Polymorphisms of genes coding for ghrelin and its receptor in relation to anthropometry, circulating levels of IGF-I and IGFBP-3, and breast cancer risk: a case–control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC)

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Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, has two major functions: the stimulation of the growth hormone production and the stimulation of food intake. Accumulating evidence also suggests a role of ghrelin in cancer development. We conducted a case–control study on 1359 breast cancer cases and 2389 matched controls, nested within the European Prospective Investigation into Cancer and Nutrition, to examine the association of common genetic variants in the genes coding for ghrelin (*GHRL*) and its receptor (*GHSR*) with anthropometric measures, circulating insulin growth factor I (IGF-I) and insulin-like growth factor-binding protein 3 and breast cancer risk. Pair-wise tagging was used to select the 15 polymorphisms that represent the majority of common genetic variants across the *GHRL* and *GHSR* genes. A significant increase in breast cancer risk was observed in carriers of the *GHRL* rs171407-G allele (odds ratio: 1.2; 95% confidence interval: 1.0–1.4; $P = 0.02$). The *GHRL* single-nucleotide polymorphism rs375577 was associated with a 5% increase in IGF-I levels ($P = 0.01$). A number of *GHRL* and *GHSR* polymorphisms were associated with body mass index (BMI) and height (P between <0.01 and 0.04). The false-positive report probability (FPRP) approach suggests that these results are noteworthy (FPRP < 0.20). The results presented here add to a growing body of evidence that *GHRL* variations are associated with BMI. Furthermore, we have observed evidence for association of *GHRL* polymorphisms with circulating IGF-I levels and with breast cancer risk. These associations, however, might also be due to chance findings and further large studies are needed to confirm our results.

Introduction

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHSR), is a 28-amino residue peptide predominantly produced by the stomach (1). In addition to the mature form of ghrelin, several posttranscriptional and posttranslational variants have been reported (2). Two molecular forms (ghrelin and des-acyl ghrelin), resulting from a different posttranscriptional modification of the protein, are observed in human plasma. An alternative processing of the prepropeptide generates also an aminated 23-aminoacid peptide, called obestatin, which binds to a different receptor, and has been shown in experimental animal models to oppose the effect of ghrelin (3). The ghrelin receptor has two known isoforms: one which is functional (GHSR-1a) and one spliced variant (GHSR-1b) with no known function (4). Only the acylated form of ghrelin can bind the GHSR-1a receptor (1).

Two main functions of ghrelin are documented: first, to stimulate growth hormone (GH) production through the activation of GHSR-1a in the hypothalamus (5); and second, to increase appetite and food intake (6,7) by mechanisms that could be independent of GHSR (8). Accumulating evidence also indicates a possible role of ghrelin in the control of cell proliferation, inhibition of apoptosis and cancer development (9–12).

Ghrelin production has been reported in both non-malignant breast tissues (13,14) and in breast carcinomas (15,16). The truncated isoform of the receptor, GHS-R1b, was reported to be highly expressed in breast cancer tissues compared with normal breast, and ghrelin, at

Abbreviations: BMI, body mass index; EPIC, European Prospective Investigation into Cancer and Nutrition; FPRP, false-positive report probability; GH, growth hormone; GHSR, growth hormone secretagogue receptor; IGFBP-3, insulin-like growth factor-binding protein 3; IGF-I, insulin growth factor I; SNP, single-nucleotide polymorphism.

physiological levels, has been shown to increase breast cancer cell proliferation (*in vitro*) (16). So far, only one epidemiological study examined the risk of breast cancer associated with polymorphisms of the genes coding for ghrelin (*GHRL*) or its receptor (*GHSR*) (17).

The role of the GH axis in breast cancer has been well documented (11,18). GH is the main endocrine stimulus of hepatic and tissue production of insulin growth factor I (IGF-I) and insulin-like growth factor-binding protein 3 (IGFBP-3). A number of epidemiological studies have shown an increased risk of breast cancer with elevated IGF-I levels (19,20). However, several studies observed a negative correlation between ghrelin and IGF-I levels in children and adolescents (21–25) or in adult subjects (26), indicating a possible inhibitory effect of ghrelin on the GH–IGF-I axis. Therefore, genetic variants in *GHRL* or *GHSR* that could alter GH-releasing activity might ultimately affect IGF-I levels in tissues and in the circulation. Only few studies, however, have examined the association between polymorphisms of these genes and IGF-I-circulating levels (27–29).

Weight gain and obesity are well-known risk factors for postmenopausal breast cancer (30). In addition to its GH-releasing activity, ghrelin is also known to induce a positive energy balance by stimulating appetite. Stimulation of food intake by ghrelin has been observed in both animals (7,31,32) and humans (6) and ghrelin administration has been shown to induce weight gain in rodents (33,34). The impact of ghrelin on obesity is less clear (9). Paradoxically, plasma ghrelin levels are lower in obese patients than in lean or anorexic individuals (21,35–38). On the other hand, a recent study showed an elevated expression of ghrelin in the hypothalamus of obese patients, indicating a more important role of central rather than peripheral ghrelin in obesity (39). The *GHSR* and *GHRL* genes are both located on chromosome 3, in regions which have been linked to obesity by several genome-wide linkage scans (40,41). A number of studies attempted to identify polymorphisms in the *GHRL* gene as determinants of obesity (27,29,42–46). One particular variant, Leu72-Met (rs696217), was associated with early onset of obesity. Three studies investigated the relation between genetic polymorphisms in *GHSR* and obesity (28,47,48) with contradictory results.

We conducted a case–control study of 1359 breast cancer cases and 2389 controls, nested within the European Prospective Investigation into Cancer and Nutrition (EPIC), to examine the association of 15 common genetic variants in the *GHSR* and *GHRL* genes with anthropometric measures, circulating IGF-I and IGFBP-3 concentrations and breast cancer risk.

Material and methods

The EPIC study

The EPIC cohort consists of ~366 500 women and 153 500 men, aged 35–69 years, recruited between 1992 and 1998 in 23 research centers in 10 Western European countries. The vast majority (>97%) of subjects recruited in the EPIC cohort are of European Caucasian origin. All subjects provided extensive standardized questionnaire data on diet and non-dietary variables, as well as anthropometric measurements. About 80% of the participants also provided a blood sample.

Cases of cancer occurring after recruitment into the cohort are identified through local and national cancer registries in 7 of the 10 countries and in France, Germany and Greece by a combination of contacts with national health insurances and/or active follow-up through the study subjects or their next of kin. Follow-up on vital status, to monitor the population remaining at risk for cancer, is achieved through record linkage with mortality registries. A fully detailed description of the EPIC study has been published elsewhere (49,50).

Questionnaire data and anthropometry

Questionnaire data on non-dietary lifestyle and health factors included menstrual and reproductive history, current and previous use of oral contraceptives and postmenopausal hormone replacement therapy, history of previous illness and disorders or surgical operations, lifetime history of tobacco smoking and consumption of alcoholic beverages, physical activity, level of education and socioeconomic status and brief occupational history. In all countries included in the present analysis, except part of the cohort recruited through the Oxford research centre, height, weight and waist and hip circumferences were measured according to standardized protocols in light dressing. In part of the Oxford cohort, height, weight and body circumferences were self-reported.

Selection of cases and controls

Cases were selected among women who developed breast cancer after their recruitment into the EPIC study, before the end of the study period (for each study center defined by the latest end-date of follow-up) and who had no previous diagnosis of cancer (except non-melanoma skin cancer). For each case subject with breast cancer, two control subjects were chosen at random from cohort members alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case. Control subjects were matched to the cases by study center where the subject was enrolled in the cohort, as well as by menopausal status, age (±6 months) at enrollment, fasting status (<3, 3–6 and >6 h), time of the day of blood donation (±1 h), exogenous hormone use and phase of the menstrual cycle (51).

Approval for the study was given by the relevant ethical committees, both at the International Agency for Research on Cancer and in each of the EPIC recruitment centers.

DNA extraction

For 7 of the 10 countries participating in EPIC (UK, Germany, The Netherlands, Spain, Italy, France and Greece), buffy coat samples for the study subjects were retrieved from the EPIC biorepository and DNAs were extracted on an Autopure instrument (Gentra Systems, Minneapolis, MN) with Puregene chemistry (Gentra Systems). Because of small DNA quantity, whole-genome amplification was performed in Denmark and Sweden. Norway was not included in the study because blood samples have been collected only recently on a subsample of cohort participants, and so far only very few cases of breast cancer have been accumulated after blood collection.

Identification, selection and genotyping of single-nucleotide polymorphisms

We collected data on polymorphisms from publicly available databases, such as dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), SNPper (http://snpper.chip.org/) and Frequency Finder (http://bluegenes.bsd.uchicago.edu/frequencyfinder/). We complemented databases searches with literature review and, for *GHRL*, with analysis of 95 subjects from the EPIC population by denaturating high-performance liquid chromatography. We included only polymorphisms whose existence in Caucasians is documented, either according to literature data or to our own experimental analysis by denaturating high-performance liquid chromatography. All new single-nucleotide polymorphisms (SNPs) identified in our laboratory by denaturating high-performance liquid chromatography searches have been deposited in dbSNP (http://www.ncbi.nlm.nih.gov/SNP). Among all polymorphisms thus identified, we retained only those with a minor allele frequency ≥5% in Caucasians. To this list, we then added the SNPs genotyped by the HapMap project (Phase II v20) in the *GHRL* and *GHSR* genomic regions. We used the R^2 based Tagger (http://www.broad.mit.edu/mpg/tagger/), to choose a representative set of 'tagging' SNPs so that variations of all SNP alleles within the gene were captured with an $R^2 > 0.8$ (52). We used R^2 pair-wise tagging for all SNPs, with the exception of rs26311,

Table I. Details of the polymorphisms used in the present study

SNP ID	Location	Codon change	MAF
GHRL			
rs171336	3′ flanking region		0.36
rs171407	3′ flanking region		0.47
rs35684	3′ flanking region		0.27
rs4684677	Exon 4	Gln90Leu	0.06
rs2075356	Intron 3		0.10
rs696217	Exon 3	Leu72Met	0.08
rs26802	Intron 1		0.33
rs27647	5′ flanking region/promoter		0.41
rs3755777	5′ flanking region/promoter		0.23
rs27498	5′ flanking region/promoter		0.37
rs10490815	5′ flanking region/promoter		0.26
GHSR			
rs2948694	Intron 1		0.09
rs495225	Exon 1	Arg159Arg	0.29
rs572169	Exon 1	Gly57Gly	0.27
rs2922126	5′ flanking region/promoter		0.36

in *GHRL*, which was represented by the 'aggressive' tagging method of Tagger, using two marker tagging between markers rs27498 and rs10490815.

Genotyping was performed by the 5′ nuclease assay (TaqMan). TaqMan probes were synthesized by Applied Biosystems, Foster City, CA (with minor groove binder chemistry). One TaqMan assay for rs519384 could not be designed; however, this SNP was moderately tagged by rs572169 (R^2 = 0.66). Positions and functions of the SNPs genotyped in this study are reported in Table I. Sequences of genotyping primers and probes are available upon request. Laboratory personnel were kept blinded to case–control status throughout the study. Repeated quality control genotypes (8% of the total) showed >99% concordance for all assays. Subjects with a completion rate <75% were excluded (N = 102). Genotyping call rates ranged between 93.5 and 97.5%. After genotyping of the samples, five SNPs were out of Hardy–Weinberg equilibrium in Denmark and therefore participants from this country were excluded from the present study. A total of 1359 cases and 2389 controls were included in the study.

In this group of controls, the distributions of genotypes of all polymorphisms were in agreement with the Hardy–Weinberg equilibrium (P value > 0.01), with the exception of one polymorphism (rs35683, P value = 0.005). After exclusion of rs35683, all *GHRL* polymorphisms can still be captured by the remaining tagging SNPs with a $R^2 > 0.80$. Two SNPs, rs35681 and rs35680, were then captured by the aggressive tagging method of Tagger, using two other markers (rs171407 and rs35684).

Hormone measurements

For a subgroup of 3276 women (including 1113 cases and 2163 controls) that were not using exogenous hormones at the time of blood collection (oral contraceptive or hormonal replacement therapy for menopause), measurements of circulating levels of IGF-I and IGFBP-3 were performed in the laboratory of the Hormones and Cancer Team, at International Agency for Research on Cancer, using enzyme-linked immunosorbent assays from Diagnostic System Laboratories (Webster, TX). For 127 subjects, measurements of IGFBP-3 were missing because of lack of serum for this particular study. The IGF-I assays included an acid–ethanol precipitation step to eliminate IGF-I-binding proteins, to avoid their interference with the IGF-I measurement. Measurements were performed on never-thawed serum sample aliquots. The mean intra- and interbatch coefficients of variation were 6.2 and 16.2%, respectively, for IGF-I and 7.2 and 9.7%, respectively, for IGFBP-3.

Statistical analyses

Relationships of polymorphic gene variants with serum levels of IGF-I and IGFBP-3, or with anthropometric variables, were estimated by standard normal regression models and percentage of difference in geometric means in each genotype category compared with the major homozygote category was calculated. All the analyses on IGF-I and IGFBP-3 were adjusted for age at blood donation, body mass index (BMI), laboratory batch and breast cancer case–control status. The analyses on anthropometric measures were adjusted for age at blood donation, country and case–control status. Subjects with BMI ≥30 were defined as obese and <25 as normal weight. Relationships of polymorphic variants with obesity [odds ratios (ORs) for obese versus normal weight] were estimated using unconditional logistic regression and adjusted for age at blood donation, country and breast cancer case–control status. The relationships with breast cancer risk were estimated using conditional logistic regression models, applied on the matched case–control sets. Analyses were performed under the recessive, dominant and codominant models.

We performed subgroup analyses on women with a breast cancer diagnosis either before or after 55 years (menopausal status at diagnosis is not available but 99% of the women enrolled in the EPIC cohort after age 55 declared themselves postmenopausal) and by categories of BMI (<25, 25–29 and 30+). Possible heterogeneity of effect between these groups and between EPIC countries was tested using χ^2 tests, comparing the deviations of logistic β-coefficients observed in each subgroup relative to the overall β-coefficient.

Results

A total of 1359 breast cancer cases and 2389 matched controls were included in the present study. Baseline characteristics of breast cancer cases and controls are presented in Table II. The mean age at blood donation was 55 years (5th–95th percentiles: 40–68 years). The mean age at diagnosis of breast cancer was 57 (5th–95th percentiles: 43–71 years). At the time of diagnosis, 40% of the subjects were premenopausal and 60% postmenopausal. In all, 653 subjects (17% of the breast cancer cases and controls) had a BMI ≥30 and were classified as obese. After adjustment for laboratory batch, age at blood donation and BMI, circulating levels of IGF-I and IGFBP-3 were higher among breast cancer cases than among controls (geometric means: 229 ng/ml among cases versus 225 ng/ml among controls, for IGF-I; 3457 ng/ml among cases versus 3393 ng/ml among controls, for IGFBP-3) (Table II). This association was mostly driven by the effect observed in older women (>55 at diagnosis), as reported previously in a more detailed analysis of the relationship of IGF-I and IGFBP-3 levels with breast cancer risk in the EPIC study (20).

Table II. Baseline characteristics of breast cancer cases and controls

Variable	Cases	Controls	P_{diff}
N	1359	2389	
Women <55 at diagnosis, *n*	533	951	
Women >55 at diagnosis, *n*	826	1438	
Age at blood donation, mean (5th–95th percentiles)	55 (20–68)	55 (40–68)	
Age at diagnosis, mean (5th–95th percentiles)	57 (43–71)	—	
BMI <25, *n*	639	1132	0.43[a]
BMI 25–30, *n*	495	829	
BMI ≥30, *n*	225	428	
Height (cm), mean (5th–95th percentiles)	161 (151–172)	161 (150–172)	0.01[b]
BMI (kg/m^2), mean (5th–95th percentiles)	26 (20–34)	26 (20–35)	0.82[b]
IGF-I (ng/ml), geometric mean (95% CI)	229 (225–233)	225 (222–228)	0.08[b]
IGFBP-3 (ng/ml), geometric mean (95% CI)	3457 (3410–3503)	3393 (3360–3427)	0.03[b]

[a]Chi-square test.
[b]Paired *t*-test.

The relationships between *GHRL* and *GHSR* polymorphisms and anthropometry are presented in Table III. Two *GHRL* polymorphisms showed a statistically significant association with reduced BMI for carriers of two copies of the minor allele, compared with non-carriers (−1.6 and −1.9% for rs171336 and rs171407, respectively). For rs171407, significance is reached for the dominant and for the codominant model. A statistically significant increased risk of obesity was observed for carriers of the minor allele of rs3755777 and rs10490815 [OR: 1.6; 95% confidence interval (CI): 1.1–2.4 and OR: 1.4; 95% CI: 1.0–2.1, respectively]. Heterozygotes of the *GHSR* SNP rs2922126 had 1.3% lower BMI and 19% reduced risk of being obese than homozygotes for the major allele. Carriers of two copies of the minor allele of three *GHRL* polymorphisms (rs171336, rs171407 and rs27647) were associated with a 0.4% greater height when compared with non-carriers while heterozygotes for rs3755777 and rs27498 showed a lower height (−0.4%). One *GHSR* polymorphism, rs572169, showed a statistically significant association with height (+0.3% for heterozygotes, $P_{codominant}$ = 0.02). When the analyses were stratified by breast cancer case–control status, the associations observed were in the same direction although not always statistically significant because of the smaller number of observation and loss of power.

The *GHRL* polymorphism rs3755777 showed a significant positive association with IGF-I levels (+5.5% for homozygotes of the minor allele, $P_{recessive}$ = 0.01). A borderline significant association with IGFBP-3 levels was observed for rs2075356 (−7.5% for homozygotes of the minor allele, $P_{codominant}$ = 0.05) (Table IV). No further significant association was observed after stratification by breast cancer case–control status or after exclusion of cases diagnosed in the first 2 years following blood donation.

The results of the association between breast cancer risk and *GHRL* and *GHSR* polymorphisms are presented in Table IV. In the *GHRL* gene, we observed a significant increase in breast cancer risk among carriers of the minor allele (G) of rs171407 (OR: 1.2; 95% CI: 1.0–1.4; $P_{dominant}$ = 0.02). Homozygotes for the G allele of the *GHSR* SNP rs2948694 had also a significant increased risk of breast cancer

Table III. Association of *GHRL* and *GHSR* SNPs with anthropometry

SNP	Allele	N[a]	Height				BMI				Obesity				
			%[b]	Pr[c]	Pd[d]	Pc[e]	%[b]	Pr[c]	Pd[d]	Pc[e]	Obese/normal weight[f]	OR (95% CI)[g]	Pr[c]	Pd[d]	Pc[e]
GHRL															
rs171336	GG	1481	Ref.	0.01	0.46	0.08	Ref.	0.06	0.19	0.06	260/700	1.0	0.09	0.63	0.23
	GT	1646	−0.0				−0.4				287/761	1.0 (0.8–1.3)			
	TT	480	+0.4				−1.6				77/244	0.8 (0.6–1.1)			
rs171407	AA	1028	Ref.	<0.01	0.26	0.02	Ref.	0.09	0.01	0.01	197/472	1.0	0.19	0.11	0.08
	AG	1763	+0.0				−1.3				294/845	0.9 (0.7–1.1)			
	GG	844	+0.4				−1.9				139/406	0.8 (0.6–1.0)			
rs35684	AA	1958	Ref.	0.51	0.36	0.66	Ref.	0.28	0.60	0.39	338/941	1.0	0.21	0.90	0.53
	AG	1421	−0.2				+0.1				244/659	1.0 (0.8–1.2)			
	GG	283	+0.1				+1.1				55/134	1.2 (0.9–1.8)			
rs4684677	TT	3243	Ref.	0.17	0.87	0.90	Ref.	0.22	0.73	0.58	561/1535	1.0	0.63	0.90	0.99
	AT	398	+0.1				−0.1				71/188	1.0 (0.8–1.4)			
	AA	17	−1.2				−4.5				2/15	0.7 (0.1–3.3)			
rs2075356	AA	2994	Ref.	0.72	0.28	0.28	Ref.	0.53	0.08	0.08	516/1426	1.0	0.72	0.52	0.60
	GA	636	−0.2				+1.1				114/296	1.1 (0.9–1.4)			
	GG	32	−0.3				+1.9				4/15	0.8 (0.3–2.7)			
rs696217	GG	3121	Ref.	0.98	0.17	0.19	Ref.	0.46	0.45	0.39	544/1480	1.0	0.59	0.90	0.82
	GT	535	−0.2				+0.5				88/252	1.0 (0.8–1.3)			
	TT	23	−0.1				+2.5				3/11	0.7 (0.2–2.7)			
rs26802	TT	1656	Ref.	0.98	0.77	0.82	Ref.	0.56	0.82	0.92	293/791	1.0	0.41	0.55	0.40
	GT	1599	−0.0				−0.2				276/750	1.0 (0.8–1.2)			
	GG	422	−0.0				+0.3				69/201	0.9 (0.6–1.2)			
rs27647	TT	1284	Ref.	0.02	0.44	0.07	Ref.	0.47	0.12	0.15	242/605	1.0	0.64	0.21	0.27
	CT	1766	+0.0				−0.8				291/835	0.9 (0.7–1.1)			
	CC	636	+0.4				−0.9				106/307	0.9 (0.7–1.2)			
rs3755777	CC	2190	Ref.	0.41	<0.01	0.01	Ref.	0.38	0.72	0.97	373/1031	1.0	0.01	0.54	0.13
	CG	1274	−0.4				−0.4				210/609	1.0 (0.8–1.2)			
	GG	213	−0.3				+0.8				53/100	1.6 (1.1–2.4)			
rs27498	GG	1462	Ref.	0.78	0.01	0.04	Ref.	0.96	0.21	0.38	243/698	1.0	0.64	0.41	0.41
	AG	1703	−0.4				+0.7				298/799	1.1 (0.9–1.3)			
	AA	487	−0.2				+0.4				86/235	1.1 (0.8–1.5)			
rs10490815	AA	1963	Ref.	0.95	0.06	0.13	Ref.	0.33	0.85	0.59	336/928	1.0	0.04	0.42	0.14
	GA	1421	−0.2				−0.1				237/672	1.0 (0.8–1.3)			
	GG	263	−0.1				+0.9				61/122	1.4 (1.0–2.1)			
GHSR															
rs2948694	AA	2974	Ref.	0.20	0.85	0.64	Ref.	0.17	0.36	0.24	517/1415	1.0	0.43	0.77	0.65
	AG	623	+0.0				−0.5				103/289	1.0 (0.8–1.3)			
	GG	34	−0.8				+3.8				9/12	1.5 (0.6–3.9)			
rs495225	AA	1852	Ref.	0.14	0.24	0.12	Ref.	0.44	0.87	0.65	320/900	1.0	0.18	0.98	0.55
	AG	1542	−0.1				+0.0				267/711	1.0 (0.9–1.3)			
	GG	284	−0.4				−0.7				49/131	0.8 (0.5–1.2)			
rs572169	CC	1988	Ref.	0.39	0.01	0.02	Ref.	0.29	0.21	0.15	344/929	1.0	0.13	0.55	0.27
	CT	1377	+0.3				+0.5				230/666	1.0 (0.8–1.3)			
	TT	317	+0.3				+1.2				62/151	1.3 (0.9–1.9)			
rs2922126	AA	1537	Ref.	0.66	0.20	0.25	Ref.	0.65	0.04	0.21	286/710	1.0	0.43	0.13	0.48
	AT	1656	−0.2				−1.3				262/796	0.8 (0.7–1.0)			
	TT	441	−0.2				−0.3				87/208	1.0 (0.8–1.4)			

[a]Number of subjects (cases and controls) excluding duplicates (cases used as controls or controls used twice).
[b]Percentage change in geometric mean BMI and height, adjusted for age at blood donation, country and breast cancer case–control status.
[c]*P* value recessive model.
[d]*P* value dominant model.
[e]*P* value codominant model.
[f]Normal weight: BMI <25; obese: BMI ≥30.
[g]OR: odds ratio (obese versus normal weight), CI: confidence interval; logistic regression adjusted for age at blood donation, country and breast cancer case–control status.

(OR: 2.2; 95% CI: 1.1–4.3; $P_{recessive} = 0.03$). Adjustment for BMI or IGF-I did not affect the risk estimates. No heterogeneity of the effect was observed after stratification by age at diagnosis of breast cancer (<55 and 55+ years), by categories of BMI (<25, 25–29 and 30+) or by EPIC country.

Discussion

To our knowledge, this is the first large-scale study to analyze simultaneously common variations in the *GHRL* and *GHSR* genes and association with breast cancer risk, IGF-I and IGFBP-3 levels and anthropometry.

In previous studies, no association was found between *GHSR* and *GHRL* polymorphisms and IGF-I levels among Caucasian normal weight subjects (27–29). Based on a much larger number of polymorphisms and subjects, our study showed that one *GHRL* polymorphism (rs3755777) was associated with 5% lower IGF-I-circulating levels.

So far, no study has been performed on *GHRL* and *GHSR* polymorphism and IGFBP-3-circulating levels. However, in some studies ghrelin-circulating levels have been positively associated with

Table IV. Association of *GHRL* and *GHSR* SNPs with IGF-I and IGFBP-3 levels and breast cancer risk

SNP	Allele	IGF-I					IGFBP-3					Breast cancer				
		N[a]	%[b]	Pr[c]	Pd[d]	Pc[e]	N[a]	%[b]	Pr[c]	Pd[d]	Pc[e]	Cases/controls	OR (95% CI)[f]	Pr[c]	Pd[d]	Pc[e]
GHRL																
rs171336	GG	1294	Ref.	0.89	0.80	0.80	1252	Ref.	0.89	0.62	0.77	506/954	1.0	0.96	0.13	0.29
	GT	1446	+0.3				1389	−0.5				607/1015	1.1 (1.0–1.3)			
	TT	412	+0.4				394	−0.1				174/303	1.1 (0.9–1.3)			
rs171407	AA	898	Ref.	0.69	0.99	0.81	867	Ref.	0.95	0.86	0.94	339/683	1.0	0.59	0.02	0.08
	AG	1547	−0.2				1493	−0.2				654/1095	1.2 (1.0–1.4)			
	GG	729	+0.4				699	−0.1				309/528	1.2 (1.0–1.4)			
rs35684	AA	1708	Ref.	0.32	0.71	0.48	1638	Ref.	0.49	0.53	0.43	719/1237	1.0	0.41	0.41	0.31
	AG	1251	+0.1				1207	+0.4				506/924	1.0 (0.8–1.1)			
	GG	234	+2.1				230	+1.3				95/182	0.9 (0.7–1.2)			
rs4684677	TT	2825	Ref.	0.55	0.48	0.43	2721	Ref.	0.76	0.19	0.19	1152/2086	1.0	0.23	0.19	0.14
	AT	359	−1.0				346	+1.7				151/245	1.1 (0.9–1.4)			
	AA	14	−4.8				14	+2.0				8/8	1.8 (0.7–5.0)			
rs2075356	AA	2595	Ref.	0.61	0.13	0.20	2499	Ref.	0.08	0.09	0.05	1094/1907	1.0	0.54	0.51	0.45
	GA	566	−2.3				549	−1.4				224/418	0.9 (0.8–1.1)			
	GG	29	+2.5				28	−7.5				10/22	0.8 (0.4–1.7)			
rs696217	GG	2709	Ref.	0.48	0.33	0.44	2604	Ref.	0.14	0.18	0.12	1131/1988	1.0	0.89	0.46	0.47
	GT	475	−1.7				463	−1.2				185/357	0.9 (0.8–1.1)			
	TT	21	+4.5				20	−7.3				8/15	0.9 (0.4–2.2)			
rs26802	TT	1445	Ref.	0.84	0.40	0.47	1389	Ref.	0.29	0.16	0.59	598/1064	1.00	0.75	0.89	0.96
	GT	1388	−0.9				1340	−1.6				583/1028	1.0 (0.9–1.2)			
	GG	369	−0.8				357	+0.6				149/273	1.0 (0.8–1.2)			
rs27647	TT	1116	Ref.	0.45	0.42	0.34	1074	Ref.	0.35	0.59	0.89	461/829	1.0	0.95	0.74	0.79
	CT	1551	+0.7				1495	−0.8				641/1139	1.0 (0.9–1.2)			
	CC	544	+1.5				524	+0.6				233/408	1.0 (0.8–1.2)			
rs3755777	CC	1907	Ref.	0.01	0.62	0.55	1832	Ref.	0.03	0.45	0.81	773/1437	1.0	0.90	0.10	0.20
	CG	1112	−1.5				1079	−1.3				486/797	1.1 (1.0–1.3)			
	GG	184	+5.5				175	+3.5				75/135	1.0 (0.8–1.4)			
rs27498	GG	1268	Ref.	0.85	0.64	0.67	1218	Ref.	0.44	0.27	0.68	534/925	1.0	0.51	0.59	0.95
	AG	1484	−0.5				1440	−1.3				597/1103	0.9 (0.8–1.1)			
	AA	432	−0.6				414	+0.2				181/306	1.0 (0.8–1.3)			
rs10490815	AA	1721	Ref.	0.19	0.92	0.65	1653	Ref.	0.12	0.73	0.36	679/1273	1.0	0.41	0.14	0.13
	GA	1236	−0.6				1197	−0.1				527/891	1.1 (0.9–1.3)			
	GG	232	+2.5				222	+2.4				98/160	1.2 (0.9–1.5)			
GHSR																
rs2948694	AA	2590	Ref.	0.94	0.41	0.44	2494	Ref.	0.45	0.74	0.63	1061/1890	1.0	0.03	0.48	0.23
	AG	545	+1.2				524	+0.2				228/398	1.0 (0.9–1.2)			
	GG	31	+0.6				31	+3.2				18/16	2.2 (1.1–4.3)			
rs495225	AA	1600	Ref.	0.93	0.21	0.30	1546	Ref.	0.71	0.37	0.58	658/1193	1.0	0.46	0.49	0.82
	AG	1355	−1.4				1303	−0.9				572/979	1.1 (0.9–1.2)			
	GG	249	−0.8				239	+0.2				97/190	0.9 (0.7–1.2)			
rs572169	CC	1761	Ref.	0.42	0.17	0.16	1693	Ref.	0.67	0.19	0.23	711/1284	1.0	0.07	0.87	0.36
	CT	1175	+1.4				1131	+1.1				487/895	1.0 (0.8–1.1)			
	TT	274	+2.1				268	+1.1				129/189	1.2 (1.0–1.6)			
rs2922126	AA	1333	Ref.	0.06	0.23	0.97	1281	Ref.	0.23	0.16	0.66	575/965	1.0	0.79	0.28	0.36
	AT	1436	+2.2				1389	+1.7				585/1064	0.9 (0.8–1.1)			
	TT	397	−1.9				379	−0.6				153/282	0.9 (0.8–1.2)			

[a]Number of subjects (cases and controls) excluding duplicates (cases used as controls or controls used twice) and with IGF-I ($N = 3276$) or IGFBP-3 ($N = 3149$) levels.
[b]Percentage change in geometric mean IGF-I and IGFBP-3 levels, adjusted for age at blood donation, BMI, laboratory batch and breast cancer case–control status.
[c]*P* value recessive model.
[d]*P* value dominant model.
[e]*P* value codominant model.
[f]OR: odds ratio; CI: confidence interval; logistic regression conditional on matching factors.

IGFBP-3-circulating levels (53,54). In the present study, one SNP of the *GHRL* gene (rs2075356) has been found to be associated with IGFBP-3-circulating levels although the trend was only borderline significant.

Among the 15 SNPs studied here, 2 showed a significant association with lower BMI and 5 showed an association with height. Two *GHRL* polymorphisms were observed more often in obese compared with normal weight subjects. Several studies have examined the possible implication of the *GHRL* gene polymorphisms in obesity. One particular SNP, Leu72Met (rs696217), has been associated with an early-onset obesity or with BMI (29,42,44,55), whereas no association was observed in other studies (43,45,46,56,57). In our study, this polymorphism is associated with a non-significant higher BMI. Another polymorphism (rs2075356) that was described to be marginally associated with BMI in young Japanese women (57) was also related to BMI in our study although the trend was not statistically significant.

Two studies have examined the association between obesity and *GHSR* gene variants among adults with conflicting results. Vartiainen *et al.* (28) found no association with BMI, whereas Baessler *et al.* (48) observed a 1.5-fold increased risk of obesity among carriers of five *GHSR* polymorphisms. Among those SNPs, one was also included in the present study (rs572169) and showed a significant greater height and a borderline significant 30% increase in risk of obesity.

For breast cancer, we observed a 20% increase in risk for carriers of the G allele of one *GHRL* SNP (rs171407) and a 2-fold increase for homozygotes of the *GHSR* polymorphism rs2948694. This later association, however, was based on a homozygote group of only 18 cases. These two polymorphisms are not located within coding regions and therefore do not lead to a change in amino acid sequence. One other study, including 405 breast cancer cases and 460 matched controls, investigated the association between genetic polymorphisms of *GHRL* and *GHSR* genes and breast cancer risk (17). This study showed an increased risk for the *GHSR* SNP rs495225 under a recessive model (OR = 2.5; 95% CI: 1.4–4.5), but the distributions of this particular SNP did not fall inside that expected by Hardy–Weinberg equilibrium. In our study, no effect of this SNP on breast cancer risk was observed.

Although >97% of the EPIC subjects are estimated to be of Caucasian origin, differences in allelic frequencies across Europe could in theory cause confounding by population stratification. However, we did not observe major variations in allele frequencies across countries for the SNP studied here. Moreover, cases and controls were systematically matched for EPIC recruitment center and regression analyses relating IGF-I, IGFBP-3 or anthropometry measurements to genetic variants were adjusted for the factor 'country of recruitment'.

With the tagging approach used in our study (pair-wise tagging), we captured most of the haplotype information for the SNPs genotyped in HapMap. In fact, with the high r^2 threshold used in our study (>0.8), our tagSNPs can resolve >80% of all existing haplotypes, as described by Carlson *et al.* (52). However, we cannot rule out the possibility that it might still exist untyped variants in these genes that the haplotype approach would capture better. We therefore performed also haplotype analyses [using the method described by Stram *et al.* (58)]. The results were very similar to the SNP results presented here and we thought that presenting both would have been overwhelming for the reader.

The large number of statistical tests performed in our study raises the question of potential false-positive results. To estimate the 'noteworthiness' of our results, we computed the false-positive report probability (FPRP) defined by Wacholder *et al.* (59). Using a prior probability of 0.1, noteworthy FPRPs <0.2 (the appropriate threshold for a study of this size) were observed for the association with breast cancer risk of rs171407 (FPRP = 0.15, with a power of 0.996 to detect an OR of 1.5), for the association with BMI levels of rs2922126 and rs171407 (FPRP = 0.08, with >90% power to detect a β-coefficient of −0.01) and for the association with IGF-I of rs3755777 (FPRP = 0.16, with a power of 0.98 to detect a β-coefficient of 0.05). FPRPs were also <0.20 for most of the associations with height (only the association between height and rs27647 was not noteworthy with an FPRP of 0.24). Higher FPRPs (>25%) were observed for the other associations with obesity and breast cancer, indicating that these results might be false positive. When using a prior probability of <0.01 (moderate), we obtained high FPRP values for all the associations tested here, all >0.47, and therefore, we cannot rule out the possibility that chance led to these associations.

Ghrelin has been implicated in cell proliferation (16), maintenance of the IGF-I–GH axis (5) and traits associated with obesity (9). Functionally, consequent genetic variation in the *GHRL* or *GHSR* genes would therefore be expected to manifest as associations across a number of these traits. It is then intriguing that the *GHRL* SNP rs171407 which we found associated with a lower BMI (−1.9%) and a greater height (+0.4%) was also associated with breast cancer risk (+20%). Similarly, the *GHSR* polymorphism rs572169 was associated with a 20% increase risk of breast cancer and greater height (+0.3%) but not with BMI. This might reflect an effect of these polymorphisms on breast cancer through the GH axis, independently of BMI or obesity and then independently of the action of ghrelin on appetite and food intake. It is also worth to note that, in addition to being associated with height and risk of obesity, the *GHRL* polymorphism rs3755777 was also associated with higher circulating IGF-I levels. These traits are interrelated, and therefore these associations may also reflect similar biological actions (i.e. altered height level might be influenced by changes in IGF-I); nevertheless, the associations we have observed would appear to be driven by differences in the biology rather than multiple testing.

The results presented here add to the accumulating body of evidence that genetic variation in *GHRL* and *GHSR* is associated with BMI. Furthermore, we have observed evidence for association of *GHRL* polymorphisms with circulating IGF-I levels and with breast cancer risk. Nevertheless, particularly as some of our findings involve recessive models with relatively few individuals, further large studies will be needed to confirm our results.

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