)	0.85 (0.17)
16–35	10.75 (2.03)	14.3 (4.02)	0.18 (0.03)	0.06 (0.01)	0.4 (0.04)	0.47 (0.09)	0.46 (0.04)	0.66 (0.14)
Kovács et al. [17]								
1	14.58 (3.81)	16.30 (2.56)	0.12 (0.09)*	0.03 (0.01)	0.63 (0.16)	0.51 (0.24)	0.91 (0.23)*	0.75 (0.33)
4	13.89 (3.24)	16.81 (3.12)	0.06 (0.03)	0.05 (0.06)	0.56 (0.15)**	0.34 (0.12)	0.76 (0.22)*	0.52 (0.19)
7	14.22 (3.77)	17.76 (5.82)	0.08 (0.03)	0.09 (0.13)	0.54 (0.18)**	0.28 (0.13)	0.75 (0.21)**	0.42 (0.16)
14	14.72 (4.50)	19.49 (5.15)	0.08 (0.03)	0.05 (0.04)	0.46 (0.15)*	0.27 (0.08)	0.57 (0.20)	0.43 (0.16)
21	18.83 (5.77)	18.65 (7.44)	0.07 (0.02)	0.11 (0.09)	0.47 (0.19)*	0.25 (0.08)	0.59 (0.22)*	0.35 (0.13)

n.d. denotes not determined. For other symbols, abbrevations and number of samples analysed see Table 1.

Discussion

In the present review, we identified 3 independent studies (55;57;58) covering 3 different time points of lactation were the 95% CIs of the significant difference of mean DHA values (preterm minus full-term) were entirely in the positive range, i.e. the percentage contribution of DHA is higher in preterm than full-term milk. In contrast, data for AA were rather controversial: in the study of Kovács et al (58) the 95% CIs for AA values were entirely in the positive range at 3 different time points of lactation, whereas in the study of Rueda et al (57) the 95% CIs for AA values were entirely in the negative range at 2 different time points of lactation, i.e. both significantly higher and lower AA levels were reported in preterm compared to full-term human milk.

The LCPUFA content of human milk is closely related to maternal LCPUFA body stores (110-112). Mothers of premature infants transfer less LCPUFAs to the fetus during a shorter pregnancy, and also the volume of human milk consumed by their infants tends to be much smaller particularly during the first weeks after birth (56), which would lead to higher maternal LCPUFA stores and thus may explain higher LCPUFA values in preterm than in full-term human milk (58). Due to the shorter period and lesser extent of intrauterine LCPUFA accumulation, preterm infants should have higher postnatal LCPUFA requirements than full-term infants. It is tempting to speculate whether there may be other adaptive or regulative mechanism to indicate higher LCPUFA contents in preterm milk in response to higher physiological needs of preterm infants, but the occurrence of a significant survival rate of small preterm infants is a very new phenomenon relative to the time scale of human evolution and hence it appears unlikely that such mechanisms specific to preterm birth would have developed.

Nonetheless, higher DHA values in preterm than in full-term human milk indicate a likely benefit of utilising own mother's milk for feeding preterm infants. Moreover, these data should lead to a reconsideration of the DHA levels in formulae for preterm infants, which currently tend to mimic DHA levels of full-term human milk and thus tend to differ from the fatty acid composition of human milk of mothers giving birth to preterm infants.

4. Prevalence of metabolic syndrome in European obese children

Context

The rapid rising prevalence of childhood obesity is related to increased risk of obesity-related diseases during adulthood. The aim of the present study was to review the data concerning the prevalence of metabolic syndrome (MS) in European children and adolescents (Part a) and to determine and compare the prevalence of MS among overweight and obese children, and adolescents in five European countries using four MS definitions (Part b).

a.) Review of the data concerning the prevalence of metabolic syndrome in European children and adolescents

Methods

We performed an electronic literature search in (Medline (www.pubmed.com) and Medscape (www.medscape.com)) databases from their start dates to February 2008 onwards. The searching expressions were as follows: metabolic syndrome, cardiovascular syndrome, children, adolescents. We also checked the list of references in the publications identified.

Results

In **Table 12**, we summarize the studies on the prevalence of MS in children and adolescents performed in Europe combined with our own, recent results. Only few studies investigated the prevalence of MS in European countries as a primary goal. The studies were performed in Middle and South part of Europe (Spain, Italy, France, Germany, and Hungary), and only one in the North (UK). Except of two studies (113;114), the investigations were performed only on overweight and obese children and adolescents with a small number of subjects included. The used definitions were either based on the adult definitions from WHO or NCEP, modified with children specific cut-offs, or previously published definitions used to define MS in children. A big variance in the prevalence of MS (8.5% to 50%) can be observed among the studies; however, the results are not comparable since the used definitions were different.

A low degree of overlap among the different MS definitions is pointed out in two studies (Reinehr et al (114), and for our results see part b), where more definitions were used.

Table 12. The prevalence of metabolic syndrome in childhood and adolescence in studies performed in Europe

Study	Study population	Definition	Prevalence (%)
Csábi et al. (Hungary; 2000)	8-18-y old, 180 obese and 239 control	WHO*	obese: 8.9
Viner et al. (UK; 2005)	2-18-y old, 103 obese	WHO*	33
Invitti et al. (Italy; 2006)	6-16-y old, 588 obese	WHO*	23,3
Druet et al. (France 2006)	308 obese and overweight	NCEP*	15,9
López-Capapé et al. (Spain; 2006)	4-18-y old, 429 obese and overweight	Cook	18,0
Bueno et al. (Spain; 2006)	Age: 10.8 (2.3) mean (SD), 103 obese	Cook	29,9
		de Ferranti	50
Sartorio et al. (Italy 2007)	8-18-y old, 439 obese	Weiss	n.d.
		Cook	EPS: 29.9 ♂, 19.2 ♀
			LPS: 52.1 ♂, 28.9 ♀
		Italian Society for Pediatrics	n.d.
Reiner (23) (Germany 2007)	9.5-13.3-y old, n=1205 overweight and obese; 84 normal weight	Cook	21
		De Ferranti	39
		Viner	18
		Weiss	29
		EGIR	8
		WHO	6
		NCEP	13
		IDF	14
Our results (part b) (Hungary, France, Italy, Greece, Poland)	4.5-18.2-y old, 1241 obese and overweight	De Ferranti	35,7
		WHO*	31,4
		NCEP*	20,3
		IDF for children	16,4

WHO, World Health Organisation; NCEP, National Cholesterol Education Program; EGIR, European Group for the Study of Insulin Resistance; IDF, International Diabetes Federation. * modified with children specific cut off values, EPS: early-mid pubertal stage, LPS: late pubertal stage, n.d.: data not shown.

b.) To determine and compare the prevalence of MS among overweight and obese children and adolescents in five European countries using four MS definitions

Materials and Methods

The investigations were carried out in 1241 overweight children and adolescents from five European countries (Hungary: n=449, France: n=283, Italy: n=274, Greece: n=145 and Poland: n=90), referred for clinical evaluation because of their overweight to the Department of Pediatrics, University of Pécs, Hungary; Saint Vincent de Paul Hospital, AP-HP, Paris and CTP, Margency, France; Department of Pediatrics, University of Rome „Sapienza", Italy; Department of Endocrinology, „P. & A. Kyriakou" Children's Hospital, Athens, Greece; Department of Pediatric Endocrinology & Diabetes, Medical University of Silesia, Katowice, Poland.

None of the subjects had chronic disease, secondary obesity, received drug treatment or were on restricted diet. We considered children as obese if their body mass index exceeded the value of Cole et al. (6) corresponding to 25 at the age of 18 years. The clinical characteristics of patients in the five different countries are shown in **Table 13**. Anthropometric measurements were performed according to harmonized methods as described by Lohman et al. (115). Body mass index was computed as weight (kilogram) divided by squared height (m^2). Blood glucose, serum insulin and lipid levels were determined from blood samples taken after an overnight fast. Plasma glucose concentrations were determined by the glucose oxidase or hexokinase method. Triglyceride and cholesterol concentrations were measured with an enzymatic kit and HDL-C using a corresponding non precipitating method. Plasma insulin concentrations were measured with commercially available radioimmunoassay or chemiluminescence method. A comparison test was performed between the two methods. The correlation between the results was calculated: $y = 0.99x$, with $r = 0.95$. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated with the following formula: (fasting insulin x fasting glucose)/22.5. Blood pressure (BP) was measured after 5 min rest, while sitting to the nearest 2 mmHg, using a standard mercury-gravity manometer with proper cuff size in each subject three times. The average of three measurements obtained on different days was used in the analysis (116).

Table 13. Clinical and biological characteristics of obese children according to nationality. Data are expressed as mean (SD).

	Whole group (n = 1241)	Polish (n = 90)	Greek (n = 145)	Italian (n = 274)	French (n = 283)	Hungarian (n = 449)	p
Boys/Girls	560/681	39/51	74/71	135/139	91/192	221/228	< 0.001
Age (years)	12.9 (2.5)	12.9 (3.3)	11.3 (2.5)	12.1 (1.6)	14.5 (1.6)	12.9 (2.7)	< 0.001*
Weight (kg)	80.53 (24.1)	78.7 (24.5)	70.4 (20.9)	63.9 (13.9)	102.4 (19.9)	80.5 (21.7)	< 0.001**
Height (cm)	158.2 (12.8)	158.4 (16.0)	151.3 (14.6)	153.6 (9.4)	164.5 (8.4)	159.4 (13.5)	< 0.001**
Waist cc (cm)	91.9 (12.9)	93.9 (12.7)	95.3 (13.1)	83.9 (10.6)	102.1 (11.3)	89.2 (10.4)	< 0.001**
BMI (kg/m^2)	31.5 (6.1)	30.8 (4.9)	30.1 (4.9)	26.8 (3.6)	37.7 (5.9)	31.1 (4.8)	< 0.001**
Triglyceride (mmol/l)	1.2 (0.9)	1.2 (0.6)	1.3 (0.3)	1.1 (0.6)	1.0 (0.5)	1.5 (1.22)	< 0.001***
HDL-cholesterol (mmol/l)	1.2 (0.3)	1.4 (0.3)	1.3 (0.3)	1.3 (0.3)	1.2 (0.4)	1.18 (0.30)	< 0.001***
Glucose (mmol/l)	4.8 (0.6)	4.9 (0.4)	5.2 (0.5)	4.9 (0.5)	4.5 (0.5)	4.8 (0.8)	< 0.001***
Insulin (μU/ml)	23.0 (15.7)	14.3 (7.9)	19.4 (13.9)	20.7 (11.8)	18.1 (12.8)	30.2 (17.9)	< 0.001***
SBP (mmHg)	120.6 (13.4)	111.3 (13.4)	121.9 (12.5)	115.9 (11.3)	119.7 (14.8)	125.4 (11.9)	< 0.001***
DBP (mmHg)	70.9 (10.5)	72.4 (9.5)	62.9 (10.9)	76.9 (9.6)	67.5 (10.1)	71.8 (8.8)	< 0.001***

BMI, body mass index; HDL-cholesterol, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure. p values: comparison among countries, * adjusted for nationality and gender, ** adjusted for nationality, age and gender; *** adjusted for nationality, age, gender and BMI.

Statistical analysis

Results were evaluated with SPSS for Windows, Release 11.5 (SPSS Inc., Chicago, IL, USA). All data are presented as mean (SD). The clinical features of obese children stratified according to nationality were compared with analysis of variance followed by post-hoc LSD test. Because the age and BMI differed significantly between groups we adjusted the biochemical parameters and blood pressures for nationality, age, gender and BMI, and compared the adjusted parameters with LSD test. Results were regarded as statistically significant at $p<0.05$.

Definition of Metabolic Syndrome

Children were classified as displaying MS according to four different definitions. Two definitions were child-specific (Ferranti et al. (79), and International Diabetes Federation (78)) while the other two (World Health Organisation (73) and National Cholesterol Education Program Adult Treatment Panel III (75)) adult-specific.

In the case of WHO criteria we omitted microalbuminuria because measurements were available only for a subset of children and we replaced waist-hip ratio with waist circumference since sex and age specific reference values were not available for waist-hip ratio. The highest quartile for insulin was 15 μU/ml (the highest 25% value of 100 healthy adolescents). The WHO definition was applied only in 1201 children because of missing plasma insulin values in 40 children. Age and gender specific reference values were used in all definitions for the diagnosis of high blood pressure and increased waist circumference (116;117).

Results

Table 13 shows the clinical features of obese children according to nationality after adjustment for nationality, age gender and BMI. Triglyceride and insulin levels and the systolic blood pressure were highest in Hungarian, while fasting plasma glucose in Greek, and diastolic blood pressure in Italian children were the highest as compared to children from other countries.

The prevalence of MS in the investigated cohort was 35.7%, 31.4%, 20.3%, and 16.4% according to the Ferranti, WHO, NCEP and IDF definitions, respectively (**Figure 8**).

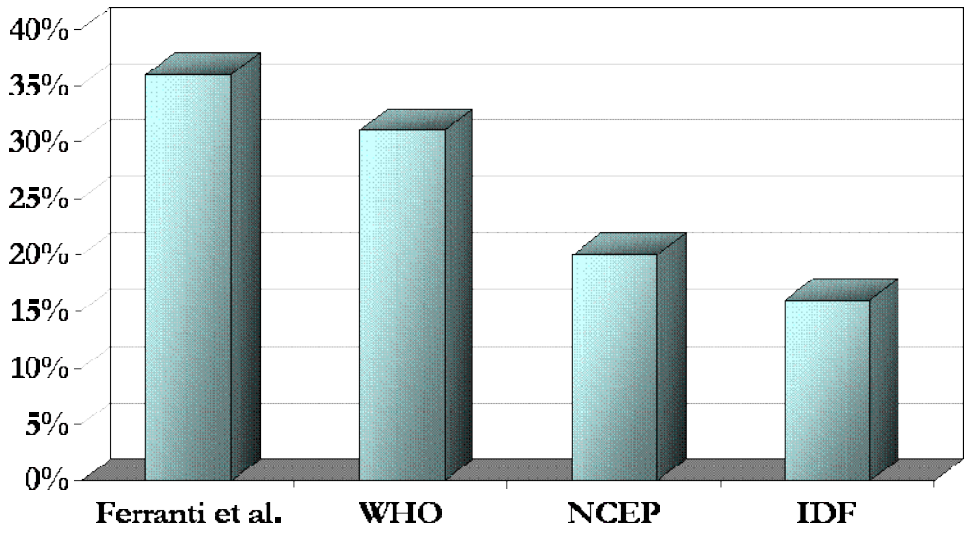

Figure 8. The prevalence of metabolic syndrome in the investigated cohort. WHO, World Health Organisation; NCEP, National Cholesterol Education Program; EGIR, European Group for the Study of Insulin Resistance; IDF, International Diabetes Federation

The prevalence of MS was not different between genders. The prevalence of risk factors according to the different definitions is shown in **Table 14**.

The prevalence of increased waist circumference was high (71.4%). Due to different cut off values the occurrence of low HDL levels varied according to definitions. We found, as expected, the highest prevalence of elevated triglyceride levels according to Ferranti et al. and of elevated fasting plasma glucose according to the definition of IDF because of the lower cut off values applied for these risk factors.

The clustering of risk factors in the investigated cohort is shown in **Table 14**. Only 6.3-8.8% of obese adolescents were free from any risk factor, and the clustering of three or more risk factors was very high (20.3-35.7%) (depending on the type of definition).

Table 14. Prevalence and clustering of risk factors according to the criteria of Ferranti et al., WHO, NCEP and IDF

	Ferranti et al	WHO	NCEP	IDF
Increased waist circumference	71.4%	71.4%	71.4%	71.4%
High fasting plasma glucose	2.3%	2.3%	2.3%	8.6%
High fasting plasma insulin*		64.7%		
High triglyceride	42.8%	15.1%	15.1%	15.1%
Low HDL-cholesterol	48.5%	13.0%	46.4%	27.6%
High blood pressure	38.1%	38.1%	38.1%	38.1%
No risk factor	6.3 %	6.5 %	8.8 %	11.3 %
1 risk factor	25.1 %	26.2 %	32.2 %	37.9 %
2 risk factors	32.9 %	34.7 %	38.7 %	33.5 %
3 or more risk factors	35.7 %	32.6 %	20.4 %	17.3 %

NCEP, National Cholesterol Education Program; WHO, World Health Organisation; IDF, International Diabetes Federation, HDL-cholesterol: high-density lipoprotein cholesterol * in n = 1201 investigated subjects.

We calculated the number of children who had MS by all four, three, two, one and none of the definitions (**Figure 9**). It is worth mentioning that 12.2% (n=147) of children had MS and 55.8% (n=670) were free of MS according to all four definitions.

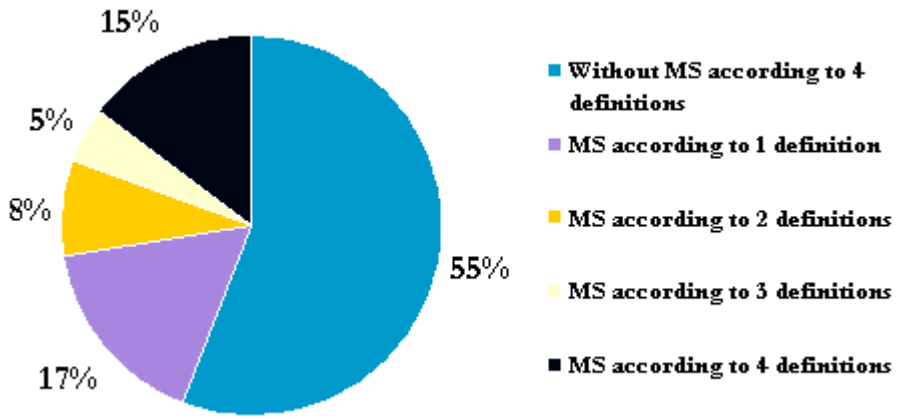

Figure 9. Frequency of metabolic syndrome if one, two, three or four definitions are applied.

The prevalence of MS was significantly influenced by the degree of obesity, characterised by BMI or waist circumference (**Table 15**), but not by age.

Table 15. Effect of age, BMI and waist cc on the prevalence of MS according to all four definition (n = 1201).

	<Q1	<Q3	>=Q3
age	49 (15.8%)	133 (14.8%)	43 (14.1%)
BMI	18 (5.9%)[a]	110 (12.2%)[b]	66 (22.2%)
waist cc	22 (7.9%)[a]	112 (12.1%)[b]	64 (23.4%)

Q1: quartile1, Q3: quartile3; a, b $p < 0.001$ compared to >Q3

Discussion

The results showed the high prevalence of MS ranging from 16.4% to 35.7% in overweight and obese children referred to outpatient clinics. The prevalence of MS was highly dependent on the definition used. The accordance among the definitions was small, only 12.2% of investigated subjects had MS according to all 4 definitions.

The observed prevalence of MS in our study is in agreement with earlier reports investigating the prevalence of childhood MS (79;113;114;118-123). However, the results of these studies are not comparable since the definitions they used for the childhood MS were different.

The disparity between different definitions is related to the different cut off values and pooling strategies of MS criteria. The highest prevalence of MS (35.7%) was found according to the definition of Ferranti et al. (79) using the lowest cut off values for triglyceride and the highest for HDL-cholesterol levels. The WHO (73) was the only definition including fasting insulin as a criterion. The high prevalence of hyperinsulinemia (64.7%) explains why WHO definition identified more children with MS than the IDF and NCEP.

Our results demonstrated that only 6.3-11.3% of overweight and obese children were free from any risk factors, and 17.3-35.7% had already more than 3 risk factors, underlining the fact that clustering of risk factors starts in childhood. The prevalence of high waist circumference was 71%, showing that the majority of children had

central obesity and consequent insulin resistance (64.7% hyperinsulinemic). The prevalence of high blood pressure was also frequent – mounting to 38% in the cohort.

There was a significant difference in the biological characteristics of children according to nationality, even if we adjusted the values for nationality, age, gender and BMI. These differences can be attributed to different referral rules. Different dietary and lifestyle habits can also contribute to the difference in the MS prevalence between the 5 cohorts.

In the present cohort, the prevalence of MS increased with the increasing degree of obesity (BMI and waist circumference) in accordance with previous studies (69).

There are certain limitations of the present study. Our cohort is not a population-based sample and might not even reflect the European obese children's population. However, it represents a large proportion of European obese children referred to obesity centres. Further weakness of our study is that the different biochemical parameters were not determined in one laboratory, however the methodology was the same in all five centres. Though the comparability of the two methods used for the determination of insulin was high, the variability between kits still has to be considered. Finally, the effect of pubertal development on prevalence of MS could not be analysed since data on pubertal stage were not available.

New findings of the study

1. The results of the present study on the common single nucleotide polymorphism at CD36 locus showed that rs3211908 was consistently associated with obesity and BF% in adolescents. The rs3211867, rs3211883 and rs1527483 were also related to obesity risk and BF% but the associations were weaker. The later SNPs are in linkage disequilibrium with rs3211908 ($0.43< r^2 <0.64$) suggesting that these associations reflect a single signal. In contrast, rs1527479, rs3211816 and rs3211931 were not linked to excess fat depot phenotype. Further analyses identified a haplotype with the minor allele of rs3211908 (frequency 4%) that is linked to increased risk of obesity in the case-control study and excess adiposity in the cross-sectional study suggesting that rs3211908 is responsible for the excess risk among other SNPs. Collectively, these findings suggest that *CD36* gene variability may contribute to the risk of body fat accumulation in adolescents.

2. In the present study we observed significant differences in the values of important n-6 LCPUFAs according to -866G/A genotypes of the human uncoupling protein 2 gene in obese children. Furthermore, by correlating the fatty acid levels with the insulin response and the values calculated for insulin area under the curve during OGTT we observed considerable differences in relations to -866 polymorphism of the UCP2.

3. In the present review, we identified 3 independent studies covering 3 different time points of lactation were the 95% CIs of the significant difference of mean DHA values (preterm minus full-term) were entirely in the positive range, i.e. the percentage contribution of DHA is higher in preterm than full-term milk. This finding supports the concept that fomuale for preterm infants should be supplemeneted with DHA. Moreover, the significantly higher contribution of DHA to the fatty acid composition of preterm than to that of full-term human milk questions the present practice to use the fatty acid composition of full-term human milk as model for the DHA content of formulae for preterm infants.

4. The results showed the high prevalence of MS ranging from 16.4% to 35.7% in overweight and obese children referred to outpatient clinics. The prevalence of MS

was highly dependent on the definition used. The accordance among the definitions was small, only 12.2% of investigated subjects had MS according to all 4 definitions. Our results demonstrated that only 6.3-11.3% of overweight and obese children were free from any risk factors, and 17.3-35.7% had already more than 3 risk factors, underlining the fact that clustering of risk factors starts in childhood.

Clinical consequencies of the study

1. Because the CD36 receptor is theoretically considered to play an important role in determining the susceptibility to the development of obesity, genetic characterisation of subgroups with actually increased risk of obesity may contribute to our better understanding of the link between receptor and clinical outcome.

2. The association between the availability of n-6 polyunsaturated fatty acids and glucose induced insulin secretion may open further opportunity to the dietary amelioration of metabolic consequences of obesity. The data obtained in the present study may provide some general messages for research on fatty acids in obesity as well. Controversial results seen in previous investigations on LCPUFAs in apparently similar groups of obese subjects may, at least in part, originate from the different genetic background including *UCP2* polymorphism.

3. Higher DHA values in preterm than in full-term human milk indicate a likely benefit of utilising own mother's milk for feeding preterm infants. Moreover, these data should lead to a reconsideration of the DHA levels in formulae for preterm infants, which currently tend to mimic DHA levels of full-term human milk and thus tend to differ from the fatty acid composition of human milk of mothers giving birth to preterm infants.

4. The prevalence of metabolic syndrome is high in European overweight and obese children referred to obesity centres, whatever definition is used. Overweight and obese children, especially those with high waist circumference, have to be screened for MS. An unified childhood-specific definition of MS is needed in order to gain comparable study results and to avoid the possibility of different diagnosis of the same individual depending on the country he/she lives in and on the criteria used.

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Papers

1. Bokor Szilvia, Csernus Katalin, Erhardt Éva, Molnár Dénes, Decsi Tamás: Role of the -866 G/A polymorphism of the uncoupling protein 2 gene in the metabolic consequences of obesity. (Az uncoupling protein 2 gén -866 G/A polimorfizmusának szerepe az elhízás metabolikus szövődményeinek kialakulásában.) Gyermekorvos továbbképzés V/4: 268-271; 2006
2. Bokor Szilvia, Csernus Katalin, Erhardt Eva, Burus István, Molnár Dénes, Decsi Tamás: Association of n-6 long-chain polyunsaturated fatty acids to -866 G/A genotypes of the human uncoupling protein 2 gene in obese children. Acta Paediatrica 96(9):1350-4; 2007. **Impact factor: 1.411**
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1. Sz. Bokor, K. Csernus, É. Erhardt, I. Burus, D. Molnár, T. Decsi: The 866A/A but not the 866 G/A genotype of uncoupling protein 2 gene is associated with reduced dihomo-gamma-linolenic acid values in obese children. Paediatric Research 58:362, 2005
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1 Bokor Szilvia, Csernus Katalin, Erhardt Éva, Molnár Dénes, Decsi Tamás: Trp64Arg polymorphism of the béta-3 adrenergicreceptor gene and erythrocyte membrane fatty acid composition in obese children. (A béta-3 adrenoreceptor gén Trp64Arg polimorfizmusának összefüggése a zsírsavellátottsággal elhízott gyermekekben.) Gyermekgyógyászat 57: 125-129; 2006

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