

Breastfeeding modulates the influence of the peroxisome proliferator-activated receptor gamma (*PPARG2*) Pro12Ala polymorphism on adiposity in adolescents: the HELENA Cross-Sectional Study

Short running title: Breastfeeding, BMI and PPARG polymorphism

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Objective: The peroxisome proliferator-activated receptor gamma 2 (*PPARG2*) Pro12Ala polymorphism has been associated with a higher body mass index (BMI) and a lower risk of type 2 diabetes in adulthood. The association between adiposity and *PPARG* variants can be influenced by environmental factors like early growth, dietary fat and (as recently shown) breastfeeding. The study objectives were to assess (i) the influence of the *PPARG2* Pro12Ala polymorphism on adiposity markers in adolescents and (ii) a possible modulating effect of breastfeeding on these associations.

Methods: Data on breastfeeding duration, BMI and genotypes for the Pro12Ala polymorphism were available for 945 adolescents (mean age 14.7 y). The breastfeeding duration was obtained from parental records. We measured weight, height, waist circumference and 6 skinfold thicknesses.

Results: No significant associations between the Pro12Ala polymorphism and any of the above-mentioned anthropometric parameters were found. There were significant interactions between the *PPARG2* Pro12Ala polymorphism and breastfeeding with regard to adiposity measurements (all adjusted $p < 0.05$). Indeed, in children who had not been breastfed, Ala12 allele carriers had higher adiposity parameters (e.g. delta BMI: +1.88 kg/m², adjusted -for age, gender and center- $p = 0.007$) than Pro12Pro adolescents. In contrast, in breastfed subjects, there was no significant difference between Ala12 allele carriers and Pro12Pro children in terms of adiposity measurements, whatever the duration of breastfeeding.

Conclusion: Breastfeeding appears to counter the deleterious effect of the *PPARG2* Pro12Ala polymorphism on anthropometric parameters in adolescents.

The peroxisome proliferator-activated receptor gamma (PPAR γ) transcription factor is primarily expressed in adipocytes. It is a member of the nuclear hormone receptor family which influences whole body energy homeostasis via 3 main metabolic pathways: adipocyte differentiation, insulin sensitivity and lipoprotein metabolism. The *PPARG* gene (located on chromosome 3) gives rise to two different proteins: PPAR γ 1 and PPAR γ 2. The PPAR γ 2 protein is the more abundant isoform in adipose tissue, whereas PPAR γ 1 is ubiquitous. Of the several identified variants in the *PPARG* gene, one of the most common (minor allele frequency of ~10 % in Caucasians) is the Pro12Ala (rs1801282) substitution at codon 12 in *PPARG2*. This polymorphism has been shown to be associated with reduced ability to transactivate responsive promoters and thus with lower PPAR γ 2 transcriptional activity (1).

In adults, the Pro12Ala polymorphism has been associated with higher BMI, waist circumference and obesity risk (2-4). Even though a recent meta-analysis of genome-wide association studies for BMI failed to find any association between the Pro12Ala polymorphism and childhood or adult obesity (5), another meta-analysis showed that in selected subgroups, such as Caucasians and obese subjects, the Ala12 allele was associated with greater BMI and greater insulin sensitivity (6), suggesting that if this variant does influence obesity predisposition, it may do so through context-dependent mechanisms. This illustrates the importance for appropriate stratification of analyses by environmental or other

genetic factors when studying *PPARG* variants. More consistently, the Pro12Ala polymorphism has been associated with a lower risk of type 2 diabetes in a meta-analysis of genome-wide association studies (7).

Data in children are scarcer. In 311 Finnish children aged 7, the Ala12 allele was associated with a higher ponderal index at birth and higher waist circumference in adulthood, relative to Pro12Pro subjects (8). In Greek girls aged 3 to 4, adiposity was higher in Ala12 allele carriers than in Pro12Pro carriers (9).

Eriksson and colleagues showed that the well-known association existing between low birth weight and insulin resistance later in life was seen only in Pro12Pro individuals (10). Moreover, Meirhaeghe *et al.* showed that individuals carrying the Ala12 allele had lower birth weight (due to shorter gestational duration and a higher risk of preterm birth) than Pro12Pro subjects (11). However, this result was not confirmed in 5652 individuals from the Northern Finland Birth Cohort of 1966 (12). Labayen *et al.* showed that low birth weight may program a lower fat-free mass in adolescents carrying the Ala12 allele (13).

Lastly, certain environmental factors (such as dietary fat and physical activity) interact with the *PPARG* polymorphism's effect on adiposity. Mook-Kanamori *et al.* showed that the growth rate from birth to 18 months of age was higher in Ala12Ala carriers than in Pro12Pro carriers when the duration of breastfeeding was between 0 and 4 months, whereas the Pro12Ala polymorphism was not associated with early growth rate in infants breastfed for longer than 4 months (14).

The aims of the present study were to (i) assess the influence of the *PPARG2* Pro12Ala polymorphism on BMI, waist circumference and the sum of 6 skinfold thicknesses in a sample of 945 European adolescents and (ii) test the modulating effect of breastfeeding on these associations.

RESEARCH DESIGN AND METHODS

Subjects: The current report is based on data derived from the Healthy Lifestyle in Europe by Nutrition in Adolescence cross-sectional study (HELENA-CSS), which aimed at obtaining a broad range of standardized, reliable and comparable nutrition- and health-related data from a random sample of European adolescents aged 12.5 to 17.5 y. Data collection took place during 2006 and 2007 in 10 European cities. A detailed description of the HELENA-CSS sampling has been published elsewhere (15).

All the adolescents meeting the general HELENA inclusion criteria and having data for age, gender and BMI were considered in the final sample (n=3546). In order to investigate biochemical assays and genetic analyses, one third of the cohort was randomly selected for blood collection (resulting in a total of 1155 subjects). Of the latter, the 945 adolescents with data on the *PPARG2* Pro12Ala polymorphism, BMI and breastfeeding information were included in the present study.

After receiving comprehensive information on the study's aims and methods, all adolescents and their parents or guardians signed informed consent forms. The study was performed according to the ethical guidelines of the Edinburgh revision of the 1961 Declaration of Helsinki (2000), good clinical practice and the legislation on

clinical research in each of the participating countries. The protocol was approved by the investigational review boards at the participating university medical centers.

The harmonized, standardized anthropometric measurements were strictly monitored. Participants were barefoot and in underwear and anthropometric measurements were taken by trained researchers. Weight was measured with an electronic set of scales (Type SECA 861; precision 0.05 kg) and height was measured in the Frankfurt plane with a height gauge (Type SECA 225; precision 1 mm). Waist circumference was measured with a non-elastic tape (Seca 200; precision 1 mm) to the nearest 0.1 cm. Skinfold thicknesses were measured at the left biceps, triceps, subscapular area, supra-iliac area, thigh and calf with a Holtain caliper (precision 0.2 mm), according to Lohman's anthropometric standardization reference manual. The overall score was calculated by summing the six skinfold thicknesses. Mean skinfolds and circumferences were calculated from three consecutive measurements.

Identification of sexual maturation (Tanner & Whitehouse stages I-V) was assessed by a physician.

Weight and height at birth and the durations of gestation and breastfeeding were collected via a parental questionnaire. The duration of gestation was stratified into 3 categories: less than 35, between 35 and 40 and over 40 weeks. The total duration of breastfeeding was recoded from 6 categories into 4: never, < 3, 3-5 and ≥ 6 months. The duration of exclusive breastfeeding (defined by the World Health Organization (WHO) as no liquid or solid nutrition other than breast milk) was recoded in a similar manner.

An uni-axial accelerometer (ActiGraph™ GT1M, Pensacola, FL, USA, www.theactigraph.com) was used to assess physical activity. Adolescents were instructed to place the monitor underneath the clothing, at the lower back, using an elastic waist band and wear it for seven consecutive days. They were also instructed to wear the accelerometer during all time awake and only to remove it during water based activities. At least three days of recording with a minimum of 8 hours registration per day, was set as an inclusion criterion. In this study, the time sampling interval (epoch) was set at 15 seconds. A measure of total volume of activity (hereafter called average physical activity) was expressed as the sum of recorded counts per epoch divided by total daily registered time expressed in minutes (cpm) (16).

The socioeconomic level was assessed in terms of the maternal educational level and was coded into 4 categories (elementary, lower secondary, higher secondary or higher education).

Preparation of genomic DNA from whole blood and genotyping: Blood samples were drawn at school after a 10-hour, overnight fast and according to a standardized collection protocol; blood for DNA extraction was collected in EDTA K3 tubes. DNA was extracted from white blood cells with the Puregene kit (QIAGEN, Courtaboeuf, France) and stored at -20°C. Genotyping of the Pro12Ala polymorphism was performed on an Illumina system using GoldenGate technology (Illumina Inc., San Diego, CA, USA). The genotyping success rate was 99.4%.

Statistical analyses: Statistical analyses were performed with SAS software (SAS Institute Inc., Cary, NC, USA). The deviation from Hardy-

Weinberg equilibrium was tested using the χ^2 test (1 d.f.). The BMI and the sum of the six skinfolds were normalized by log-transformation. We compared groups in terms of genotype and allele distributions by using χ^2 tests. Inter-group comparisons of quantitative variables were performed using a general linear model (GLM). Reported p values were systematically adjusted for confounding variables. Data on anthropometric phenotypes were adjusted for age, gender and center. Data on weight and height at birth were adjusted for age, gender, center and gestational duration. Study center was used as a surrogate estimate of ethnicity. The presence of interaction between polymorphism and breastfeeding for anthropometric variables was tested with a GLM adjusted for age, gender and center. The threshold for statistical significance was set to $p \leq 0.05$. Power calculations were performed using Quanto v1.2.4 (17).

RESULTS

Within our sample, 81.7% of adolescents had been breastfed (Table 1). A quarter of the adolescents had finished puberty (28.3%). There were 746 (78.9%) Pro12Pro, 187 (19.8%) Pro12Ala and 12 (1.3%) Ala12Ala subjects (Ala12 allele frequency= 0.11) in the sample. This distribution respected the Hardy-Weinberg equilibrium in the HELENA-CSS ($p=0.94$) and in each center separately (data not shown).

Table 2 presents the association between the *PPARG2* Pro12Ala polymorphism and the neonatal characteristics and adiposity measurements. No significant associations were found between the Pro12Ala polymorphism and BMI, waist circumference and the sum of skinfolds.

Accordingly, underweight (n=61), normal weight (n=663), overweight (n=164) and obese children (n=57) did not differ significantly in terms of the genotype distribution of the *PPARG2* Pro12Ala polymorphism ($p=0.82$) (data not shown). However, Ala12Ala subjects had lower weight ($p=0.03$) and height ($p=0.02$) at birth than subjects carrying the Pro12 allele (Table 2), independently of the duration of gestation. The genotype distribution of the polymorphism did not differ between subjects born before 35 weeks, between 35 and 40 weeks or after 40 weeks of pregnancy ($p=0.98$).

After checking that the distribution of the Pro12Ala polymorphism was similar in all four breastfeeding categories (never breastfed, < 3 months, 3-5 months and ≥ 6 months) ($p=0.73$), breastfeeding was introduced into the analysis. We detected significant interactions between the Pro12Ala polymorphism and breastfeeding when considering BMI (adjusted -for age, gender and center- $p=0.004$), waist circumference (adjusted $p=0.03$) or skinfolds (adjusted $p=0.03$). Indeed, in children who had not been breastfed (n=173), Ala12 allele carriers had higher BMI (+1.88 kg/m², adjusted $p=0.007$, Figure 1A), higher waist circumference (+3.8 cm, adjusted $p=0.02$, Figure 1B) and higher skinfold thicknesses (+16.3 mm, adjusted $p=0.03$, Figure 1C) than Pro12Pro subjects. This association was not altered by further adjustment for maternal educational level, Tanner & Whitehouse stage, average physical activity level, birth weight or duration of gestation (data not shown). In contrast, in children who had been breastfed, there was no significant difference in adiposity measurements between Ala12 allele carriers and Pro12Pro subjects, whatever the duration of breastfeeding. It is noteworthy that our

analyses yielded similar results when using the duration of exclusive breastfeeding (data not shown). Furthermore, there was no significant interactions with gender ($p>0.90$) and the associations were similar in boys and girls (data not shown).

We performed power calculations in the whole sample (n=945) using a dominant or a recessive model, and in the non-breastfed children sub-sample (n=173) using a dominant model only (Table 3). As an example, the whole sample had sufficient power (>80%) to identify significant effect sizes of at least 0.75 kg/m² for BMI, 2.1 cm for waist circumference, 9.6 mm for skinfold thicknesses, 140g for birth weight and 0.7 cm for birth height using a minor allele frequency of 0.11 under a dominant model.

DISCUSSION

In the present study, the *PPARG2* Ala12 allele was associated with higher adiposity indices (BMI, waist circumference and the sum of skinfolds) in children who had not been breastfed. However, this association was not seen in children who had been breastfed (even for a short period).

Our results are in agreement with Mook-Kanamori *et al.*, who showed that the Ala12 allele was associated with increased weight gain in early infancy in non-breastfed children (14). We observed similar findings for BMI, waist circumference and skinfolds, even later in life (i.e. adolescence). This result supports the hypothesis whereby breastfeeding has a beneficial effect on the obesity risk later in life in a genetically-predisposed group.

Our study illustrates an association between an environmental factor (breastfeeding) and the phenotypic

expression of a gene (*PPARG*'s modulation of anthropometric parameters) and thus suggests that phenotypes modulated by *PPARG2* polymorphisms can be influenced by gene-environment interactions early in life. Barker and others have explained the impact of pre- and postnatal nutrition later in life by the theory of "nutritional programming": what is beneficial *in utero* and during the postnatal period in cases of undernutrition could become deleterious in the event of an excessive nutritional environment (i.e. metabolic diseases) (18). The exact mechanisms involved in this type of phenomenon are still subject to speculation; they may begin to operate during fetal life and continue until the early neonatal period. A recent meta-analysis performed by the WHO including 33 studies concluded that breastfed individuals were less likely to be overweight and/or obese in childhood and adolescence (19). Some studies but not all showed a dose-response effect, with a more pronounced effect associated with a long duration of breastfeeding (20). The reason for the absence of dose-response effect on the *PPARG2* Ala12 allele in our study is unclear, one possible explanation could be that the programming effect of breastfeeding is more strongly influenced by gene*nutrient interactions at an early age rather than a quantitative process linked to the duration of the exposition.

A number of mechanisms can potentially explain how breastfeeding could counterbalance the deleterious effect of the Ala12 allele in adolescents. It has been shown that the association between dietary fat and BMI is influenced by *PPARG2* genotypes. Memisoglu *et al.* found that monounsaturated fat-rich diets were inversely associated with BMI in Ala12 allele carriers but the authors did

not find any association in Pro12Pro women (21). Similarly, Luan *et al.* showed that for a diet with a low polyunsaturated-to-saturated fat ratio, Ala12 allele carriers had a greater BMI than Pro12Pro carriers (22). Considering that breast milk constitutes a diet with specific fat intake (with a higher proportion of polyunsaturated fatty acids than formula milk (23)), our results appear to be in line with those reported by Luan *et al.*, albeit their study was conducted in adults. Moreover, one potential hypothesis is that breast milk or breastfeeding supply factors such as prostaglandin J2 (24), a natural PPAR γ ligand. The decrease in PPAR γ 2 transcriptional activity observed in Ala12 allele carriers could be therefore compensated for by breast milk. The latter also contains a number of adipokines. It is known that PPAR γ agonists (such as the thiazolidinediones) can downregulate leptin expression (25); however, the presence of this compound in breast and/or formula milk has yet to be established and would require further investigation.

We also showed in the present study that Ala12Ala subjects had a lower body weight (-430 g) and height (-2.7 cm) at birth than subjects carrying the Pro12 allele, independently of the duration of gestation. Although these results need to be considered with caution (as they concern only 12 homozygote children) and replicated, they are in line with previous data. Indeed, in 2 Irish population samples, we have previously shown that the *PPARG2* Ala12 allele was associated with lower birth weight (primarily caused by shorter gestational duration) (11).

The present study has certain limitations. First, the duration of gestation was coded into 3 categories rather than

being specified in weeks and was obtained from questionnaires filled out by the parents (rather than from a national health registry). Therefore, the accuracy of the data on gestational duration needs to be considered with circumspection. Second, the "being small for gestational age" phenotype could not be assessed. However, since the duration of gestation did not influence the effect of the *PPARG2* polymorphism in the present study, we believe that this factor did not bias our results. Likewise, we lacked information on singleton or multiple pregnancies, which have different growth patterns. Other factors (such as parental weight status, food preferences or smoking status) known to influence the effect of breastfeeding on the subject's subsequent BMI could not be assessed in our study. However, the main factors known to influence fat mass were available and did not alter the observed associations when used as confounders. Third, study center was used as a surrogate estimate of ethnicity which is not ideal and might induce misclassification. Last, the subgroup of non-breastfed children was relatively small (n=173), which might prone to identify false positive associations. However, we feel quite confident of our data as they are in line with the data of Mook-Kanamori *et al.* (14).

In conclusion, our results suggest that breastfeeding can counterbalance the deleterious impact of the *PPARG2* Pro12Ala polymorphism on adiposity in

adolescents. These findings confirm the importance of taking account of gene-environment interactions in association studies and the possible effect of early, diet-based prevention programs in population subgroups. At a time when the prevalence of obesity in children and adolescents continues to increase, our results may constitute a new argument in favor of the public health benefits of breastfeeding.

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DISCLOSURE

All authors state there are no actual or potential conflicts of interest to disclose.

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Table 1. Descriptive characteristics of the HELENA-CSS study sample

Neonatal data	n	Mean	SD
Birth weight (kg)	914	3.33	0.58
Birth height (cm)	882	50.4	3.2
Duration of total breastfeeding	n	%	
Never breastfed	173	18.3	
< 3 months	279	29.5	
3-5 months	237	25.1	
≥6 months	256	27.1	
Duration of pregnancy			
<35 weeks	49	5.4	
35-40 weeks	574	63.6	
>40 weeks	280	31.0	
Clinical Characteristics	n	%	
Boys	434	45.9	
Girls	511	54.1	
Pubertal status			
Tanner stage 2	12	1.4	
Tanner stage 3/4	601	70.3	
Tanner stage 5	242	28.3	
	n	Mean	SD
Age (years)	945	14.7	1.4
BMI (kg/m ²)	945	21.3	3.8
Waist circumference (cm)	935	72.2	9.3
Sum of 6 skinfolds (mm)	887	92.0	41.4
Physical activity (counts per min.)	638	434	151

Table 2. Association between the *PPARG2* Pro12Ala polymorphism and body composition and neonatal characteristics in the HELENA-CSS

Genotype (n)	Pro12Pro (746)		Pro12Ala (187)		Ala12Ala (12)		p^a	p^a X/Ala12 vs Pro12Pro	p^a Ala12Ala vs X/Pro12
	Mean	SD	Mean	SD	Mean	SD			
BMI (kg/m ²)	21.3	3.6	21.4	4.3	20.2	2.5	0.55	0.98	0.29
Waist circumference (cm)	72.1	9.2	72.8	10.0	69.8	7.5	0.50	0.78	0.29
Sum of 6 skinfolds (mm)	92.2	41.1	92.5	43.5	73.2	24.0	0.52	0.79	0.31
Birth weight (kg)	3.33	0.57	3.34	0.57	2.90	1.08	0.10 ^b	0.43 ^b	0.03^b
(n)	(689)		(177)		(10)				
Birth height (cm)	50.4	3.1	50.4	2.7	47.7	6.1	0.07 ^b	0.43 ^b	0.02^b
(n)	(666)		(171)		(10)				
Duration of gestation	n (frequency)		n (frequency)		n (frequency)		p		
<35 weeks	38 (0.78)		10 (0.20)		1 (0.02)		0.98		
35-40 weeks	454 (0.79)		113 (0.20)		7 (0.01)				
>40 weeks	219 (0.78)		58 (0.21)		3 (0.01)				

^a adjusted for age, gender and center. ^b adjusted for age, gender, center and gestational duration.

Table 3. Power calculation for the *PPARG2* Pro12Ala polymorphism effects

	Mean delta using a dominant model	Mean delta using a recessive model
In the HELENA-CSS (n=945)		
BMI, kg/m ²	0.75	3.15
Waist circumference, cm	2.1	7.7
Sum of 6 skinfolds (mm)	9.6	36
Birth weight (kg)	0.14	0.50
Birth height (cm)	0.7	2.8
In non-breastfed children (n=173)		
BMI, kg/m ²	2.1	NC
Waist circumference, cm	5.0	NC
Sum of 6 skinfolds (mm)	23.1	NC

NC: not calculated.

Figure legend

Figure 1A. Mean BMI as a function of the breastfeeding duration in *PPARG2* Pro12Pro (black bars) versus Ala12 allele carriers (white bars).

** p=0.007 (adjusted for age, gender and center).

Figure 1B. Mean waist circumference as a function of breastfeeding duration in *PPARG2* Pro12Pro (black bars) versus Ala12 allele carriers (white bars).

* p=0.02 (adjusted for age, gender and center).

Figure 1C. Mean sum of skinfolds as a function of breastfeeding duration in *PPARG2* Pro12Pro (black bars) versus Ala12 allele carriers (white bars).

p=0.03 (adjusted for age, gender and center).

Figure 1

