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ARTICLE

# Controversial association results for *INSIG2* on body mass index may be explained by interactions with age and with *MC4R*

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**Among the single-nucleotide polymorphisms (SNPs) previously reported to be associated with body mass index (BMI) and obesity, we focus on a common risk variant rs7566605 upstream of the insulin-induced gene 2 (*INSIG2*) gene and a rare protective variant rs2229616 on the melanocortin-4 receptor (*MC4R*) gene. *INSIG2* is involved in adipogenesis and *MC4R* effects hormonal appetite control in response to the amount of adipose tissue. The influence of rs2229616 (*MC4R*) on BMI and obesity has been confirmed repeatedly and insight into the underlying mechanism provided. However, a main effect of rs7566605 (*INSIG2*) is under debate because of inconsistent replications of association. Interaction of rs7566605 with age may offer an explanation. SNP–age and SNP–SNP interaction models were tested on independent individuals from three population-based longitudinal cohorts, restricting the analysis to an observed age of 25–74 years. KORA S3/F3, KORA S4/F4 (Augsburg, Germany, 1994–2005, 1999–2008), and Framingham-Offspring data (Framingham, USA, 1971–2001) were analysed, with a total sample size of $N = 6926$ in the joint analysis. The effect of interaction between rs7566605 and age on BMI and obesity status is significant and consistent across studies. This new evidence for rs7566605 (*INSIG2*) complements previous research. In addition, the interaction effect of rs7566605 with the *MC4R* variant rs2229616 on BMI was observed. This effect size was three times larger than that in a previously reported single-locus main effect of rs2229616. This leads to the conclusion that SNP–age or SNP–SNP interactions can mask genetic effects for complex diseases if left unaccounted for.**
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## INTRODUCTION

Obesity is prevalent in the Western world, posing a serious health risk.[1] Genes are known to contribute to its pathogenesis.[2] The discovery of genetic interactions with age, other genes or the environment may improve consistency of associations as well as the understanding of the mechanisms leading to or underlying obesity. Here we present longitudinal interaction analyses for two candidate single-nucleotide polymorphisms (SNPs); one located upstream of the insulin-induced gene 2 (*INSIG2*) on chromosome 2 and the other in the melanocortin-4 receptor gene (*MC4R*) on chromosome 18. *INSIG2* rs7566605 and *MC4R* rs2229616 were the first SNPs with a replicated polygenic effect on body mass in the general population[3,4] and known functional implications.[5,6]

The *INSIG2* region has been connected with obesity in human linkage studies[7] as well as in one of the first genome-wide association studies (GWAS)[3] to address obesity. This GWAS identified common variant rs7566605, located 10-kb upstream of the *INSIG2* transcription start site. Elevated body mass index (BMI) or increased obesity risk was found for minor allele homozygotes ($\sim$11% Caucasians) in at least six independent studies.[3,8] *INSIG2* rs7566605 interaction analyses are of particular interest, as its main effect on BMI or obesity status was questioned following equal numbers of replications and non-replications of association in $>20$ well-powered data sets.[3,8–17] Combining all study results on *INSIG2* suggest age as the most probable interaction candidate. A large meta-analysis[18] (34 studies, 74 345 individuals) confirmed association of *INSIG2* with obesity across study designs when comparing extremes (eg, BMI $\geq 32.5\,kg/m^2$ *versus* BMI $<25\,kg/m^2$). No heterogeneity in *INSIG2* main effect estimates was found between studies with higher compared with lower mean subject age; however, contrasted studies overlapped considerably in age range, with mean ages lying between 41 and 58 years.[18] Nevertheless, this meta-analysis revealed an increased obesity risk for rs7566605 minor allele homozygotes in general population studies (odds ratio 1.092, $P = 0.035$, 48 844 subjects from 16 studies), but a decreased obesity risk in population-based studies with subjects selected for better health

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status (odds ratio 0.796, $P = 0.028$, 7640 subjects from 5 studies). Six other studies also indicated a tendency toward a *protective* effect of rs7566605 *risk* genotype CC on BMI or obesity status,[3,9,10,12,14] or on waist-to-hip ratio.[16] The effect strength of rs7566605 association varied between the first six longitudinal examinations on unrelated Framingham-Offspring subjects.[8] Tests for rs7566605–age interaction were not significant for early childhood to middle age (4–50 years).[19,20] However, a lower gain in weight-for-length was reported for genotype CC babies between birth and the age of 6 months.[21] These findings suggest an *INSIG2*–age interaction with weak marginal effect because of crossing BMI-age trends for different *INSIG2* genotypes at a mid-life age (Figure 1, left). In this case, contrasting BMI extremes is robust but the heterogeneity test will not detect this, and power and sign of marginal effect estimates depend on the age range studied.[22,23]

*INSIG2* fulfils a role complementary to insulin-induced gene 1 (*INSIG1*) in the regulation of cholesterol and triglyceride levels.[24,25] However, both genes also have separate additional functions. *INSIG2* (but not *INSIG1*) is predominant in adipogenesis with a marked increase in gene expression during adipocyte differentiation.[5] Multi-marker tag-SNP haplotypes in the close neighbourhood of rs7566605 associate with waist-to-hip ratio and computed tomography measures of visceral and subcutaneous adipose tissue.[26] This suggests that rs7566605 tags a variant for altered adipogenesis.

This interaction analysis also examined the functional SNP rs2229616 in *MC4R*, which contributes to hormonal appetite control in response to the amount of adipose tissue.[1,27] *MC4R* harbours several susceptibility loci for obesity.[6,28,29] Known functional SNPs in *MC4R* are all rare, have been found mostly in obesity studies and mostly associate with elevated BMI. The missense variant V103I (rs2229616), however, is relatively frequent and well-studied in population-based samples.[4,30–34] Minor allele carriers (~3% Caucasians) have lower BMI,[30,31] lower risk of obesity[4,32,33] and metabolic syndrome,[34] and beneficially altered triglyceride and HDL-cholesterol levels.[34,35] Concurrently, functional studies demonstrated that V103I alters MC4R receptor responsiveness to its endogenous antagonist agouti-related protein.[6]

In this candidate study, we tested *INSIG2* interaction with age and *MC4R* variant V103I on individual-level BMI data of unrelated adults (25–74 years) from three large longitudinal population-based cohorts. No other SNPs or hypotheses were tested.

## MATERIALS AND METHODS

### Samples

We analysed independent individuals from the KORA S3/F3 and KORA S4/F4 cohorts[30,36] (from the KORA study, http://www.helmholtz-muenchen.de/en/kora), and from the Framingham Heart Study Offspring cohort (FHS-Off)[37] (Genetic Analysis Workshop 16 Framingham data, accession number phs000128.v1.p1 from the *Database of Genotypes and Phenotypes* (dbGaP), http://www.ncbi.nlm.nih.gov/gap). Data were retrieved and analysed in compliance with the Declaration of Helsinki. All studies were approved by local, regional and national ethics committees as required.

The KORA platform (Cooperative Research in the Region of Augsburg) recruited from the adult general population of the southern German city of Augsburg and its two adjacent districts.[30,36] The aim was to describe the prevalence of common diseases and their risk factors. We analysed baseline survey S3 (1994–1995) with 10-year follow-up F3 (KORA3), and baseline survey S4 (1999–2001) with 7-year follow-up F4 (KORA4).

Furthermore, we analysed independent subjects from the FHS-Off[37] (26 years of follow-up: examinations 1 (1971–1975), 3 (1983–1987), 5 (1991–1995), 7 (1998–2001)). Subjects were adults recruited from the town of Framingham, MA, USA, with the identification of common factors in cardiovascular disease as objective. We excluded subjects on cholesterol treatment, as *INSIG2* contributes to the regulation of cholesterol synthesis.

BMI is known to exhibit a gain phase up to late mid-life (55–62 years, dependent on study and sex); followed by diminished gain and a plateau or even slight decline at older age.[38] We analysed subjects (Table 1) whose examinations occurred between 25 and 74 years of age, and at least half their examinations within the BMI gain phase (age $\leq$62 years). For FHS-Off with 26-year follow-up and four examinations, this restricted the selected baseline age range to 25–46 years (accounting for any discrepancy between actual age on examination and scheduled age). For KORA3 and KORA4 with 10 or 7-year follow-up and two examinations, this restricted the selected baseline age range to the whole adult BMI gain phase (25–62 years). The cohorts are otherwise comparable, including minor allele frequencies of considered SNPs and duration between examinations.

### Statistical analysis

We performed longitudinal interaction analyses of rs7566605 with age and rs2229616 on BMI and obesity status. An additive model is not powerful for a recessive SNP (*INSIG2*)[3] and its interaction with a dominant SNP (*MC4R*).[4]

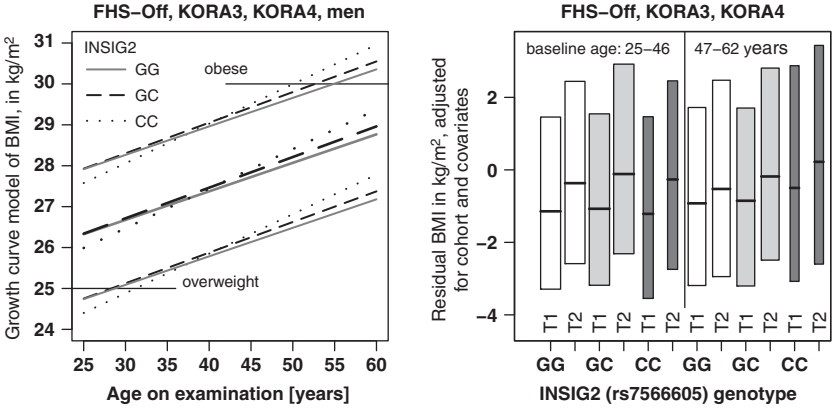


**Figure 1** *INSIG2*–age interaction on longitudinal BMI. Left: growth curves of raw BMI for *INSIG2* genotypes in men (joint analysis: FHS-Off, KORA3, KORA4). Displayed is a corridor of ±1 SD of the random subject-effect on intercept. Random subject-effects on the regression coefficient were small. Similar results were obtained for women and in analyses of rank-normalized BMI (Table 2). Right: *INSIG2*–age interaction on residual BMI (joint model: FHS-Off examinations 3 (1983–1987) and 5 (1991–1995); KORA3, KORA4). Displayed are the median and interquartile of residual BMI, after adjusting raw BMI for sex, baseline age, education and cohort. *INSIG2* genotype is displayed by box colour (GG = white, GC = light grey, CC = dark grey), baseline age class by figure column. Longitudinal examinations T1 (KORA baseline, FHS-Off examination 3) and T2 (KORA follow-up, FHS-Off examination 5) are presented adjacent to each other.

**Table 1 Age-restricted, population-based cohorts analysed**

| *Cohort* | *FHS-Off* | *KORA3* | *KORA4* |
|---|---|---|---|
| Population | US American | German | German |
| Sample size *n*[a] | *n* = 821 | *n* = 3161 | *n* = 2944 |
| Recruitment, *n*[a] | 1971/75, *n* = 821 | 1994/95, *n* = 3153 | 1999/01, *n* = 2936 |
| Follow-up[b], *n*[a] | 12 years, 1983/87, *n* = 698<br>19 years, 1991/95, *n* = 768<br>26 years, 1998/01, *n* = 765 | 10 years, *n* = 2198 | 7 years, *n* = 2205 |
| *Minor allele frequency (in percent) ± 95% confidence interval* | | | |
| *INSIG2* rs7566605, C allele | 34.2 ± 2.3 | 33.6 ± 1.2 | 33.3 ± 1.2 |
| *MC4R* rs2229616, A allele | 1.2 ± 0.5 | 1.9 ± 0.3 | 1.8 ± 0.3 |
| Sex (% male) | 42.5 | 48.8 | 49.3 |
| Baseline age range | 25–46 years | 25–62 years | 25–62 years |
| Follow-up duration[b] | 26 years (median[b]) | 10 years (scheduled[b]) | 7 years (scheduled[b]) |
| Maximal age | 74 years (exact[b]) | 72 years (according to schedule[b]) | 69 years (according to schedule[b]) |
| *Percentage of obese subjects ± 95% confidence interval (age range)* | | | |
| 1. Exam | 10.2 ± 2.1 (25–46 years) | 18.1 ± 1.3 (25–62 years) | 20.3 ± 1.5 (25–62 years) |
| 2. Exam | 14.3 ± 2.6 (36–59 years) | 24.5 ± 1.8 (35–72 years) | 24.7 ± 1.8 (32–69 years) |
| 3. Exam | 21.2 ± 2.9 (43–67 years) | | |
| 4. Exam | 26.1 ± 3.1 (49–74 years) | | |
| Education level (% lower) | Not available | 51.5 | 48.5 |
| *Individual average of longitudinal BMI: mean (SD), in kg/m², stratified by* | | | |
| Sex | | | |
| Men | 27.5 (3.8), *n* = 349 | 27.4 (3.8), *n* = 1542 | 27.4 (4.2), *n* = 1450 |
| Women | 25.2 (4.6), *n* = 472 | 26.4 (5.0), *n* = 1619 | 26.5 (5.2), *n* = 1494 |
| Baseline age | | | |
| 25–33 years | 25.8 (4.2), *n* = 423 | 24.8 (4.0), *n* = 691 | 25.5 (4.4), *n* = 645 |
| 34–46 years | 26.6 (4.5), *n* = 398 | 26.6 (4.4), *n* = 1095 | 26.3 (4.6), *n* = 1033 |
| 47–62 years | — | 28.2 (4.3), *n* = 1375 | 28.2 (4.7), *n* = 1266 |
| Education | | | |
| Basic | Not available | 27.8 (4.6), *n* = 1627 | 28.0 (4.9), *n* = 1427 |
| Higher level | Not available | 25.9 (4.1), *n* = 1534 | 25.9 (4.4), *n* = 1517 |
| *INSIG2* | | | |
| GG | 25.9 (3.9), *n* = 370 | 27.0 (4.5), *n* = 1377 | 26.8 (4.7), *n* = 1302 |
| GC | 26.3 (4.8), *n* = 341 | 26.7 (4.4), *n* = 1442 | 27.0 (4.6), *n* = 1326 |
| CC | 26.8 (4.8), *n* = 110 | 26.9 (4.7), *n* = 342 | 27.3 (5.3), *n* = 316 |
| *MC4R*[c] | | | |
| GG | 26.1 (4.3), *n* = 801 | 26.9 (4.5), *n* = 3040 | 27.0 (4.8), *n* = 2840 |
| GA | 27.8 (6.6), *n* = 20 | 26.5 (3.9), *n* = 121 | 26.5 (4.2), *n* = 104 |

Abbreviations: FHS-Off, Framingham-Offspring cohort; *n*, sample size; SD, standard deviation.
[a]Baseline age, sex, *INSIG2* and *MC4R* genotypes known (FHS-Off, KORA), as well as level of education (grouped into two strata; KORA). BMI was computed from measured body mass and height. Baseline BMI is missing in rare instances. Reductions in sample size are due to loss-to-follow up.
[b]FHS-Off: age at follow-up was available. Listed are median follow-up spans compared with baseline. KORA3 and KORA4: age at follow-up was not available. Listed is protocol follow-up span compared with baseline. Actual follow-up span and actual maximum age may be slightly larger.
[c]The samples contained no *MC4R* rs2229616 minor allele homozygotes.

We therefore used co-dominant SNP coding. All analyses were adjusted for sex, age, study and education (see Appendix for details). All *P*-values are nominal and two sided.

BMI is distributed right-skewed. Rank normalization of longitudinal BMI (joint Blom-transformation[39] of all examinations; all cohorts in the joint analysis) removed this skewness but maintained the longitudinal rank correlation and order of the BMI data. Rank-normalized BMI was analysed using growth curve models.[40] The resulting relative estimates of effect size were converted into absolute values (BMI units) by multiplying with the sample SD of BMI (KORA3: 4.5 kg/m², FHS-Off, KORA4: 4.8 kg/m², joint analysis: 4.7 kg/m²). Longitudinal obesity status (BMI $\geq$30 kg/m²) was analysed by logistic regression, accounting for dependence by random subject intercept or by solving generalized estimating equations with an unstructured working correlation matrix. The longitudinal nonparametric rank-sum test LNPT[41,42] validated the parametric analyses. LNPT resembles special repeated-measures ANOVA on rank order data[42] of BMI, necessitating conversion of predictors and covariates into factors. Hence, baseline age is categorized for LNPT; cutoffs (Tables 2 and 3) ensure balanced classes for each cohort. LNPT was applied on raw BMI (adjusting for covariates within LNPT) and on residual BMI (after *a priori* covariate adjustment of raw BMI, see Appendix). FHS-Off and KORA cover the same age range. LNPT models the longitudinal study design by the factor *longitudinal time course* (examination number) and adjusts for baseline age class. However, power of the parametric models is optimized by using the covariable age on examination directly.[42]

## RESULTS

### *INSIG2* rs7566605–age interaction on BMI

We detected SNP–age interaction for *INSIG2* rs7566605, but not for *MC4R* rs2229616. The BMI growth curve (Table 2, upper panel) of

**Table 2** ***INSIG2*** **rs7566605–age interaction effect on longitudinal BMI in age-restricted population-based cohorts**

| | | *Single-cohort analyses* | | | | | | | | | *Joint analysis* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *FHS-Off* | | | *KORA3* | | | *KORA4* | | | *KORA3, KORA4, FHS-Off* | | |
| | | *USA, 1971–2001* | | | *Germany, 1994–2005* | | | *Germany, 1999–2008* | | | | | |
| | | *n = 821; 26 years record* | | | *n = 3161; 10 years record* | | | *n = 2944; 7 years record* | | | *n = 6926* | | |
| *BMI*[a] | | *4 examinations* | | | *2 examinations* | | | *2 examinations* | | | *All available examinations (≤4)* | | |
| *Growth curve* | *INSIG2* | *Effect* | *98.75% CI* | P | *Effect* | *98.75% CI* | P | *Effect* | *98.75% CI* | P | *Effect* | *98.75% CI* | P |
| Intercept (kg/m$^2$) | GC | −0.85 | −2.06, 0.36 | 0.078 | −0.82 | −2.10, 0.45 | 0.106 | 0.71 | −0.74, 2.16 | 0.219 | −0.30 | −1.13, 0.52 | 0.359 |
| | CC | −0.25 | −1.99, 1.49 | 0.718 | **−2.72** | **−4.79, −0.64** | **0.001** | 0.33 | −1.98, 2.65 | 0.720 | *−1.19* | *−2.50, 0.12* | *0.024* |
| × age | GC | **0.03** | **0.004, 0.05** | **0.003** | 0.01 | −0.01, 0.04 | 0.171 | −0.008 | −0.04, 0.02 | 0.481 | 0.009 | −0.008, 0.03 | 0.170 |
| kg/(m$^2$ year) | CC | 0.02 | −0.01, 0.05 | 0.100 | **0.05** | **0.008, 0.09** | **0.003** | 0.002 | −0.05, 0.05 | 0.916 | **0.03** | **0.003, 0.06** | **0.006** |
| *BMI*[b] | | *4 examinations* | | | *2 examinations* | | | *2 examinations* | | | *KORA+ FHS-Off exams 3, 5* | | |
| *Rank-sum test LNPT* | | | | | | | | | | | | | |
| | *INSIG2* | | | P | | | P | | | P | | | P |
| | Average effect | | | 0.1598 | | | 0.5881 | | | 0.5205 | | | 0.3210 |
| | × examination | | | 0.6198 | | | 0.0596 | | | 0.7611 | | | 0.0846 |
| | × age | | | 0.7378 | | | **0.0044** | | | 0.4964 | | | 0.0912 |
| | × age × examination | | | **0.0079** | | | 0.8526 | | | 0.2568 | | | 0.4674 |
| *Obesity status*[c] | | *4 examinations* | | | *2 examinations* | | | *2 examinations* | | | *All available examinations (≤4)* | | |
| *Logistic model* | *INSIG2* | *OR* | *98.75% CI* | P | *OR* | *98.75% CI* | P | *OR* | *98.75% CI* | P | *OR* | *98.75% CI* | P |
| Intercept | GC | 0.92 | 0.06, 14.20 | 0.942 | 0.63 | 0.08, 4.75 | 0.564 | 2.97 | 0.27, 32.3 | 0.255 | 0.90 | 0.24, 3.37 | 0.850 |
| | CC | 0.10 | 0.002, 6.04 | 0.163 | 0.07 | 0.002, 2.07 | 0.050 | 2.74 | 0.07, 109.7 | 0.495 | 0.22 | 0.03, 1.67 | 0.062 |
| × age | GC | 1.02 | 0.98, 1.08 | 0.216 | 1.01 | 0.97, 1.05 | 0.633 | 0.99 | 0.94, 1.04 | 0.537 | 1.01 | 0.98, 1.03 | 0.420 |
| | CC | *1.07* | *1.00, 1.16* | *0.013* | 1.05 | 0.98, 1.12 | 0.071 | 1.00 | 0.93, 1.07 | 0.924 | **1.04** | **1.00, 1.08** | **0.0125** |

Abbreviations: CI, confidence interval; FHS-Off, Framingham-Offspring cohort; *n*, sample size; OR, odds ratio.
Analyses of age-restricted samples, adjusted for age, sex, education and study. Multiple-testing adjusted significances are set in bold, all other $P \leq 0.05$ in italic.
[a]Growth curve analyses of rank-normalized BMI (population-level estimates of effect size, compared with *INSIG2* reference genotype GG, Bonferroni $P \leq 0.05/4$).
[b]Longitudinal rank-sum test LNPT for single cohorts (on raw BMI, covariate adjustment within LNPT) and for joint analysis (on residuals of *a priori* covariate adjusted raw BMI); Bonferroni adjustment $P \leq 0.05/3$ for the three interaction tests of *INSIG2* with examination or baseline age class (FHS-Off: age 25–33/34-46 years, all other: 25–46/47–62 years). Joint analysis comprises KORA and FHS-Off examinations 3 (1983–1987) and 5 (1991–1995).
[c]Longitudinal logistic regression of obesity status (BMI ≥ 30 kg/m$^2$); model with random subject intercept and *INSIG2* reference genotype GG, Bonferroni $P \leq 0.05/4$.

**Table 3 *MC4R—INSIG2* interaction effect on longitudinal BMI in age-restricted population-based cohorts**

| | | *Single-cohort analyses* | | | | | | *Joint analysis* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *KORA3* | | | *KORA4* | | | *KORA3&4, FHS-Off* | | |
| | | *Germany, 1994–2005* | | | *Germany, 1999–2008* | | | *Germany; USA* | | |
| | | n = *3161; 10 years* | | | n = *2944; 7 years* | | | n = *6926* | | |
| *BMI*[a] | | *2 examinations* | | | *2 examinations* | | | *All examinations (≤4)* | | |
| *Growth curve* | | *Effect* | P | $P_{type3}$ | *Effect* | P | $P_{type3}$ | *Effect* | P | $P_{type3}$ |
| *INSIG2* intercept (kg/m$^2$) | GC | −0.69 | 0.174 | **0.007** | 0.73 | 0.211 | 0.443 | −0.21 | 0.521 | 0.099 |
| | CC | **−2.69** | **0.001** | | 0.27 | 0.774 | | *−1.17* | *0.027* | |
| *INSIG2* × age kg/(m$^2$ year) | GC | 0.01 | 0.216 | *0.013* | −0.009 | 0.463 | 0.723 | 0.008 | 0.231 | *0.031* |
| | CC | *0.05* | *0.003* | | 0.002 | 0.933 | | *0.03* | *0.009* | |
| *MC4R* (kg/m$^2$) | GA | 0.22 | 0.650 | 0.651 | −0.37 | 0.483 | 0.705 | 0.15 | 0.666 | 0.845 |
| *INSIG2* × *MC4R* (kg/m$^2$) | GC × GA | **−1.83** | **0.006** | **0.004** | −0.007 | 0.993 | 0.267 | **−1.26** | **0.009** | **0.001** |
| | CC × GA | 0.68 | 0.484 | | 1.59 | 0.128 | | 0.97 | 0.148 | |

| *BMI*[b] | *2 examinations* | *2 examinations* | *KORA + FHS-Off exam 3, 5* | |
|---|---|---|---|---|
| *Rank-sum test LNPT* | $P_{Heterozygotes}$ | $P_{Heterozygotes}$ | $P_{Heterozygotes}$ | $P_{all}$ |
| *INSIG2* average effect | *0.036* | 0.208 | 0.444 | 0.062 |
| × examination | 0.116 | 0.369 | 0.067 | 0.336 |
| × age | 0.784 | 0.564 | 0.473 | 0.102 |
| *MC4R* average effect | 0.282 | 0.343 | 0.215 | 0.734 |
| *MC4R* × *INSIG2* average effect | *0.036* | 0.565 | 0.139 | 0.058 |

Abbreviations: FHS-Off, Framingham-Offspring cohort; *n*, sample size.
Analyses of age-restricted samples, adjusted for age, sex, education and study. Multiple-testing adjusted significances are set in bold, all other $P \leq 0.05$ in italic.
[a]Growth curve analyses of rank-normalized BMI: population-level estimates of effect size with *P* value (*P*), compared with *INSIG2* and *MC4R* reference genotypes GG. Type 3 tests ($P_{type3}$) examine the significance of each model component. Bonferroni $P \leq 0.05/4$ and the closed testing principle were applied to adjust for multiple testing.
[b]Longitudinal rank-sum test LNPT on residuals of covariate-adjusted raw BMI. Tested were *INSIG2* interaction on BMI with examination and with baseline age class (age 25–46/47—62 years), as well as average effects where the average is over all examinations and both age classes. Joint analysis comprises KORA and FHS-Off examinations 3 (1983–1987), 5 (1991–1995). *INSIG2* genotype CC was either excluded ($P_{Heterozygotes}$, compare with growth curve parameter tests' *P* for heterozygotes (*INSIG2*: GC, *INSIG2* × *MC4R*: GC × GA)), or included for joint analysis ($P_{all}$, compare with growth curve $P_{type3}$).

*INSIG2* minor allele carriers has a lower intercept and significantly increased regression coefficient compared with the GG genotype (FHS-Off, KORA3, joint analysis). KORA3 and KORA4 contributed 46% and 43% of the total sample size to the joint analysis. Longitudinal logistic regression of obesity status (Table 2, bottom panel) confirmed a SNP-age effect. Expectedly, binary trait obesity is less powerful compared with quantitative BMI; however, evidence of SNP–age interaction is consistent between both phenotype models. The initially lowered obesity odd for genotype CC increases significantly with age.

Interestingly, estimates of *INSIG2* CC genotype interaction with age were greater in the full KORA4 cohort (0.02 kg/m$^2$ per year more than for GG genotype subjects, $P = 0.124$, with negative intercept estimate), in contrast to the age-restricted KORA4 sample (Table 2), in which 835 subjects with baseline age >62 years were excluded. An *INSIG2* main effects model also estimated greater CC genotype effect in the full KORA4 cohort (0.64 kg/m$^2$, $P = 0.0015$, in agreement with Herbert *et al*[3]) when compared with the age-restricted KORA4 sample (0.42 kg/m$^2$, $P = 0.0656$). The GC genotype's main effect, however, was stable (full cohort: 0.29 kg/m$^2$, $P = 0.0203$; age-restricted sample: 0.32 kg/m$^2$, $P = 0.0258$; compared with the GG genotype). This strongly implies that *INSIG2* genotype CC also interacts with age in KORA4.

SNP–age effect estimates were stable for different models of longitudinal correlation (comparing unstructured estimation of longitudinal covariance (data not shown) with parametric covariance models (Tables 2 and 3: autoregressive covariance (growth curve), or random subject intercept (logistic regression))). The interaction was also confirmed in raw BMI, albeit with weaker power (Supplementary Table). Further validation comes from the rank-sum test LNPT, which is model-free and free of distributional assumptions.[42] LNPT on raw BMI (Table 2, middle panel, single cohorts) agreed strongly with LNPT on residuals of covariate-adjusted raw BMI (data not shown). LNPT tested two-way interactions of *INSIG2* with baseline age (averaging over repeated examinations), or with longitudinal time course (averaging over the baseline age classes), and three-way interaction of *INSIG2* with longitudinal time course and baseline age class. Power of the interaction tests with longitudinal time course[42] is aided by the long-term follow-up (26 years) and relatively strong longitudinal autocorrelation of BMI ($\rho = 0.38$) in FHS-Off. However, the two-way interaction test with baseline age is expected to have better power[42] in KORA3 and KORA4, because of short-term follow-up (10, 7 years) and lower autocorrelation ($\rho = 0.16, 0.27$). The three-way interaction tested for differential effects in older compared with younger subjects (which were suspected previously[18]). LNPT agreed with parametric results. *INSIG2*–age interaction on BMI was significant for FHS-Off (26 years follow-up, $P = 0.0079$, three-way interaction with longitudinal time course and baseline age) and for KORA3 (10 years follow-up, $P = 0.0044$, two-way interaction with baseline age) but not for KORA4. The three-way interaction in FHS-Off is due to a significant *INSIG2* effect on longitudinal time course of BMI in older subjects ($P = 0.022$, baseline age 34–46years), while no significance was reached in younger FHS-Off subjects ($P = 0.16$, baseline age 25–33 years). LNPT joint analysis used FHS-Off examinations 3 and 5 to have balanced age classes (≤46 *versus* >46 years KORA baseline age, or FHS-Off age on examination 3) in each study. *INSIG2* interaction with age on examination (Figure 1, left: growth curve) is consistent with *INSIG2* interaction with longitudinal time course and age class

(Figure 1, right: residuals of covariate-adjusted raw BMI, as analysed by LNPT). Growth curves of *INSIG2* genotypes cross (Figure 1, left). Mean BMI of genotype CC subjects (bold dotted line) is initially lower compared with the other genotypes, but increases more rapidly with age and is elevated for ages $\geq 40$ years. Consistently, LNPT detected no *INSIG2* average effect but a tendency toward different marginal effects over the two baseline age classes ($\leq 46$ years/other; two-way interaction with age; joint analysis Table 2). *P*-values were also close to 0.05 for the interaction with examination (Table 2: joint analysis, comparable to KORA3).

### *MC4R–INSIG2* interaction on BMI

The final genetic interaction model contains the *INSIG2*–age interaction and tests for *INSIG2–MC4R* interaction effects on intercept (because of power considerations[22]). This model was tested in the large KORA cohorts and jointly over all three cohorts (ensuring $>10$ observations for all two-locus genotypes and all examinations). *INSIG2–MC4R* interaction was significant ($P=0.001$, Table 3, upper panel). Compared with additive SNP effects, we found an enhanced protective effect of the *MC4R* variant in *INSIG2* heterozygotes ($-1.26\,kg/m^2$ on intercept (95% CI: $-2.21$, $-0.31$), $P=0.009$) and a tendency toward an enhanced detrimental effect in *INSIG2* minor allele homozygotes who also have the *MC4R* variant ($0.97\,kg/m^2$ on intercept, $P=0.148$). A *MC4R* rs2229616 main effect was found in large, single-locus meta-analyses[4,30–33] but was not detected by our interaction model. *INSIG2* interaction with age was consistent between Tables 2 and 3. Growth curve results again were consistent with LNPT analyses of residuals of covariate-adjusted raw BMI (Table 3, Figure 2). However, LNPT had weaker power. LNPT *P*-values for the *INSIG2–MC4R* interaction were close to 0.05 whenever the growth curve model revealed significance (KORA3: $P=0.036$ interaction test for GC-GA subjects, excluding *INSIG2* genotype CC, remaining subgroups have sufficient sample size; joint analysis: $P=0.058$ global test over all genotypes). Previous literature[3,4] on main effects reported that the *INSIG2* SNP is recessive and the *MC4R* SNP is dominant. A recessive-dominant growth curve model of BMI confirmed the interaction of *INSIG2* with *MC4R* and age (increased intercept of *INSIG2–MC4R* CC-GA genotype by $1.32\,kg/m^2$ (95% CI: 0.10, 2.54), $P=0.034$; and increased regression coefficient for *INSIG2* CC genotype by $0.02\,kg/m^2$ per year, $P=0.028$).

## DISCUSSION

Previous literature (Table 4) reported a recessive main effect[3] of $0.60\,kg/m^2$ for *INSIG2* rs7566605 genotype CC in KORA4, with replication failures[8] in FHS-Off and KORA3. In contrast, we provide consistent evidence of rs7566605–age interaction with both major statistical interaction models, testing interaction on both the additive scale (growth curve, LNPT) and multiplicative scale (logistic

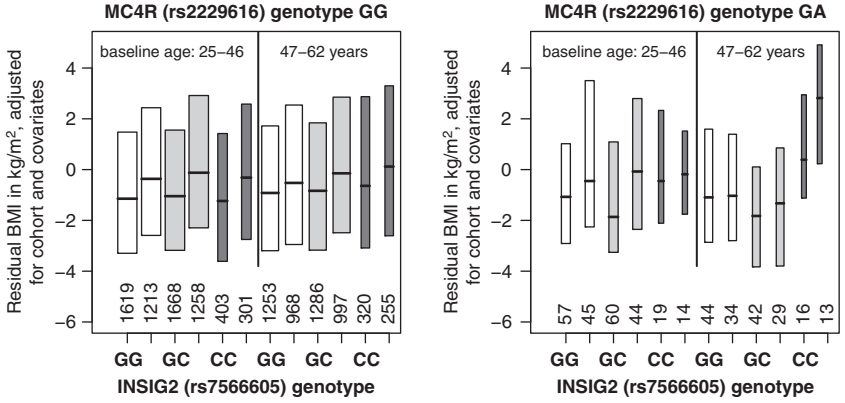


**Figure 2** *MC4R–INSIG2* interaction on longitudinal BMI in population-based cohorts (joint model: FHS-Off examinations 3 (1983–1987) and 5 (1991–1995); KORA3, KORA4). Displayed are the median and interquartile of residual BMI, after adjusting raw BMI for sex, baseline age, education and cohort. *INSIG2* rs7566605 genotype is displayed by box colour (GG = white, GC = light grey, CC = dark grey) in *MC4R* rs2229616 homozygotes (left) and heterozygotes (right); stratified by baseline age class (figure columns). Longitudinal examinations T1 (KORA baseline, FHS-Off examination 3) and T2 (KORA follow-up, FHS-Off examination 5) are displayed adjacent to each other. Sample sizes are given at the bottom of the graphs.

**Table 4 This article compared with previous research: *INSIG2* rs7566605 association with BMI and obesity**

| | | *FHS-Off* | *KORA3* | *KORA4* |
|---|---|---|---|---|
| *Phenotype* | *Study* | *USA, 1971–2001* | *Germany, 1994–2005* | *Germany, 1999–2008* |
| BMI | Literature[a] | n.s. | n.s. | Genotype CC: higher BMI |
| | This article[b] | Genotype CC: significantly higher regression coefficient | | n.s. |
| Obesity | Literature[a] | Genotype CC: OR > 1, exam 3 (but exams 1,2,4,5,6: n.s.) | n.s. | OR > 1 for genotype CC |
| | This article[b] | Genotype CC: OR increases significantly with age | | n.s. |

Abbreviations: FHS-Off, Framingham-Offspring cohort; n.s., not significant; OR, odds ratio.
[a]Previous literature: Lyon *et al.*[8] (FHS-Off, KORA S3), Herbert *et al.*[3] (KORA S4), no age restriction, cross-sectional analyses with a recessive SNP main effect model (KORA baseline surveys, FHS-Off single examinations).
[b]This article: age restriction to BMI gain phase, longitudinal analysis with a co-dominant SNP–age interaction model.

regression). *MC4R* rs2229616 was previously analysed for baseline examinations KORA S3 and S4, demonstrating a protective main effect[30] of $-0.52\,kg/m^2$ for minor allele carriers. This may be a weaker marginal effect from a stronger *INSIG2–MC4R* interaction shown here (Table 3).

We found that *INSIG2* minor allele homozygotes accumulate on average an excess of $1.86\,kg/m^2$ in 62 years but have an *MC4R* genotype-dependent intercept ($-0.05\,kg/m^2$ for CC-GA genotype, $-1.17\,kg/m^2$ for CC-GG genotype), compared with GG-GG genotype subjects. The *INSIG2*–age effect is observed directly in *INSIG2–MC4R* CC-GA genotype subjects with a mean BMI elevated by $1.81\,kg/m^2$ at the age of 62 years ($\sim$0.4% Caucasians). In contrast, the mean BMI of CC-GG genotype subjects is lowered by $-0.42\,kg/m^2$ at the age of 25 years and raised by $0.69\,kg/m^2$ at the age of 62 years ($\sim$10.8% Caucasians). In contrast, mean BMI for GC-GA genotype subjects is lowered by $-1.32\,kg/m^2$ with no significant genotype–age effects ($\sim$1.6% Caucasians). Hence, elderly *INSIG2–MC4R* CC-GA genotype subjects are particularly at risk of obesity. This also may explain previous *INSIG2* main effect replication failures, suggesting that the proportion of younger and elderly subjects in a study can influence association outcome when not accounting for SNP–age interaction.

The strengths of this study are the modelling of individual participant data from high quality, well-characterized, population-based studies (no meta-analysis). The presented effects were robust with respect to different modelling approaches. In contrast to previous analyses, we restricted our analysis to subject age, motivated by the general dependence of BMI on age.[38] No interaction was found earlier between *INSIG2* rs7566605 and the GWAS identified common SNP rs17782313 near *MC4R*.[17] This interaction analysis, however, examined the functional SNP[6] rs2229616 in *MC4R*.

A few study limitations exist. As with every other joint analysis, we had to accommodate differences in study design (baseline age and follow-up duration) and different covariate information. Follow-up in KORA is relatively short and sample size for *INSIG2–MC4R* interaction analysis is still relatively small because of the rare rs2229616 variant. Our finding of *INSIG2*–age interaction (altered adipogenesis) and of *INSIG2–MC4R* interaction (interplay between adipogenesis and hormonal appetite control) would require validation by an independent study. We conclude that gene–gene or gene–age interaction may explain differences in genetic main effects across studies. More elaborate modelling could tackle this important question in future, large, well-characterized studies. Our results also encourage investigation into interactions between other obesity genes and with *MC4R*.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## APPENDIX

## STATISTICAL ANALYSIS

### Parametric longitudinal models of BMI and obesity status

Rank-normalized BMI $Y_{it}^{Blom}$ of subject $i$ at examination $t$

$$Y_{it}^{Blom} = b_{0i} + b_{1i} \times age_{it} + \varepsilon_{it} \quad (1)$$

depends on age on examination $age_{it}$ with multivariate normal errors $\varepsilon_{it}$ with zero mean and longitudinal covariance $\sum$. Unstructured estimation of $\sum$ from the data (data not shown) yielded similar estimates but weaker power compared with an autoregressive covariance model (growth curve, Tables 2 and 3). Between-subjects model equations 2 and 3

$$\begin{aligned} b_{0i} =& \beta_{00} + \beta_{01} \times INSIG2_i + \beta_{02} \times sex_i + \beta_{03} \times het5_i \\ &+ \beta_{04} \times sex_i \times het5_i + v_{0i} \end{aligned} \quad (2)$$

$$b_{1i} = \beta_{10} + \beta_{11} \times INSIG2_i + \beta_{12} \times sex_i + v_{1i} \quad (3)$$

distinguish population-level estimates $\beta$ for fixed effects of genes, confounders and individual random effects $v$ (bivariate normally distributed with zero mean, unstructured covariance matrix). Study-specific differences were adjusted by five-level factor *het*5 constructed from the available information on education and study. Factor *het*5 distinguished FHS-Off (education not available) and two education strata in KORA3 and KORA4. Using three levels (FHS-Off, and two education strata in pooled KORA3, KORA4) yielded results identical to Tables 2 and 3 because of a nonsignificant study effect between age-restricted KORA cohorts ($P = 0.071$). SNP–SNP interaction analysis extended the individual intercept model equation 2 by a *MC4R* main-effect and an age-independent *MC4R–INSIG2* interaction. Obesity status was modelled analogous with a logistic link function for binary trait BMI $\geq 30\,kg/m^2$, accounting for longitudinal dependence by random subject intercepts $v_{0i}$ (Table 2; maximum-likelihood solutions obtained with $v_{1i} = 0$). These estimates were verified by solving generalized estimating equations with an unstructured working correlation matrix (data not shown).

### Nonparametric longitudinal rank-sum test LNPT

LNPT compares BMI distributions $\mathbf{F} = \{F_t^{g,a}\}$ at repeated examinations $t$ for groups of subjects with genotype $g$ and covariate $a$ (presented as factors). LNPT resembles heteroscedastic repeated-measures ANOVA on mid-ranks of longitudinal BMI.[42] LNPT is adjusted for all possible interactions between the included factors (SNP genotype, categorical covariates, examination number), to ensure reliable type-1 error control.[42] Subjects with incomplete longitudinal BMI can be included. We applied LNPT on raw BMI for single studies, adjusting for covariates within LNPT by the factors sex, education and baseline age class. To better incorporate differences in the cohort designs, we also performed *a priori* covariate adjustment on raw BMI $\mathbf{Y}_i$ with a longitudinal fixed effects model

$$\begin{aligned} Y_i =& b_0 + b_s \times sex_i + b_a \times baselineage_i + b_h \times het3_i \\ &+ b_{sa} \times sex_i \times baselineage_i + b_{hs} \times sex_i \times het3_i + Y_i^{res} \end{aligned} \quad (4)$$

Three-level factor *het*3 adjusted for FHS-Off and two education strata in pooled KORA3, KORA4 (given a nonsignificant study effect between age-restricted KORA cohorts). Residual BMI $Y_i^{res}$ was analysed by LNPT for genetic effects. Removal of a population trend with baseline age does not remove subgroup trends in residual BMI.