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Polymorphisms in the *NPY* and *AGRP* genes and body fatness in Dutch adults

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Abstract

Objective:

To investigate the association between DNA polymorphisms in the *NPY* and *AGRP* genes and body fatness.

Design and methods:

The association between the *AGRP* Ala67Thr or the *NPY* Leu7Pro polymorphisms and indicators of body fatness (baseline leptin levels, body mass index (BMI) values and prevalence of overweight) are investigated in 582 participants of two large cohorts in The Netherlands (total 18 500 adult men and women), aged 20–40 years whose weight remained relatively constant or whose weight increased substantially (range 5.5–47 kg) during a mean follow-up of 7 years.

Results:

No consistent associations were found for the indicators of body fatness for men and women. Among women, BMI values, leptin levels and prevalence of overweight were not statistically different for carriers of the mutant alleles compared to that of the non-carriers. Among men, carriers of the Thr67-allele of the *AGRP* gene had similar leptin levels, but higher BMI values compared to those with the genotyping Ala67/Ala67: mean adjusted BMI 25.6 kg/m^2 (95% CI 24.3–27.0) vs 23.9 kg/m^2 (23.6–24.3). Also, the risk of being overweight at baseline tended to be higher for male carriers of the Thr67-allele of the *AGRP* gene (OR 2.52; 95% CI 0.86–7.4). Furthermore, male carriers of the Pro7-allele of the *NPY* gene had on average higher leptin levels and BMI values vs non-carriers of this allele: 4.7 μg/l (95% CI 3.7–6.0) and 25.7 kg/m^2 (95% CI 24.4–27.0) vs 3.1 μg/l (95% CI 2.9–3.4) and 23.9 kg/m^2 (95% CI 23.5–24.3), respectively. These male carriers had also a higher risk on being overweight at baseline (OR 3.3 (95% CI 1.2–8.9)) compared to non-carriers of the Pro7-allele.

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Conclusion:

The consistent findings among men suggest that the *NPY* Leu7Pro polymorphism (or another linked marker) might be involved in the development of obesity at younger ages. The findings for the *AGRP* Ala67Thr were less consistent and need further investigation. Among women, these polymorphisms do not play an important role.

Introduction

Obesity is increasingly common especially but not exclusively in affluent societies and is a risk factor for a number of chronic diseases.[1] Several behavioural and genetic factors are involved in the development of obesity.

Leptin is a hormone that is mainly produced by adipose tissue and binds to leptin receptors which have a high density in the hypothalamus.[2] An increase in body fat leads to an increased plasma level of this hormone. In response to this adiposity signal of leptin, the NPY/AGRP neurones in the area of the hypothalamus are inhibited, resulting in reduced food intake. Or *vice versa*: in case of depletion of body fat stores and/or reduced leptin/insulin signalling, gene expression and secretion of the AGRP or NPY peptides in the hypothalamus are increased, resulting in an increased energy intake.[2] However, the responsivity to leptin between individuals is variable and obese individuals in general appear to be leptin resistant.[2] A failure in one of the neuronal systems downstream of the leptin signal, for instance due to variations in the *NPY* or *AGRP* genes, may be one of the explanations for this.

Polymorphisms in these genes that have been described in the literature are the *AGRP* Ala67Thr and the *NPY* Leu7Pro polymorphisms, but published association studies on these polymorphisms are limited in number. The Thr67 variant was associated with a higher prevalence of anorexia nervosa, a lower body weight, body mass index (BMI), fat mass, and abdominal visceral fat mass,[3, 4, 5] and Kallio *et al.*[6] have reported that carriers of the Pro7-allele of the *NPY* gene had higher NPY levels. Although also in some studies no association was found,[7, 8, 9, 10] we expected higher body fatness in carriers of the Ala67-allele of the *AGRP* gene or the Pro7-allele of the *NPY* gene.

In a large general Dutch population, we have selected subjects, aged 20–40 year, whose weight remained relatively constant and subjects who gained weight over time. In a previous analysis, we found that these genetic factors were not associated with weight gain during a follow-up of about 7 years.[10] However, it is possible that the effects are more pronounced at an earlier age, or for other obesity-related phenotypes. We investigated whether genetic variation in the *NPY* or *AGRP* genes might contribute to body fatness, measured with baseline leptin levels, baseline BMI values and prevalence of overweight.

Materials and methods

Study population

Subjects were selected from two large cohorts in the Netherlands that have been carried out between 1987 and 1998 (total 17 500 adult men and women) in two Dutch towns, Maastricht and Doetinchem.[11] The selection of the subjects has been described previously. In short, we excluded all participants who reported to be on a energy- or fat-restricted diet, those using more than five glasses of

alcoholic beverages per day, those who suffered from chronic diseases, those who had changed recently their smoking habits, those who were pregnant, and those with a follow-up of less than 4 years, those who suffered from serious illnesses. From the remaining group, we selected for another research question 'weight gainers' aged 20–40 years by taking the top decile of the distribution of average weight gain per year (range 5.5–47 kg during a mean follow-up of 6.8 years) and an equal sized group of subjects (='non-weight gainers') whose weight remained relatively constant (range: -0.3 to +0.3 kg/year). The non-weight gainers were frequency matched for cohort, sex, age and smoking status with the weight gainers in a way that these two groups had not only the same marginal distributions but also the same joint distributions. Finally, genomic DNA was successfully extracted from frozen blood samples that were stored at -20°C for 4 -12 years by digestion with proteinase K, followed by salting out with potassium acetate and chloroform/isoamyl alcohol extraction. This was successfully carried out for 296 weight gainers and 286 non-weight gainers. All subjects were treated according to the Helsinki declaration and had signed an informed consent to allow the use of stored blood samples for further scientific research.

Measurements

The examinations at baseline and the second measurement included physical examinations, for example, anthropometric measurements, a self-administered questionnaire and blood sampling. However, for those in Maastricht, the second measurement included only a self-administered questionnaire.

Characteristics of the study population

The questionnaires at baseline and/or follow-up provided information about history of chronic diseases, alcohol consumption, cigarette smoking, pregnancy, and educational level.

Anthropometric measures

Weight at baseline was measured without shoes and wearing light indoor clothing at the Municipal Health Centre. For participants from Maastricht, the second measurement was a self-reported weight. It is likely that these subjects wore less clothing when measured at home compared with the measurement at the health center. To allow for the weight of clothing, we added 1.5 kg to the self-reported weight. This amount of 1.5 kg was based on some measurements performed by the investigators of the Municipal Health Center. Weight gain was defined as the difference between the weight at baseline and the weight at the second examination. As the period of follow-up varied (6 years in Doetinchem and a range from 4.0 years to 11.3 years in Maastricht), we calculated the average weight gain per year.

Measures of body fatness

Body mass index and leptin levels were assumed as two indicators of body fatness. Body mass index was calculated as baseline weight divided by squared height (in kg/m^2), and a BMI above 25 kg/m^2 was indicated as 'being overweight'. Leptin concentrations were measured in the baseline plasma samples, which had been stored at -20°C for 6–14 years. The blood samples were not taken for all (non-fasting) subjects at the same time of the day. These measurements were

carried out in duplicate by radio-immunoassay (HL-81K kit by Linco Research Inc., St Charles, USA). Leptin levels were missing for 22 subjects.

Genotyping

Genotyping was performed using PCR-restriction fragment length polymorphism (RFLP) analyses. Two investigators independently evaluated the gels. We assayed a G to A nucleotide transition resulting in an Ala to Thr substitution at codon 67 (=Ala67Thr) of the *AGRP* gene (rs28937570)[3] and we determined a T to C polymorphism which results in a substitution of leucine to proline substitution at codon 7 in the *NPY* gene (rs16139).[7] The percentage of efficiency was 100%. All genotyping methods are described in more detail elsewhere.[3, 7]

Data analyses

Characteristics of the study population were assessed with Student's *t*-tests. Since leptin concentrations were not normally distributed, this variable was natural logarithmically transformed. Leptin levels or BMI values for each genotype were analyzed with *t*-tests and regression analyses. The (adjusted) logarithmically transformed leptin values were transformed back to normal values (geometric means). In addition, we performed χ^2 analyses and logistic regression analyses in order to investigate whether overweight (BMI>25 kg/m^2) was more prevalent among carriers of the mutant alleles compared to the non-carriers of these alleles. As our study population was selected on weight gain (high weight gain vs stable weight), we stratified our analyses on the basis of this. In the (logistic) regression analyses, adjustments were done for all matching factors. As the unadjusted and the adjusted associations between weight gain and genetic factors were mostly similar, we presented the unadjusted percentages and adjusted odds ratios. Leptin levels were also additionally adjusted for BMI values. The distributions of the non-weight gainers were tested for Hardy–Weinberg equilibrium by χ^2 analyses.[12] Analyses were performed separately in men and women using the statistical package SAS (version 8.2) and a *P*-value of 0.05 was reported as statistically significant.

Results

The characteristics of the study population are shown in Table 1. Subjects were on average 29.3 (s.d.$\pm$5.9) years old and had a baseline BMI of 23.6 kg/m^2 (s.d.$\pm$3.2). Subjects with high weight gain increased on average 12.8 kg (range 5.5–47 kg) during a mean follow-up of 6.8 years, while the non-weight gainers gained on average 0.5 kg (range -2.6 to 3.1 kg).

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Table 1 - Characteristics of the study population (means (s.d.)).

	Men			*Women*		
	***High weight gain* (n=*134*)**	***Stable weight* (n=*138*)**	**P-*value of* t-*test***	***High weight gain* (n=*152*)**	***Stable weight* (n=*158*)**	**P-*value of* t-*test***
At baseline						
Age (years)	28.1 (5.9)	28.9 (5.6)	[a]	29.9 (6.0)	29.9 (5.9)	[a]
Weight (kg)	79.4 (10.8)	76.3 (10.0)	0.016	66.8 (11.9)	63.6 (8.0)	0.007
Height (cm)	1.82 (0.07)	1.80 (0.07)	0.020	1.68 (0.07)	1.67 (0.06)	0.27
BMI (kg/m^2)	24.1 (3.0)	23.7 (2.8)	0.26	23.8 (3.9)	22.9 (2.8)	0.03
At end of follow-up						
Follow-up time (years)	6.9 (1.7)	7.2 (1.9)	0.16	6.7 (1.4)	6.8 (1.6)	0.42
Weight (kg)	92.0 (11.5)	76.8 (10.0)	[b]	79.7 (13.3)	64.2 (8.0)	[b]
Weight gain (kg/year)	1.86 (0.53)	0.06 (0.17)	[b]	1.95 (0.62)	0.07 (0.16)	[b]
BMI (kg/m^2)	27.9 (3.3)	23.8 (2.8)	[b]	28.4 (4.6)	23.1 (2.8)	[b]

[a] *P*-values not given, one of the matching variables.

[b] *P*-values not given, criteria for group selection.

Furthermore, weight gainers had a higher baseline body weight compared to non-weight gainers. As male weight gainers were also 2 cm taller compared with the non-weight gainers, weight gainers did not differ in their BMI values from the non-weight gainers.

The allele frequency for the least frequent allele was 3.6, and 3.5%, for the *AGRP* Ala67Thr and *NPY* Leu7Pro polymorphisms, respectively. All genotype frequencies were found to be in Hardy–Weinberg equilibrium.

AGRP Ala67Thr

Tables 2a and 2b show the association between the *AGRP* Ala67Thr polymorphism and BMI, leptin and overweight for the weight gainers and the non-weight gainers separately, as well as the total group. Among women, BMI values, leptin levels and prevalence of overweight were not statistically different for carriers of the mutant alleles compared to that of the non-carriers.

Table 2 - (a) BMI and leptin levels (geometric means) by *AGRP* Ala67Thr polymorphism, stratified for gender and weight gain and (b) being overweight (prevalences and odds ratios) by *AGRP* Ala67Thr polymorphism, stratified for gender and weight gain.

	Men				*Women*			
	Ala/Ala		***Thr/+***	**P-*value*** [b]	***Ala/Ala***		***Thr/+***	**P-*value*** [b]
	n	***Mean (95% CI)***	**n**	***Mean (95% CI)***		**n**	***Mean (95% CI)***	**n**	***Mean (95% CI)***	
(a)										
Weight gainers										
BMI (kg/m^2)	129	24.0 (23.5–24.5)	5	24.9 (22.3–27.5)		140	23.9 (23.2–24.5)	12	22.9 (20.7–25.1)	
BMI adj. for matching factors[a] (kg/m^2)	129	24.2 (23.7–24.8)	5	24.7 (22.1–27.2)		140	23.6 (22.9–24.3)	12	22.5 (20.3–24.6)	
Leptin (μg/l)	122	3.40 (3.08–3.75)	5	3.63 (2.23–5.93)		130	10.63 (9.45–11.96)	10	10.11 (6.61–15.45)	
Leptin adj. for matching factors[a] (μg/l)	122	3.39 (3.04–3.78)	5	3.49 (2.11–5.75)		130	9.87 (8.69–11.20)	10	9.48 (6.20–14.50)	

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	Men				Women			
	Ala/Ala		*Thr/+*	P-*value* [b]	*Ala/Ala*		*Thr/+*	P-*value* [b]
	n	*Mean (95% CI)*	n	*Mean (95% CI)*	n	*Mean (95% CI)*	n	*Mean (95% CI)*
Stable weight								
BMI (kg/m^2)	127	23.5 (23.0–24.0)	11	25.4 (23.8–27.1)	0.028	144	22.9 (22.5–23.4)	14	22.6 (21.1–24.0)
BMI adj. for matching factors[a] (kg/m^2)	127	23.6 (23.1–24.1)	11	25.8 (24.1–27.4)	0.015	144	22.9 (22.4–23.3)	14	22.5 (21.0–23.9)
Leptin (μg/l)	123	2.95 (2.69–3.23)	11	3.57 (2.64–4.84)		133	9.10 (8.29–9.99)	14	10.04 (7.54–13.39)
Leptin adj. for matching factors[a] (μg/l)	123	3.01 (2.73–3.32)	11	3.77 (2.77–5.14)		133	8.78 (7.95–9.69)	14	9.81 (7.37–13.04)
Total								
BMI (kg/m^2)	256	23.8 (23.4–24.1)	16	25.3 (23.9–26.7)	0.044	284	23.4 (23.0–23.8)	26	22.7 (21.4–24.0)
BMI adj. for matching factors[a] (kg/m^2)	256	23.9 (23.6–24.3)	16	25.6 (24.3–27.0)	0.019	284	23.2 (22.8–23.6)	26	22.6 (21.3–23.9)
Leptin (μg/l)	245	3.17 (2.96–3.39)	16	3.59 (2.76–4.68)		263	9.83 (9.11–10.59)	26	10.07 (7.86–12.91)
Leptin adj. for matching factors[a] (μg/l)	245	3.19 (2.97–3.43)	16	3.74 (2.87–4.87)		263	9.31 (8.59–10.08)	26	9.84 (7.69–12.59)

(b)

	AGRP *Ala67Thr*		Men P-*value of* χ^2 *test* [b]	*OR (95% CI)*	*OR* [a] *(95% CI)*	AGRP *Ala67Thr*		Women P-*value of* χ^2 *test* [b]	*OR (95% CI)*	*OR* [a] *(95% CI)*
	Ala/Ala (%)	*Thr/+* (%)				*Ala/Ala* (%)	*Thr/+* (%)			
Weight gainers	*n*=129	*n*=5				*n*=140	*n*=12			
BMI <25 kg/m^2	69.0	60.0		1 (ref)	1 (ref)	69.3	66.7		1 (ref)	1 (ref)
BMI ≥ 25 kg/m^2	31.0	40.0		1.48 (0.24–9.23)	1.13 (0.15–8.55)	30.7	33.3		1.13 (0.32–3.95)	1.01 (0.26–3.98)
Stable weight	*n*=127	*n*=11				*n*=144	*n*=14			
BMI <25 kg/m^2	69.3	45.5		1 (ref)	1 (ref)	79.2	78.6		1 (ref)	1 (ref)
BMI ≥ 25 kg/m^2	30.7	54.5		2.71 (0.78–9.40)	3.25 (0.89–11.90)	20.8	21.4		1.04 (0.27–3.95)	1.06 (0.27–4.15)
Total	*n*=256	*n*=16				*n*=284	*n*=26			
BMI <25 kg/m^2	69.1	50.0		1 (ref)	1 (ref)	74.3	73.1		1 (ref)	1 (ref)
BMI ≥ 25 kg/m^2	30.9	50.0		2.24 (0.81–6.18)	2.52 (0.86–7.37)	25.7	26.9		1.07 (0.43–2.64)	1.12 (0.43–2.89)

95% CI, 95% confidence interval; OR, odds ratio.

[a] Adjusted for matching factors: age, town, smoking habits and weight gain (only for total group).

[b] Only *P*-values below 0.10 are shown.

Among men, carriers of the Thr67-allele had similar leptin levels but higher BMI values compared to those with the genotyping Ala67/Ala67: mean adjusted BMI=25.6 kg/m^2 (95% CI 24.3–27.0) vs 23.9 kg/m^2 (95% CI 23.6–24.3). This higher level of BMI was observed in the total male group and both strata of weight change. However, this was statistically significant for those men with a stable weight and the total group. The risk of being overweight at baseline was increased for male carriers of the Thr67-allele of the *AGRP* gene (adjusted OR 2.5; 95% CI 0.86–7.4). However, this was not statistically significant.

NPY Leu7Pro

Associations with the *NPY* Leu7Pro polymorphism are shown in Tables 3a and 3b. Also for this polymorphism, no associations with BMI, leptin or overweight were found among women. Furthermore, male carriers of the Pro7-allele of the *NPY* gene had on average higher leptin levels vs non-carriers of this allele: adjusted mean leptin level was 4.7 μg/l (95% CI 3.7–6.0) and 3.1 μg/l (95% CI 2.9–3.4) for the male carriers and non-carriers, respectively. Body mass index values were also higher among carriers of this allele compared to non-carriers: 25.7 kg/m^2 (95% CI 24.4–27.0) vs 23.9 kg/m^2 (95% CI 23.5–24.3), respectively. Table 3 shows that the prevalence of being overweight was slightly higher among carriers of the Pro7-allele of the *NPY* gene 50–60 vs 30–31%. For the total male group, this was statistically significant: the adjusted odds ratio for being overweight was 3.3 (95% CI 1.2–8.9).

Table 3 - (a) BMI and leptin levels by *NPY* Leu7Pro polymorphism, stratified for gender and weight gain and (b) being overweight (prevalences and odds ratios)by *NPY* Leu7Pro polymorphism, stratified for gender and weight gain.

	Men				***Women***			
	Leu/Leu		***Pro/+***	***P-value*** [b]	***Leu/Leu***		***Pro/+***	***P-value*** [b]
	n	***Mean (CI)***	**n** ***Mean (CI)***		**n**	***Mean (CI)***	**n** ***Mean (CI)***	
Weight gainers								
BMI (kg/m^2)	126	24.0 (23.5–24.5)	8 25.4 (23.3–27.5)		140	23.7 (23.0–24.3)	12 24.8 (22.6–27.0)	
BMI adj. for matching factors[a] (kg/m^2)	126	24.2 (23.6–24.7)	8 25.5 (23.5–27.5)		140	23.4 (22.8–24.1)	12 24.2 (22.0–26.4)	
Leptin (μg/l)	119	3.31 (3.00–3.66)	8 5.17 (3.53–7.56)	0.028	128	10.59 (9.41–11.93)	10 10.55 (7.16–15.54)	
Leptin adj. for matching factors[a] (μg/l)	119	3.30 (2.96–3.67)	8 5.15 (3.51–7.57)	0.029	128	9.89 (8.72–11.20)	10 9.23 (6.24–13.66)	
Stable weight								
BMI (kg/m^2)	128	23.5 (23.1–24)	10 25.4 (23.7–27.1)	0.044	148	22.9 (22.5–23.4)	14 22.5 (20.8–24.2)	
BMI adj. for matching factors[a] (kg/m^2)	128	23.7 (23.2–24.2)	10 25.6 (23.9–27.3)	0.038	148	22.9 (22.4–23.3)	14 22.3 (20.6–24.1)	
Leptin (μg/l)	124	2.92 (2.67–3.20)	10 4.09 (2.98–5.61)	0.047	138	9.19 (8.39–10.08)	14 9.05 (6.32–12.95)	
Leptin adj. for matching factors[a] (μg/l)	124	2.99 (2.71–3.29)	10 4.35 (3.16–5.99)	0.028	138	8.86 (8.04–9.77)	14 9.10 (6.36–13.01)	
Total								
BMI (kg/m^2)	254	23.8 (23.4–24.1)	18 25.4 (24.1–26.7)	0.020	288	23.3 (22.9–23.7)	22 23.7 (22.3–25.2)	
BMI adj. for matching factors[a] (kg/m^2)	254	23.9 (23.5–24.3)	18 25.7 (24.4–27)	0.011	288	23.1 (22.7–23.6)	22 23.4 (22–24.8)	
Leptin (μg/l)	243	3.11 (2.91–3.32)	18 4.54 (3.55–5.80)	0.004	266	9.84 (9.14–10.61)	21 9.88 (7.57–12.88)	

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	Men					Women				
	Leu/Leu		Pro/+		P-value [b]	Leu/Leu		Pro/+		P-value [b]
	n	Mean (CI)	n	Mean (CI)		n	Mean (CI)	n	Mean (CI)	
Leptin adj. for matching factors[a] (μg/l)	243	3.14 (2.92–3.37)	18	4.70 (3.68–6.00)	0.002	266	9.39 (8.68–10.16)	21	8.87 (6.81–11.55)	

(b)

	Men					Women				
	NPY Leu7Pro		P-value of χ^2 test [b]	OR (95% CI)	OR [a] (95% CI)	NPY Leu7Pro	P-value of χ^2 test [b]	OR (95% CI)	OR [a] (95% CI)	
	Leu/Leu (%)	Pro/+ (%)				Leu/Leu (%)	Pro/+ (%)			
Weight gainers	*n*=126	*n*=8				*n*=140	*n*=12			
BMI <25 kg/m^2	69.8	50.0		1 (ref)	1 (ref)	70.7	50.0		1 (ref)	1 (ref)
BMI ≥25 kg/m^2	30.2	50.0		2.32 (0.55–9.75)	2.43 (0.53–11.2)	29.3	50.0		2.41 (0.74–7.93)	2.56 (0.71–9.27)
Stable weight	*n*=128	*n*=10				*n*=148	*n*=10			
BMI <25 kg/m^2	69.5	40.0		1 (ref)	1 (ref)	79.1	80.0		1 (ref)	1 (ref)
BMI ≥25 kg/m^2	30.5	60.0	0.06	3.42 (0.91–12.81)	3.83 (0.99–14.90)	21.0	20.0		0.94 (0.19–4.67)	0.95 (0.19–4.81)
Total	*n*=254	*n*=17				*n*=288	*n*=22			
BMI <25 kg/m^2	69.7	44.4		1 (ref)	1 (ref)	75.0	63.6		1 (ref)	1 (ref)
BMI ≥25 kg/m^2	30.3	55.6	0.027	2.87 (1.09–7.56)	3.27 (1.20–8.90)	25.0	36.4		1.72 (0.69–4.26)	1.59 (0.62–4.07)

95% CI, 95% confidence interval; OR, odds ratio

[a] Adjusted for matching factors: age, town, smoking habits and weight gain (only for total group).

[b] Only *P*-values below 0.10 are shown.

Discussion

The results of our study suggest that in men aged 20–40 years, the mutant alleles Thr67-*AGRP* and Pro7-*NPY* were associated with (some) indicators of body fatness. The associations between *NPY*Leu7Pro and all indicators of body fatness were consistently in the same direction: the Pro7-allele was associated with higher body fatness. Among women, no association with body fatness were found.

A number of issues need to be addressed before the results can be interpreted. First, our study population was selected from participants of two cohorts with a participation rate of 50%.[13] However, we assumed that there was no selective participation by genotype, as the frequencies of the investigated polymorphisms were similar to those reported in other Caucasian populations.[3, 4, 5, 7, 8, 9] Furthermore, the selection was based on the largest contrast between subjects with weight gain and those with stable weight. The association between

polymorphisms and body fatness might be affected by this selection. Nevertheless, we assumed that we solved this last problem by stratification for weight gain.

Secondly, leptin levels were measured in blood samples that were not taken for all (non-fasting) subjects at the same time of the day or for women in the same phase of the menstrual cycle. All these factors may have affected the leptin levels.[14, 15, 16] Also, BMI is not a perfect measure for body fatness, but a study in Dutch adults showed a high correlation between BMI and percentage body fat ($r \approx 0.75$).[17] Thus, although both indicators are not perfect measures for body fatness, in our view, it is unlikely that these factors were strongly associated with the studied polymorphisms. Therefore, we expect that if anything, the limitations of these measures have led to an underestimation of the associations. This is confirmed by the fact that we found consistent findings for both indicators.

Finally, other factors, such as the matching factors or other linked genetic markers, could have confounded our results. Fortunately, the bias of the matching factors was of minor importance, as similar results were found with and without adjustment for these factors. To elucidate the evidence for the found associations, we compared our findings with the published literature on this genes and polymorphisms. In other words, could the found associations be explained by biological mechanisms, is it convincing that these findings are attributed to these polymorphisms or to other linked markers or is it plausible that the findings are by chance (e.g. because of the number of tested associations) or lack of statistical power?

AGRP Ala67Thr

Computational analyses have shown that the Ala67Thr polymorphism in the *AGRP* gene could affect the secondary structure of the protein.[4] However, Marks *et al.* did not observe any difference in the stability or cellular distribution of the mutant protein in a heterologous expression system. Thus, the mechanism of this effect requires further investigation. As far as we know, no other functional tests have been reported to show the impact of the *AGRP* Ala67Thr polymorphism on the activity of the AGRP protein. Published association studies on the effect of this polymorphism on obesity-related phenotypes are also limited and were more focussed on leanness in stead of obesity. Vink *et al.*[3] reported an association with anorexia nervosa, suggesting that the Thr67-variant has a potential role in the development of eating disorders Also Marks *et al.*[5] reported that the Thr67-allel was associated with a lower body weight, with the largest effect being observed on body fat mass. Argyropoulos *et al.*[4] found that the Thr67-allele of the *AGRP* gene was associated with a lower BMI, fat mass and abdominal visceral fat in a population with a mean age of 53 years, while no association was observed in the younger offspring population. In a case–control study with obese children, no association was found.[9] In contrast to these findings, we did not find an association in women and observed even higher BMI values for male carriers of the Thr67-allele. The conclusion of Argyropoulos *et al.*[4] about the larger role of this polymorphism at older ages could not be confirmed in our study population: We found a stronger effect at younger ages, as no association with weight gain was observed in a previous analyses, and baseline BMI was higher for those with the Ala67-allele. In our view, it is not yet possible to draw conclusions from these findings about the role of this polymorphism on the development of obesity, as the associations were not statistically significant for all indices of body fatness and our findings were in an opposite direction compared to findings of the other published studies. Therefore, these findings need to be verified in other general populations.

NPY Leu7Pro

It is known that the expression and secretion of the NPY peptide in the hypothalamus are increased during depletion of body fat stores and/or reduced leptin/insulin signalling to the brain, resulting in an increased energy intake. Furthermore, deletion of the *NPY* gene reduces hyperphagia and obesity in *ob/ob* mice. However, mice lacking NPY, and otherwise genetically normal, have intact feeding responses, which suggest that compensatory responses mask the consequences of NPY deficiency.[18] Although the Leu7Pro polymorphism is located in the signal peptide part of pre-pro NPY,[7] Buckland *et al.*[19] found evidence that promoter element polymorphisms, at least in brain-expressed genes, should be afforded a high priority for molecular genetic studies as they can still be functional. Kallio *et al.*[6] have reported that carriers of the Pro7-allele had higher NPY levels. Therefore, we expected higher body fatness in carriers of the Pro7-allele. Our consistent findings in men with higher body fatness, measured with leptin or BMI, for those with a Pro7-allele, are in line with this, although this finding was only observed in men. This finding together with a previous study in the same study population in which we did not find an association with weight gain,[10] suggest that the role of this polymorphism may be more pronounced at younger ages. Two other studies reported no association between between BMI and this polymorphism.[7, 8] Only Mattevi *et al.*[8] reported in one specific subgroup of premenopausal women an effect with BMI. First, these discrepancies in results may have to do with lack of statistical power, especially for a relatively rare polymorphism such as the *NPY* Leu7Pro polymorphism. Therefore, it would be useful to investigate this association in a larger population or if there is more data available in a meta-analyses. Secondly, differences in characteristics of the study population could account for the differences in findings. As mentioned before, a lack of NPY could be masked by compensatory responses. It is likely that these responses are affected by variation in other factors such as gender, other genetic factors, lifestyle, etc. More refined association studies based on larger cohorts, in which more factors are taken into account, would be helpful to elucidate these interactions.

Thus, the consistent findings among men suggest that the *NPY* Leu7Pro polymorphism (or another linked marker) might be involved in the development of obesity at younger ages in the general Dutch population. The findings for the *AGRP* Ala67Thr polymorphism were less consistent and need further investigation. Among women, we found no evidence for an important role of these polymorphisms in the development of obesity.

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