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Loss-of-function mutations in the melanocortin 4 receptor in a UK birth cohort

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Mutations in the melanocortin 4 receptor gene (*MC4R*) are associated with obesity but little is known about the prevalence and impact of such mutations throughout human growth and development. We examined the *MC4R* coding sequence in 5,724 participants from the Avon Longitudinal Study of Parents and Children, functionally characterized all nonsynonymous *MC4R* variants and examined their association with anthropometric phenotypes from childhood to early adulthood. The frequency of heterozygous loss-of-function (LoF) mutations in *MC4R* was ~1 in 337 (0.30%), considerably higher than previous estimates. At age 18 years, mean differences in body weight, body mass index and fat mass between carriers and noncarriers of LoF mutations were 17.76 kg (95% CI 9.41, 26.10), 4.84 kg m^{-2} (95% CI 2.19, 7.49) and 14.78 kg (95% CI 8.56, 20.99), respectively. *MC4R* LoF mutations may be more common than previously reported and carriers of such variants may enter adult life with a substantial burden of excess adiposity.

Mutations disrupting the leptin–melanocortin system have frequently been reported in severe, early-onset human obesity but the prevalence and extent of phenotypic impact of such mutations are unclear. The critical role of the leptin–melanocortin system in the long-term sensing of body fat stores was first established in the 1990s, with defects in this system resulting in obesity in rodents and humans[1–5]. Specifically, the melanocortin 4 receptor (MC4R) is a G-protein coupled, seven-transmembrane receptor expressed widely in the central nervous system[6,7]. The binding of its natural agonists, the pro-opiomelanocortin-derived melanocortin peptides, α- and β-melanocyte-stimulating hormone (MSH), results in the suppression of food intake and activation of a subset of autonomic neurons of the sympathetic nervous system[8–10]. Leptin acts on hypothalamic neurons to promote the release of melanocortins and suppress the secretion of the melanocortin antagonist, agouti-related peptide[9,11].

Severe early-onset human obesity associated with mutations in genes encoding leptin or the leptin receptor are very rare and observed only in individuals who are homozygous for LoF mutations in those genes[3,5]. However, in the case of mutations in the *MC4R* gene, severe early-onset obesity has been reported in multiple affected members of several families who only carried heterozygote LoF mutations[4,12]. Subsequent studies reported more severe obesity in homozygotes, suggesting a semi-dominant form of inheritance[13].

Many *MC4R* LoF mutations have been described in obese individuals and families[14–16] and the severity of disruption of *MC4R* signaling resulting from such mutations has been reported to correlate with adiposity and degree of hyperphagia[17,18]. While early reports based on clinically ascertained cohorts suggested a high penetrance of early-onset obesity, subsequent studies of less-highly selected patients demonstrated that the carriage of LoF mutations was not always associated with obesity[15,19]. For example, in a population-based cohort from Germany, Hinney et al., using a mutational scanning technique, reported a prevalence of LoF mutations in *MC4R* of ~0.1%[15]. Stutzmann et al. reported a prevalence of *MC4R* LoF mutations of 1.7% in obese European adults and that obesity in carriers of the same mutation differed across generations within the same families, providing evidence for gene–environment interactions[19]. In a study of participants in the UK Biobank based on high-density single-nucleotide polymorphism genotyping, Turcot et al. reported that, while carriers of rare (0.01%) nonsense mutations were ~7 kg heavier on average, than noncarriers (for an average 1.7-m-tall individual), the majority of carriers of this mutation were not obese[20].

Pharmacological agonists of MC4R are in clinical development for the therapy of obesity[21]. In a phase 1b trial of one such drug, setmelanotide, obese participants carrying heterozygous LoF *MC4R* mutations showed drug-induced weight loss[18]. As preventive efforts for metabolic disease are increasingly focusing on tackling obesity in childhood[22], knowledge regarding the prevalence of *MC4R* LoF mutations and their impact on body composition and growth during the first decades of life will be increasingly important and relevant to future drug development.

To determine the frequency of functionally impaired *MC4R* mutations and their clinical and phenotypic consequences throughout childhood, adolescence and early adult life in an unselected UK population, we examined the *MC4R* coding sequence in 5,724 participants from the Avon Longitudinal Study of Parents and Children (ALSPAC), a birth cohort recruited in Bristol, UK in 1990–1992 and repeatedly followed up until early adulthood[23,24]. We functionally characterized all nonsynonymous *MC4R* variants via in vitro assays and examined the relationship between carriage of LoF mutations and anthropometric variables from childhood to early adulthood.

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Table 1 | Sanger sequencing validated nonsynonymous *MC4R* mutations identified in those sequenced in ALSPAC

MC4R variant	Genomic position (bp)	Codon change	Database accession(s)	Domain	No. of carriers[a]	ALSPAC MAF
p.T5N[b]	Chr18:60372336	aCc/aAc	rs752432398	N-term	1	0.0087%
p.T11A	Chr18:60372319	Act/Gct	rs372794914	N-term	2	0.0175%
p.S30F	Chr18:60372261	tCc/tTc	rs13447323	N-term	3	0.0262%
p.S36T	Chr18:60372244	Tct/Act	rs954123325	N-term	1	0.0087%
p.T53I	Chr18:60372192	aCt/aTt	rs141148170	TM1	3	0.0262%
p.Y80C	Chr18:60372111	tAc/tGc	rs1368643838	TM2	1	0.0087%
p.S85I[b]	Chr18:60372096	aGc/aTc	rs1420993856	TM2	1	0.0087%
p.V95I	Chr18:60372067	Gtt/Att	rs13447328	TM2	1	0.0087%
p.V103I[c]	Chr18:60372043	Gtc/Atc	rs2229616	TM2	-	-
p.T112M	Chr18:60372015	aCg/aTg	rs13447329	ECL1	4	0.0349%
p.N123S[b]	Chr18:60371982	aAt/aGt	rs761982475	TM3	1	0.0087%
p.S127L	Chr18:60371970	tCg/tTg	rs13447331	TM3	1	0.0087%
p.I137T	Chr18:60371940	aTt/aCt	rs151102515	TM3	2	0.0173%
p.H158R	Chr18:60371877	cAt/cGt	rs202081467	ICL2	2	0.0173%
p.S180P	Chr18:60371812	Tca/Cca	rs193922685	TM4	1	0.0087%
p.F184L[b]	Chr18:60371800	Ttc/Ctc	-	TM4	1	0.0087%
p.F202L	Chr18:60371744	ttC/ttA	rs138281308	TM5	2	0.0175%
p.M215I	Chr18:60371705	atG/atA	rs768687497	TM5	1	0.0087%
p.A227T[b]	Chr18:60371671	Gct/Act	rs201736647	ICL3	1	0.0087%
p.R236C	Chr18:60371644	Cgc/Tgc	rs758426526	ICL3	1	0.0087%
p.G238V*fs*X4[b]	Chr18:60371636-60371637	gGt/gt	-	TM6	1	0.0087%
p.N240S	Chr18:60371631	aAt/aGt	rs202228712	TM6	2	0.0175%
p.I251W*fs*X34	Chr18:60371598-60371600	ctGAtt/cttt	rs13447339	TM6	1	0.0087%
p.I251L[c]	Chr18:60371599	Att/Ctt	rs52820871	TM6	-	-
p.G252S	Chr18:60371596	Ggc/Agc	rs13447336	TM6	1	0.0087%
p.V253I	Chr18:60371593	Gtc/Atc	rs187152753	TM6	2	0.0175%
p.C271Y	Chr18:60371538	tGt/tAt	rs121913562	ECL3	1	0.0087%
p.S295P	Chr18:60371467	Tca/Cca	rs368264587	TM7	1	0.0087%
p.G323V[b]	Chr18:60371382	gGa/gTa	rs926626133	C-term	1	0.0087%

BP, base-pair position; C-term, C terminus; ECL, extracellular loop; N-term, N terminus; TM, transmembrane. [a]Some mutations listed were carried by multiple individuals. [b]Previously uncharacterized functionally. [c]The common variants, p.V103I and p.I251L, were excluded from the main analyses.

Results

Detection of *MC4R* mutations by pooled sequencing. ALSPAC is a birth cohort originally consisting of >75% of all pregnancies delivered in the Greater Bristol area from 1990–1992. While a specific cohort, ALSPAC represents a population-based sample with deep longitudinal phenotyping suitable for the dissection of *MC4R* mutation associations. Characteristics of the sequenced set of individuals and the complete ALSPAC cohort were similar (Supplementary Table 1 and Extended Data Fig. 1), suggesting that the sequenced set were at least representative of the wider cohort, which is well described in terms of both demographic profile[23] and attrition[25].

The single exon gene, *MC4R*, was sequenced using a new, cost-effective high-throughput approach, which involved pooling DNA from 5,993 unrelated participants of ALSPAC into 120 pools (Methods). We established that this approach had a sensitivity equivalent to whole-exome sequencing (WES) of each individual DNA sample and ~90% specificity in detecting single heterozygous *MC4R* mutations in pools of up to 50 individual DNA samples (Methods). In total, 29 different nonsynonymous mutations in *MC4R* were identified during sequencing of the cohort, including two frameshift/premature stop mutations and 27 missense mutations (Table 1). Two of the missense mutations were the commonly occurring p.V103I and p.I251L variants. Sanger sequencing confirmed the presence of all rare *MC4R* mutations (minor allele frequency (MAF) < 0.1%) and that carriers were heterozygous.

The impact of *MC4R* mutations on canonical cAMP signaling. MC4R transduces external stimuli through $G\alpha_s$-mediated activation of adenylyl cyclase, resulting in the increase of cytoplasmic levels of cyclic adenosine monophosphate (cAMP). Of the 29 nonsynonymous mutations that were detected, 22 had previously been reported in terms of their ability to generate a cAMP response to melanocortin ligands and their association with human obesity (Supplementary Table 2). Of the 22 historically studied variants, 2 were reported to show complete LoF (cLoF), 9 to have a partial LoF (pLoF), 2 to show gain of function (GoF) and 9 to show wild-type (WT)-like activity (Methods and Supplementary Table 2 describe classification criteria and references).

We next generated the seven previously uncharacterized mutants by site-directed mutagenesis and, in transiently transfected COS-7 cells, measured cAMP accumulation in response to escalating doses of [Nle^4,D-Phe^7]-α-MSH (NDP-α-MSH) (Fig. 1). Of the seven variants characterized, two were cLoF mutations (p.S85I and p.G238V*fs*X4) and one was a pLoF mutation (p.F184L). The

four remaining variants (p.T5N, p.N123S, p.A227T and p.G323V) all displayed 'WT-like' activity (Fig. 1). In total, there were 14 rare *MC4R* LoF mutations (4 cLoF and 10 pLoF) identified in the study cohort (Supplementary Tables 2 and 3).

More recently, β-arrestin-2 coupling has been postulated to provide an important alternative post-receptor signal relevant to the control of body weight[26]. We examined NDP-α-MSH-induced β-arrestin-2 coupling for all 27 rare variants (the common variants p.V103I and p.I251L were excluded) in transiently transfected human embryonic kidney 293 (HEK293) cells. Using the same efficacy (relative maximal efficiency (E_{max})) and potency (half-maximum effective concentration (EC_{50}))-based criteria, we found that 10 of the 14 variants that were annotated as LoF for cAMP accumulation also showed impaired β-arrestin-2 coupling (Extended Data Fig. 2 and Supplementary Table 3). As cAMP is still considered to be the canonical signaling pathway for *MC4R*, our primary analyses of the association between in vitro function and clinical phenotype were undertaken using the cAMP-based functional classification with β-arrestin-2-based functional classification as a sensitivity analysis.

Identification of rare variant carriers. Once we completed the functional characterization of *MC4R* mutations, we unencrypted the sequenced pools to identify specific individuals carrying these mutations in ALSPAC. Of the 5,993 individuals sequenced, a total of 5,724 were used in the following analyses characterizing the prevalence and downstream effects of *MC4R* LoF mutations on anthropometric traits due to exclusions of duplications and related individuals (Methods describes the quality control process). Of these 5,724 participants, 40 individuals carrying 27 unique variants were confirmed as true positives (Methods). Of these, 17 individuals carried a heterozygous copy of 1 of the 14 LoF mutations (Table 2), giving a frequency of 0.30% of LoF mutations (approximately 1 in 337). Four participants (0.07%) carried a cLoF mutation and 13 (0.23%) carried a pLoF mutation. Twenty-one (0.37%) individuals carried WT-like mutations and two (0.03%) individuals had GoF mutations.

Age-specific associations with anthropometric traits. Age-specific analyses were conducted using linear regression across all measures of selected anthropometric traits between birth and 24 years. There was a positive association between carriage of *MC4R* LoF mutations and body mass index (BMI) in childhood, adolescence and adulthood, with the mean difference increasing over time from as early as 5 years. This effect was greatest at age 18 years (Supplementary Tables 4 and 5 and Fig. 2), where the mean difference in BMI between carriers and non-LoF carriers of *MC4R* mutations was 4.84 kg m^{-2} (95% CI 2.19, 7.49; $P=3.42\times10^{-4}$). Similarly, there was a positive association between carriage of *MC4R* LoF mutations and weight (Supplementary Table 4 and Extended Data Fig. 3), with the greatest difference between carriers and non-LoF carriers seen at 18 years (mean difference of 17.76 kg; 95% CI 9.41, 26.10; $P=3.11\times10^{-5}$). There was a smaller overall effect of *MC4R* LoF carriage on height over time (Supplementary Table 4 and Extended Data Fig. 4), with the greatest difference at 12 years (mean difference of 6.53 cm; 95% CI 2.88, 8.54; $P=4.50\times10^{-4}$). For both BMI and weight, there was an attenuation of effect of carriage of *MC4R* LoF mutations from age 18 to 24 years; however, it is worth noting that there were no individuals carrying cLoF mutations with anthropometric data at this age.

MC4R LoF also showed a positive association with fat mass measured by dual energy X-ray absorptiometry (DXA) (Supplementary Table 6), with the greatest difference between carriers and non-LoF carriers at 18 years (mean difference of 14.78 kg; 95% CI 8.56, 20.99; $P=3.27\times10^{-6}$). The positive association between *MC4R* LoF and lean mass was also consistent over time (Supplementary Table 6), with the greatest difference between carriers and non-LoF carriers seen at 12 years (mean difference of 4.28 kg; 95% CI 2.08, 6.48; $P=1.38\times10^{-4}$).

Of the four waist–hip ratio (WHR) measures available in ALSPAC, the mean difference in WHR with *MC4R* LoF was the same at ages 10, 12 and 24 years, with carriers of *MC4R* LoF mutations increasing WHR by 0.04 (Supplementary Table 6) compared to non-LoF carriers. This difference was smaller at age 8 years (0.01; 95% CI −0.01, 0.03; $P=0.48$) and increased to 0.04 at 24 years (95% CI −0.03, 0.10; $P=0.29$); however, there were no individuals carrying a cLoF mutation and a measure of WHR at age 24 years.

In contrast to the associations seen between *MC4R* LoF carriage and anthropometric traits, there were no substantive differences in BMI, weight or height among individuals carrying 'WT-like' receptors compared to non-LoF carriers not carrying WT-like mutations (Supplementary Table 7).

Previous studies have reported that carriers of *MC4R* LoF mutations have somewhat lower blood pressure (BP) than equally obese people who are WT at *MC4R*[27]. In this study, there was evidence that *MC4R* LoF mutation carriers had slightly higher systolic blood pressure and left ventricular mass index (LVMI) but almost no difference in diastolic blood pressure and central BP compared to non-LoF carriers between 8 and 18 years (Extended Data Figs. 5 and 6). These differences largely attenuated (or, indeed reversed) when adjusting for BMI at the same age. Of note, however, between ages 10 and 12 years, carriers of *MC4R* LoF mutations had a diastolic blood pressure that was ~3–5 mm Hg lower after correction for BMI and sex.

Longitudinal associations with anthropometric traits. Longitudinal analyses were conducted to examine the association between the *MC4R* LoF mutations and the trajectory of BMI, weight and height. Multilevel linear-spline models used to examine longitudinal associations between *MC4R* LoF and anthropometric traits performed well when predicting each trait (Supplementary Tables 8–10).

There was little evidence to suggest that birth weight (mean 3.44 kg) was related to *MC4R* LoF (Supplementary Table 11). The first measure (intercept of the linear-spline multilevel models) of BMI and height was at 18 months (mean 16.84 kg m^{-2} and 81.90 cm, respectively) and there was little evidence that *MC4R* LoF was associated with a difference in either BMI or height at this age in ALSPAC (Supplementary Tables 12 and 13).

The effect estimates for the mean difference in BMI change (kg m^{-2} per year in carriers versus noncarriers of *MC4R* LoF mutations) between 18 months and 18 years were nonzero and consistently positive between 18 months and 15 years, consistent

Fig. 1 | Ligand-activated cAMP accumulation of previously uncharacterized *MC4R* mutations. a, Dose–response characteristics of NDP-α-MSH-mediated cAMP production for the previously uncharacterized LoF mutations found in ALSPAC. Means ± s.e.m. are shown ($n=3$–6, each from an independent experiment). **b**, Dose–response characteristics of previously uncharacterized 'WT-like' variants. Means ± s.e.m. are shown ($n=5$–6, each from an independent experiment). **c**, E_{max} of NDP-α-MSH on *MC4R* mutants compared to WT, represented as estimated % WT response ± 95% CI (*P* values and sample sizes are listed in Supplementary Table 2). **d**, The potency of NDP-α-MSH (−logEC_{50}) on *MC4R* mutants, represented as estimated −logEC_{50} ± 95% CI (*P* values and sample sizes are listed in Supplementary Table 2). **e**, Schematic representation of *MC4R* showing all 29 mutations identified in the study cohort and their functional classification. There are two mutations at the p.I251 residue, p.I251L (WT-like) and p.I251W*fs*X34 (cLoF). *$P<0.05$ (*P* values were two-sided and not corrected for multiple comparisons).

with age-specific analyses (Fig. 3 and Supplementary Table 12). Similarly, the effect estimates for mean differences in weight change (kg per year) between birth and 18 years were consistently positive, consistent with age-specific analyses (Extended Data Fig. 7 and Supplementary Table 11). There was comparatively stronger evidence for a consistently positive effect of *MC4R* LoF on weight

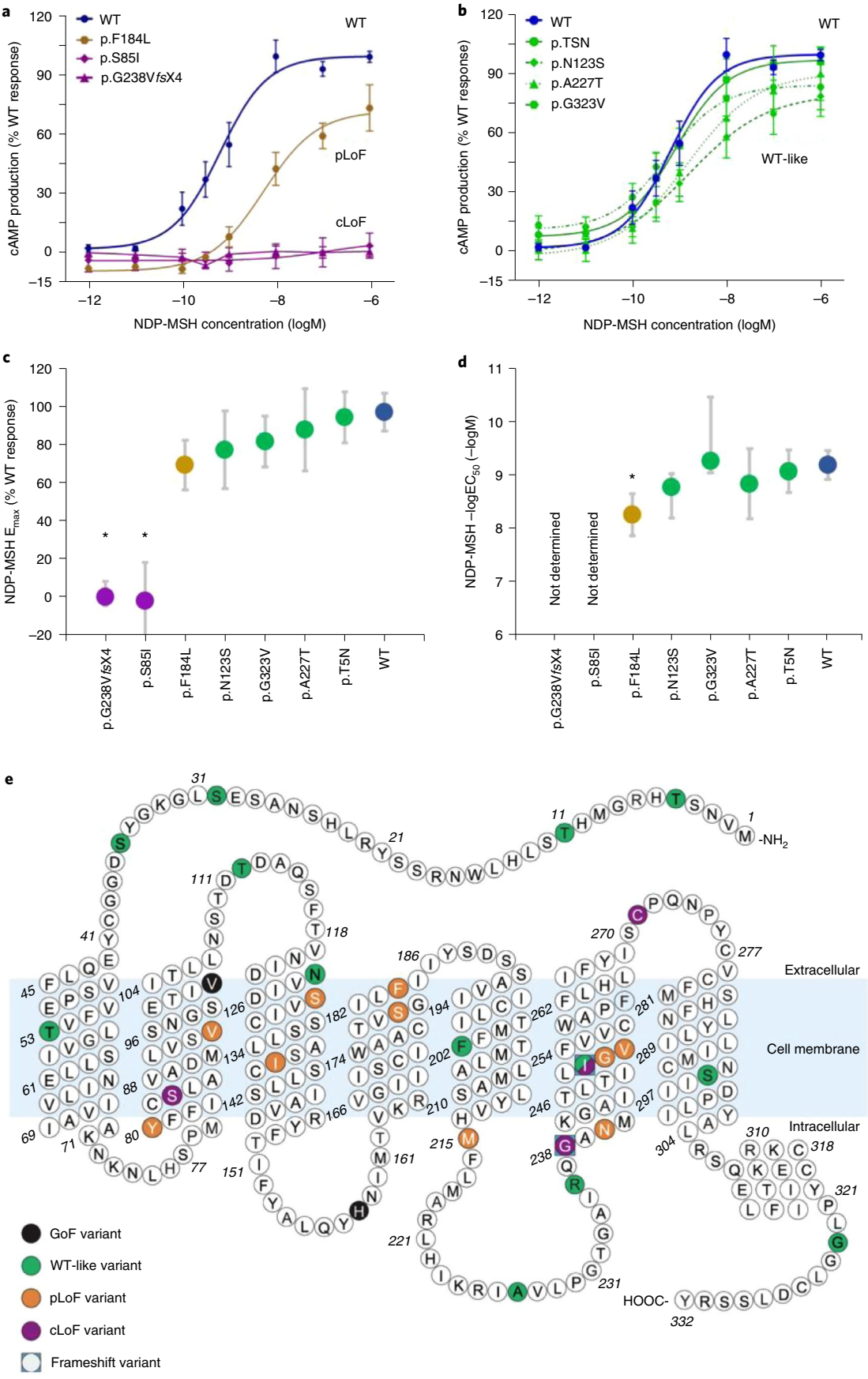

Table 2 | Prevalence estimates of *MC4R* LoF of cAMP accumulation identified in the ALSPAC sample used for analyses

Mutational characterization	Frequency	Prevalence estimate (% of total)
Synonymous, common variations or no LoF mutation	5,684	99.30
GoF	2	0.03
WT-like	21	0.37
pLoF	13	0.23
cLoF	4	0.07
Total	**5,724**	**100**

change between the ages of 12 months to 8 years (0.84 kg per year; 95% CI 0.40, 1.28; $P = 1.63 \times 10^{-4}$) and 8 and 15 years (1.33 kg per year; 95% CI 0.66, 1.99; $P = 9.62 \times 10^{-5}$).

There was a modest increase in height with the *MC4R* mutation across childhood and adolescence, with an inverse association in adulthood; however, most confidence intervals for these differences overlapped the null (Extended Data Fig. 7 and Supplementary Table 13).

Sensitivity analyses. Comparison with β-arrestin-2 coupling. The phenotypic associations of *MC4R* LoF status were largely similar independent of LoF status being defined by β-arrestin-2 coupling or cAMP accumulation assay. There was a consistently positive trend between *MC4R* LoF of β-arrestin-2 coupling and BMI from age 3.5 years and weight and height across the lifecourse (Supplementary Table 14 and Extended Data Figs. 8 and 9), where effect estimates were consistently larger with impairment of β-arrestin-2 coupling from approximately 8 years than cAMP accumulation. Associations between β-arrestin-2 coupling-based classification and fat mass, lean mass and WHR were consistently positive across all time points and had larger (or with WHR, comparable) effects than those derived with cAMP signaling data (Supplementary Table 15). However, it is worth noting that all confidence intervals of associations of impairment in cAMP accumulation and β-arrestin-2 coupling assays with anthropometric traits overlapped.

Comparison between rare and common variation. Given the considerable recent interest in the use of genome-wide polygenic risk score (PRS) to predict the development of obesity[28], we compared the impact of carriage of LoF mutations in *MC4R* with the PRS developed by Khera et al.[28] (comparing upper 10th to lower 90th percentile of PRS distribution). The magnitude of the effect estimates of *MC4R* LoF on BMI between the ages of 3 and 18 years was approximately double that of obtained by the PRS (Fig. 4a and Supplementary Table 16). Similarly, using multilevel linear-spline models, the effect sizes of the change in BMI at 18 months and between 18 months and 18 years were almost always larger with the *MC4R* LoF mutation compared to the PRS (Fig. 4b and Supplementary Tables 17 and 18). Findings from the main analyses of impact of the *MC4R* LoF mutations on BMI were also persistent, albeit slightly attenuated, even when adjusting for genome-wide PRS (Supplementary Table 19). Unsurprisingly, given the relative rarity of *MC4R* LoF compared to the common single-nucleotide polymorphisms comprising the PRS, the latter explained more of the population variance in BMI than the former (for example, 0.40% and 10% explained by *MC4R* LoF mutations and the PRS, respectively, in BMI at age 18 years).

Discussion

By studying a large, representative birth cohort in which anthropometric measures are available throughout childhood, adolescence and early adulthood, we provided estimates of likely frequency for functionally impaired mutations in the *MC4R* gene. In addition to this, we have provided estimates of the phenotypic impact of these mutations during growth and development. We found that mutations at *MC4R* are more frequent and have a consistent and sizeable association with adiposity compared to what has been suggested previously[15].

To establish the frequency of *MC4R* nonsynonymous mutations in a specific population-based study, we developed an approach based on initial pooled high-throughput sequencing. We validated this methodology against WES of individual DNA samples and showed it to have very high sensitivity and specificity. This approach, which has considerable cost advantages, is applicable to the detection of rare, including private, mutations in any gene of interest in large populations.

We estimated a frequency of heterozygous *MC4R* LoF mutations in the ALSPAC birth cohort to be 0.30% (with 0.23% carrying pLoF and 0.07% carrying cLoF mutations). Given the well-understood demographic characteristics of ALSPAC[23,24] and notwithstanding ancestry-specific deviations in frequency, it is reasonable to suggest that as many as 1 in every 337 people in the United Kingdom could carry a heterozygous LoF mutation in the *MC4R* gene. These estimates are approximately double the previous reports[15,29–31] and while they are based on assumptions on the properties of our sample, results here allow a recalibration of prevalence estimates more broadly.

MC4R deficiency in mice results in an increase in both fat and lean mass[2], with early reports suggesting that the same is true in humans[13]. Our results are consistent with these observations, with evidence to suggest a substantial impact of *MC4R* LoF carrier status on BMI, weight, fat mass and lean mass, which was detectable from as early as 5 years. For example, at age 18 years, carriage of an *MC4R* LoF mutation was associated with a 17.76 kg greater body weight, a 4.84 kg m^{-2} higher BMI and a 14.78 kg greater fat mass, with 47.1% of carriers being overweight or obese (≥25 kg m^{-2}) at that age (compared to 12.6% of non-LoF carriers). Indeed, in our study, 208 participants (3.63%) of the 5,724 individuals who were sequenced were obese (BMI > 30 kg m^{-2}) at age 18 years and, of these, 0.96% had LoF mutations in *MC4R* (compared to the 0.27% individuals who carried LoF mutations in *MC4R* out of 5,516 individuals who were not obese). *MC4R* deficiency has also been reported to be associated with an increase in linear growth velocity attributable to hyperinsulinemia and absence of suppression of growth hormone levels that is usually seen in other forms of obesity[32]. Consistent with this, we observed a trend toward increased height with *MC4R* LoF during longitudinal follow-up.

In a recent study of UK Biobank participants, Turcot et al. reported a somewhat smaller impact of carriage of a heterozygous nonsense mutation in *MC4R* on body weight and the prevalence of obesity, where the majority of carriers were not obese[20]. While we similarly found that not all carriers were, indeed, obese (in fact, distributions of carriers and noncarriers of *MC4R* LoF mutations overlapped in most cases), the impact of carriage of such LoF mutations was, as described, more substantial. However, these previous findings by Turcot et al. should be viewed in the light of the known selection bias in UK Biobank[33], whose participants are on average lighter and healthier than unselected members of the UK population and the fact that the particular subset of UK Biobank participants analyzed in this study contained a subpopulation that disproportionately represented heavy smokers.

In the current analyses, carriers of mutations that were functionally WT-like were practically indistinguishable from other non-LoF carriers or carriers of GoF mutations in their anthropometric characteristics. This emphasizes the importance of knowing the functional impact of any nonsynonymous mutation found during diagnostic testing in obesity. Indeed, databases collating information

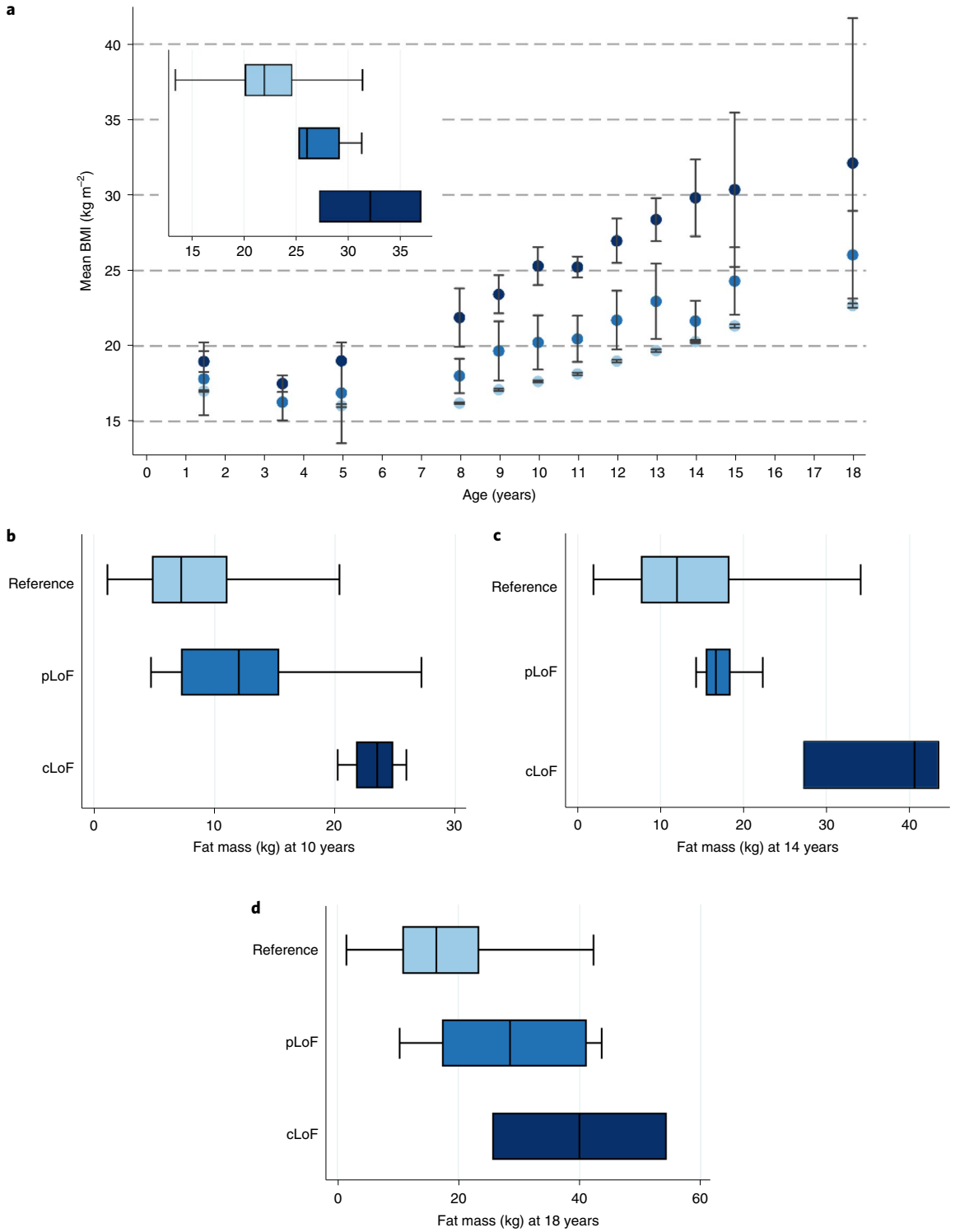

Fig. 2 | BMI at different ages with *MC4R* LoF of cAMP accumulation. a, Mean BMI ± 95% CI at different ages (sample sizes across all ages presented in Supplementary Table 4) and box plot showing distribution (median, interquartile range (IQR) and range) of BMI at age 18 years ($n = 3{,}499$). **b**–**d**, Box plots showing distribution (median, IQR and range) of the earliest (10 years; $n = 5{,}109$), middle (14 years; $n = 4{,}295$) and last (18 years; $n = 3{,}408$) measure of fat mass with *MC4R* LoF of cAMP accumulation (carriers of pLoF and cLoF) and the reference group (non-LoF carriers, combining individuals with synonymous, common variations or no LoF mutation and those with WT-like and GoF mutations). The figure only shows results where all LoF mutations (pLoF and cLoF mutations) were represented by at least one individual at all time points between birth and 24 years. Reference, pLoF and cLoF groups are depicted in light, medium and dark blue, respectively.

on the likely pathogenicity of all known mutations (https://www.mc4r.org.uk/) are very helpful to clinicians in this regard but, until every possible mutation has been systematically generated and characterized as has been undertaken with *PPARG*[34], for example, such databases will remain incomplete. The mutations found in the present study had a largely similar impact on β-arrestin-2 coupling

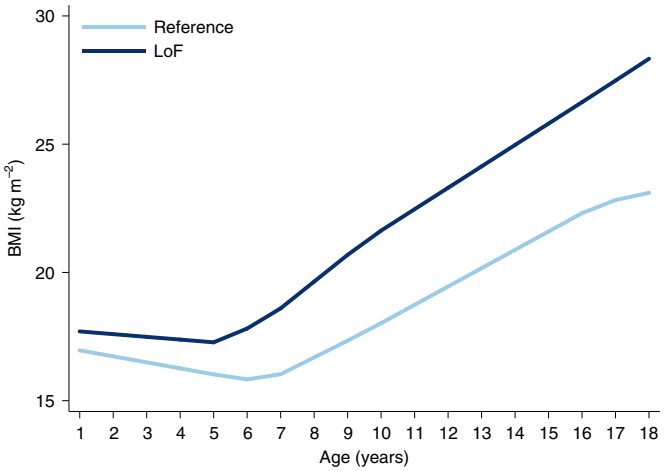

Fig. 3 | Association between *MC4R* LoF of cAMP accumulation with BMI trajectory between the ages of 18 months and 18 years using linear-spline multilevel models. Values for the reference group (all individuals with synonymous, common variations or no LoF mutation and those with WT-like and GoF mutations) and LoF mutations (combining pLoF and cLoF mutations) are depicted in light and dark blue, respectively ($n = 5,716$). Estimates and CIs of these associations are presented in Supplementary Table 12 and were generated using linear-spline multilevel models.

and cAMP accumulation (and thus, had a largely similar impact on anthropometric traits) and do not, therefore, contribute to addressing questions around the relevance of biased signaling.

We previously reported that obese patients with *MC4R* LoF mutations have a lower BP compared to similarly obese non-LoF carriers[27]. In this study, while there was a trend for an inverse association between *MC4R* LoF and BMI-adjusted measurements of both arterial and central cardiovascular health, there was no clear indication of lower BP in the *MC4R* carriers across the lifecourse. This finding, albeit restricted to a limited age range and sample size, is consistent with previous observations regarding the cardiovascular effects of *MC4R* functional impairment[27].

Genome-wide PRSs associated with BMI have recently received considerable attention as possible predictors of phenotypes such as obesity[28]. In that context, it is notable that the impact of carrying a functionally impaired *MC4R* locus on BMI was approximately double that of the common PRS used previously (comparing the lower 90th and upper 10th percentiles of the continuous PRS distribution). Indeed, this was seen to be an effect which was persistent after having adjusted for PRS. This observation is not incompatible with the possibility of a buffer or enhancer-effect being present as a result of the individual-level combination of rare genetic changes and PRS value[35]. However, results here do suggest that the rare changes at *MC4R* are likely to have a larger impact than more subtle and continuous on-average differences delivered by theoretically additive PRS contributions at an individual level, a contrast to the nature of effect when considering total population variance explained.

A particular advantage of ALSPAC is the availability of robust longitudinal phenotyping data throughout childhood, adolescence and adulthood. Childhood obesity is strongly associated with adverse cardiometabolic outcomes in later life[36]. However, it seems that long-term adverse health consequences of childhood obesity are driven by the tendency of the obese phenotype to persist into adult life[37,38]. While it is conceivable that rescue of the phenotype at this stage of development would reduce cardiometabolic risk (also suggested in our analyses when adjusting for BMI), we know from a longitudinal assessment of adult *MC4R* mutation carriers, that penetrance of the phenotype increases with age[19]. It therefore seems likely that the obese phenotype of *MC4R* LoF mutation carriers in ALSPAC cohort will persist or may even worsen with age.

The main limitations of our study broadly include the sample size and representative value of the ALSPAC study with respect to the wider population. First, there was a relatively small number of individuals carrying *MC4R* LoF mutations identified in the ALSPAC participants sequenced. Despite this, we were able to identify, validate and functionally characterize *MC4R* LoF mutations in addition to assessing their downstream impact on adiposity-related phenotypes within the current sample. The estimates presented should be taken in the context of their precision and overall pattern in these longitudinal data, which we have displayed transparently. Of course, we are likely to be underpowered to detect *MC4R* LoF mutations occurring at a lower frequency in this population, so further analyses such as those presented here conducted in larger populations are warranted.

Second, it is important to acknowledge the limited representation that ALSPAC offers to other populations, even those that are predominantly of European descent. There was evidence for differences in some lifestyle, socioeconomic and anthropometric traits between those sequenced and not sequenced and, while most differences were indeed negligible in real terms, these differences suggested an overrepresentation of healthier individuals of higher socioeconomic position. This may imply that the frequencies and associations of the *MC4R* LoF mutation presented here may not be totally representative of the wider United Kingdom and beyond; however, it is difficult to ascertain whether results presented here are over or under estimates of these characteristics. No single birth cohort, no matter how comprehensively collected, can be assumed to be representative of a complete target sample. Furthermore, given the initial sampling frame for ALSPAC, which captured >80% of all pregnancies in the Greater Bristol region of the United Kingdom in the early 1990s, the study is likely more representative of an unselected population of the wider United Kingdom than other sampling initiatives based on adult volunteers conducted within this field. While greater precision around frequency and associational estimates of *MC4R* LoF mutations described in this study would certainly be afforded by a larger sample, the accuracy of derived estimates would similarly be subject to the representation of such samples.

Our study suggests that *MC4R* LoF mutations contribute substantially to adiposity traits, with effects starting in early childhood and persisting into adult life. Estimates here are complementary to existing studies, though naturally vary given the sampling frame and data type reported here[26]. Despite this, our work suggests that heterozygous mutations that substantially impair the function of the *MC4R* gene may very well be found in several millions of people worldwide and will tend to increase the body weight and adiposity from an early age and persist across the lifecourse. Given the established association between *MC4R* LoF mutations and the complications of obesity such as type 2 diabetes and coronary artery disease, this is of substantial clinical importance to the long-term health of individual carriers who will, on average, likely enter adult life carrying ~15 kg of extra fat mass. With a prevalence of ~1 in 340, MC4R deficiency can no longer be considered a 'rare disease', the definition of which, in the United Kingdom, is a prevalence of <1 in 2,000 and in the United States, is <200,000 affected patients nationally. If the prevalence in the United States reflects that found in the United Kingdom, we would predict that there are around a million Americans whose weight is substantially increased by the carriage of an *MC4R* mutation. Efforts to reduce obesity and maintain a healthy weight in carriers of *MC4R* LoF mutations, through diet and physical activity will likely need to begin early in life and be targeted in nature, to have an optimal chance of reducing the risks of developing obesity later in life. Pharmacological enhancement of residual intact melanocortin signaling could provide a clinically

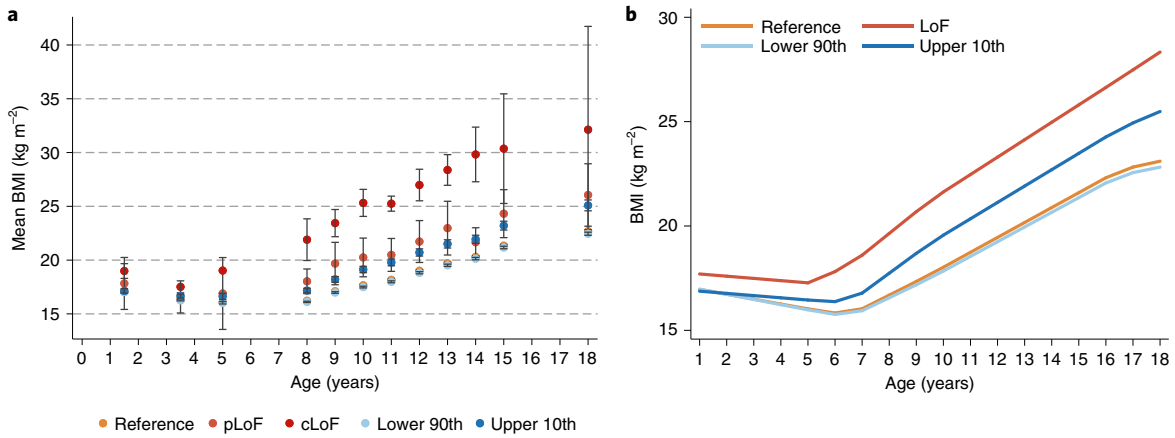

Fig. 4 | Comparison of age-specific and longitudinal associations of *MC4R* LoF of cAMP accumulation and a weighted genome-wide PRS with BMI. **a**, Mean BMI ± 95% CI across ages (sample sizes across all ages for *MC4R* LoF and weighted genome-wide PRS are presented in Supplementary Tables 4 and 16, respectively). **b**, Illustration of BMI trajectory between the ages of 18 months and 18 years using linear-spline multilevel models comparing *MC4R* LoF mutations ($n = 5{,}716$) and weighted genome-wide PRS ($n = 5{,}162$). Estimates and CIs of associations with *MC4R* LoF and weighted genome-wide PRS are presented in Supplementary Tables 12 and 18, respectively. Figures only show results where all LoF mutations for *MC4R* (pLoF and cLoF mutations) were represented by at least one individual at all time points between birth and 24 years for comparison with the weighted genome-wide PRS. The reference group included individuals with synonymous, common variations or no LoF mutation and those with WT-like and GoF mutations.

useful complement to such measures in these patients. The likely size of the population affected should help to stimulate investment in such therapeutic approaches.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-021-01349-y.

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Methods

Study sample and measures. ALSPAC is a large geographically homogeneous prospective birth cohort from the southwest of England established to investigate environmental and genetic characteristics that influence health, development and growth of children and their parents[23,24,39]. Full details of the cohort and study design have been described previously and are available at http://www.alspac.bris.ac.uk. Please note that the study website contains details of all the data that are available through a fully searchable data dictionary and variable search tool (http://www.bristol.ac.uk/alspac/researchers/our-data/).

Briefly, 14,541 pregnant women residing in the former county of Avon with an estimated delivery date of between 1 April 1991 and 31 December 1992 (inclusive) were enrolled to the study. Out of those initially enrolled, 13,988 children who were alive at 1 year of age and have been followed up to date with measures obtained through regular questionnaires and clinical visits, providing information on a range of behavioral, lifestyle and biological data. When the oldest children were approximately 7 years of age, an attempt was made to bolster the initial sample with eligible cases who had failed to join the study originally. As a result, when considering variables collected from the age of seven onwards (and potentially abstracted from obstetric notes), there are data available for more than the 14,541 pregnancies mentioned above. The number of new pregnancies not in the initial sample (known as phase I enrollment) that are currently represented on the built files and reflecting enrollment status at the age of 24 is 913 (456, 262 and 195 recruited during phases II, III and IV, respectively), resulting in an additional 913 children being enrolled. The phases of enrollment are described in more detail in the cohort profile paper[23].

The total sample size for analyses using any data collected after the age of 7 years is therefore 15,454 pregnancies, resulting in 15,589 fetuses. Of these, 14,901 were alive at 1 year of age. A 10% sample of the ALSPAC cohort, known as the Children in Focus group, attended clinics at the University of Bristol at various time intervals between 4 to 61 months of age. The Children in Focus group was chosen at random from the last 6 months of ALSPAC births (1,432 families attended at least one clinic). Those excluded were mothers who had moved out of the area or were lost to follow-up and those partaking in another study of infant development in Avon.

Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for biological samples was collected in accordance with the Human Tissue Act (2004) and informed consent for the use of data collected via questionnaires and clinics was obtained from participants following recommendations of the ALSPAC Ethics and Law Committee at the time. Written informed consent was obtained from mothers at recruitment, from the main carers (usually the mothers) for assessments on the children from ages 7 to 16 years and, from age 16 years onwards, the children gave written informed consent at all assessments.

Academic attainment was derived by a questionnaire asking whether the participant was still in full-time education (with possible answers of 'yes' and 'no'), when the participant was aged 18 years. Participant sex was measured from the birth notification as part of the cohort profile. Participant ethnicity was defined as either 'White' or 'non-White' based on a questionnaire issued at approximately 32 weeks gestation completed by the participant's mother. Participant ancestry was confirmed using multidimensional scaling on 1000 Genomes Project imputed data available in the ALSPAC sample. Household income was defined as family income (in GBP) per week when the participant was 33 months old (defined as <£100, £100–199, £200–299, £300–399 or >£400). Age of mother at the birth of her first child was taken from a questionnaire administered during the 18–20 weeks gestational period of the ALSPAC participant. Maternal pre-pregnancy BMI was derived from weight (kg) and height (cm) measures obtained from a questionnaire administered during pregnancy with the ALSPAC participant and calculated as weight divided by the square of height ($kg\,m^{-2}$). Maternal weight gain was taken from obstetric records, calculated as the absolute weight gain from the last minus the first weight measurement (kg).

Highest household social class was a derived variable reflecting the highest social class based on occupation held by the participant's mother or mother's partner at 18 weeks gestation (with levels including 'I, Professional'; 'II, Managerial and technical'; 'IIINM, Skilled non-manual'; 'IIIM, Skilled manual'; 'IV, Partly skilled' and 'V, Unskilled'). Maternal and paternal education were derived from a questionnaire administered to the participant's mother at 32 weeks gestation asking whether she and her partner had various qualifications, combined into a single variable reflecting her and her partner's highest educational qualification (with levels including 'Certificate of Secondary Education/none', 'Vocational', 'O level', 'A level' and 'Degree'). Parity was defined as the number of previous pregnancies that the participant's mother had that resulted in either a live or still birth, obtained from a questionnaire administered at 18–20 weeks gestation.

Length and weight of each participant were measured at birth and at 4, 8, 12 and 18 months. Height (to the nearest mm) and weight (to the nearest 50 g) were measured from 25 months to 24 years. For weight, the participant was encouraged to pass urine and undress to their underclothes. For height, children were positioned with their feet flat and heels together, standing straight so that their heels and shoulders came into contact with the vertical backboard. Equipment used (for example, Harpenden Neonatometer or Stadiometer, Kiddimetre and Leicester measure for height and the Fereday 100 kg combined scale, Soenhle scale, Seca scale and Tanita Body Fat Analyzer for weight) for each measurement was comparable. In addition to the height and weight measures obtained at ALSPAC clinics, weight and height measures derived from other sources (specifically, mother-completed questionnaires and health visitor records) between the ages of 4 months and when participants were 10 years were used to supplement available clinic values[40]. BMI at all ages was calculated as weight or length (kg) divided by height (m) squared.

Both waist and hip circumferences were measured when the participants were a mean age of 8, 10, 12 and 24 years. Waist circumference was measured to the nearest mm at the minimum circumference of the abdomen between the iliac crests and the lowest ribs. Hip circumference was measured to the nearest mm at the point of maximum circumference around the participant's hips. WHR was calculated as the ratio of these two measurements.

Fat and lean masses (kg) were measured when participants were a mean age of 10, 12, 14, 15, 18 and 24 years using the Lunar prodigy narrow fan beam densitometer DXA scanner. The participant was asked to lie on the machine (in light clothing without any metal fastenings) and encouraged to keep as still as possible while the arm of the machine moved over and two sources of X-ray scanned the participant.

Arterial BP was measured when participants were a mean of 3, 4, 5, 8, 10, 12, 13, 14, 15, 18 and 24 years old, with the appropriately sized cuff. Equipment used included Dinamap vital signs monitors (models 9300, 9301 and 8100) and Omron oscillometric devices (models MI-5, 705 IT, IntelliSense M6 and BP Cuff), which were comparable. Additionally, when participants were a mean age of 18 and 24 years, measures of cardiac structure and function were obtained. Of these measures, we used information about central BP and LVMI scaled by height to the power of 2.7 ($g\,m^{-2.7}$), as proxies for cardiovascular health[41]. Central BP was measured using radial artery tonometry with a SphygmoCor Px Pulse Wave Analysis System (Atcor Medical) at both age 18 and 24 years. Echocardiography was performed using a HDI 5000 ultrasound machine (Phillips) and P4-2 Phased Array ultrasound transducer (at age 18 years) and a Philips EPIQ 7G Ultrasound System (at age 24 years) using a standard examination protocol and left ventricular mass was estimated according to American Society of Echocardiography guidelines[42].

Full details of all measures used in this study are available at the online dictionary at http://www.bristol.ac.uk/alspac/researchers/our-data/.

Detection of *MC4R* mutations by pooled sequencing. The pooled *MC4R* sequencing workflow used in the study is shown in Extended Data Fig. 10. The workflow was broadly divided into the 'Discovery' phase and the 'Validation' phase. In the 'Discovery' phase', a small aliquot of the original DNA sample was combined with 49 other samples into a DNA pool and this was followed by high-throughput sequencing (HTS) of the pool to identify variation in the *MC4R* gene. Next, in the second 'Validation' phase, for each pool containing one or more variants of interest (in this case rare variants with MAF < 0.01%), we went back to all of the 50 original DNA samples and re-sequenced these variants using the traditional Sanger method. The main objectives for this phase were to (1) orthogonally validate the variant discovery from HTS; (2) identify the variant carriage; and (3) establish the zygosity of the carriage.

Pooled HTS of *MC4R*. A total of 20 ng of 5,993 DNA samples from ALSPAC were randomly combined into pools of 50 at the MRC Biorepository Unit. Then, 10 ng of pooled DNA was used for *MC4R* exon PCR with Q5 Hot Start High-Fidelity DNA polymerase (NEB) and *MC4R* exon primers −27 bp upstream and +104 bp downstream of the protein-coding region (Extended Data Fig. 10 and Supplementary Table 20). The PCR product was purified using Agencourt Ampure XP beads (Beckman Coulter) and quantified using a QuantiFluor dsDNA system (Promega) and Tecan Infinite M1000 Pro plate reader. Sequencing libraries were constructed from 1 ng of purified PCR product using the Nextera XT Library Preperation kit with Nextera XT Index V2 barcodes (Illumina) according to manufacturer's instruction. The final libraries were purified using Agencourt Ampure XP beads. Purified libraries were quantified by quantitative PCR using the Kapa Library quantification kit (Roche) on a Quantstudio 7 Flex Real-Time PCR instrument (Thermo Fisher Scientific). Finally, the libraries were combined at 10 nM for sequencing both ends for 150 bp (PE150) on the Illumina HiSeq 4000 instrument at the Cancer Research UK Cambridge Institute Genomics Core. We achieved an even coverage throughout the protein-coding region of *MC4R*, with a mean sequencing depth at 43,654 ± 356-fold per pool (Extended Data Fig. 10).

Sequencing bioinformatics. Sequence reads were mapped using BWA MEM algorithm (v.0.7.12) onto the Human GRCh38 (hg38) genome. PCR duplicates were removed using Picard v.1.127 followed by indel realignment and base quality score recalibration using GATK v.3.8 according to GATK best practices. The variant calls were generated by Varscan v.2.4.2 mpileup2snp and mpileup2indel function with the following criteria: variant allele frequency (VAF) ≥ 0.05%, coverage ≥ 100, $P < 0.05$ and strand filter set to 'on'. To maximize variant detection sensitivity, we started with an initial VAF cutoff at 0.5%, which was lower than the theoretical value of 1% to allow for technical errors and experimental bias, with an

expectation of detecting false positives. The cutoff was readjusted using validation results from Sanger sequencing (see below).

Variant validation and rare variant carrier identification. Original DNA samples from all rare variant containing pools (except p.V103I and p.I251L) were retrieved for variant validation using traditional Sanger sequencing. The *MC4R* coding region was amplified using GoTaq Green (Promega) Master Mix with 10 ng DNA per 10 µl PCR reaction and *MC4R* exon primers used in next-generation sequencing (Supplementary Table 20). *MC4R* PCR cycling conditions were as follows: one cycle of Hot Start at 95 °C for 5 min, then 35 cycles of the following: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 2 min. Then, there was one cycle of final extension at 72 °C for 5 min.

Unincorporated primers and dNTPs were removed from the PCR reactions by digesting with exonuclease I (Exo) (NEB) and shrimp alkaline phosphatase (SAP) (NEB) as follows: 20 units of Exo and 1 unit of SAP were added directly to the 10 µl PCR reaction; the Exo/SAP reaction was then incubated at 37 °C for 20 min and then enzymes were deactivated by incubating at 80 °C for 15 min. This Exo/SAP PCR reaction was then used as the template for the Sanger sequencing reaction.

Sanger sequencing reactions were set up using BigDye Terminator v.3.1 Cycle Sequencing kit (Thermo Fisher Scientific) in a 10-µl reaction using 0.5 µl of BigDye Terminator v.3.1, 2 µl 5× sequencing buffer, 0.5 µM sequencing primer and 1 µl of the Exo/SAP PCR product, which was made up to 10 µl using nuclease-free water. The Sanger sequencing cycling conditions were as follows: denaturation at 95 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C for 4 min. This program was continued for 24 cycles in total.

Sanger sequencing reactions had unincorporated dye and primers and dNTPs removed using AxyPrep MAG PCR Clean-Up kit (Axygen) according to manufacturer's instructions. Purified sequencing products were resuspended in 30 µl nuclease-free water. Sanger sequencing reactions were analyzed on a 3730 DNA Analyzer (Thermo Fisher Scientific). Sanger sequencing data files were analyzed using Sequencher v.4.8 Build 3767 (Gene Codes Corporation).

Specificity and sensitivity of pooled sequencing. Excluding p.V103I and p.251L, the initial screen using a VAF 0.5% cutoff resulted in a total of 38 rare, nonsynonymous *MC4R* variants with an estimated carriage of 57 individuals. Of these, 40 individuals carrying 27 unique variants were confirmed as true positives (TPs) by Sanger sequencing (Extended Data Fig. 10). The mean (± s.d.) VAF detected for TP was 1.18 (± 0.39%). We found a strong relationship between VAF and specificity, where all variants called at VAF < 0.60% were false positives (Extended Data Fig. 10), this indicates the likelihood of missing any true potential variants at VAF of <0.5% was extremely low. We also performed receiver operating characteristic curve analysis using GraphPad Prism v.6 and showed that VAF was a strong predictor for variant detection (area under the curve (AUC) = 0.976; Extended Data Fig. 10). Using findings from receiver operating characteristic curves, we adopted a final VAF cutoff at ≥0.60% and improved specificity to 88.89%, while retaining all 40 TP calls for downstream analysis.

To establish method sensitivity, we compared our *MC4R* variant call set with another ALSPAC WES study of 2,971 individuals. The *MC4R* locus in this study was sequenced at a depth of 28.15×. Within the overlap of 2,451 (out of 5,724) unique individuals sequenced in both studies (Extended Data Fig. 10), we found that the TP variant call sets were 100% concordant (Extended Data Fig. 10). This implies the sensitivity from our new pooled sequencing method was on par with standard WES.

Functional characterization of MC4R mutations in vitro. *cAMP accumulation assay.* CV-1 in Origin with SV40 genes 7 (COS-7) cells were maintained in a growth medium containing low-glucose Dulbecco's modified Eagle's medium (Invitrogen), 10% fetal bovine serum (Invitrogen), 1% Glutamax (Invitrogen), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Sigma-Aldrich). COS-7 cells were kept at 37 °C humidified air with 5% CO_2.

Site-directed mutagenesis was performed on WT human N-FLAG-MC4R PCDNA3.1(+) using an Agilent QuikChange Lightning kit to generate all seven previously uncharacterized *MC4R* variants for cAMP activity measurement.

Thirty nanograms of plasmid carrying *MC4R* WT and variants was transfected into COS-7 cells using (Lipofectamine 2000, Invitrogen) for 24 h. NDP-α-MSH, dissolved in 0.1% bovine serum albumin and 1 mM acetic acid at a stock concentration of 5 mM, was added to cells at increasing final concentrations of 10^{-12} to 10^{-6} M in growth medium for 2 h, before intracellular cAMP concentration measurement using a luminescence-based HitHunter cAMP assay for small molecules (cat. no. DiscoverX 90-0075SM25 Eurofins DiscoverX) and a Tecan Spark 10M microplate reader. The baseline and maximal luminescence signal was normalized to *MC4R* WT and a four-point sigmoidal dose–response curve was fitted to normalized values from all replicates to determine the E_{max} and $logEC_{50}$ using GraphPad Prism v.7. Differences in estimated $logEC_{50}$ and E_{max} were determined using an extra sum-of-squares F-test. Due to the lack of response, we did not perform a curve fit for cLoF variants and only determined the relative E_{max} based on cAMP level measured at 10^{-6} M NDP-α-MSH and the difference was measured using a two-sided Student's *t*-test.

β-arrestin-2 coupling assay. To examine interactions between MC4R and β-arrestin-2, we used the NanoBiT protein/protein interaction assay (Promega). *MC4R* WT and variants were cloned into the pBiT1.1-C(TK/LgBiT) vector. Fifty nanograms of *MC4R*-LgBiT and *ARRB2*-SmBiT were co-transfected into HEK293 cells as described by Lotta et al.[26]. HEK293 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Invitrogen), 10% fetal bovine serum (Invitrogen), 1% Glutamax (Invitrogen), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Sigma-Aldrich). HEK293 cells were kept at 37 °C humidified air with 5% CO_2. At 24 h after transfection, culture medium was replaced with Opti-MEM I medium (Invitrogen) 30 min before luciferase activity was measured by the Tecan Spark 10M microplate reader set at 37 °C and 5% CO_2. After 2.5 min, 20 µl of Nano-Glo Live Cell Assay System (Promega) was added and luciferase activity was measured for 10 min to generate the baseline signal. Cells were then stimulated with NDP-α-MSH at 10^{-12} to 10^{-6} M and luciferase activity was monitored for another 30 min. The AUC above the baseline was then used to determine coupling between MC4R and β-arrestin-2. For each individual experimental replicate, AUC values were normalized to % maximum AUC of *MC4R* WT from the same experiment and a three-point sigmoidal dose–response curve was fitted to determine E_{max} and $logEC_{50}$. The average E_{max} and $logEC_{50}$ values were used for LoF determination. The $logEC_{50}$ was not used for cLoF mutants that exhibited no response. All calculations were performed with GraphPad Prism v.6. Differences in mean $logEC_{50}$ and E_{max} values were determined using a two-sided Student's *t*-test.

Identification of rare variant carriers. Once we completed the functional characterization of *MC4R* mutations, we unencrypted sequenced pools to identify specific individuals carrying these mutations. This allowed the phenotypic characterization of such functional impairment of *MC4R*. Of the 5,993 individuals sequenced, 5 had missing identifier information for linkage with the wider ALSPAC dataset and 214 were duplicated; therefore, these exclusions left 5,774 participants in the sequenced set (note that none of the excluded individuals had an *MC4R* LoF mutation). After merging in all required clinic and questionnaire data from the ALSPAC cohort, there were related individuals (siblings) within the total sample. For appropriate comparisons between those included within and excluded from the sequenced set, all related individuals were excluded. Specifically, there were one set of quadruplets (none of whom were in the sequenced set), four sets of triplets (one full set of which was in the sequenced set) and 255 sets of twins (48 complete sets of which were in the sequenced set). In addition to these 48 pairs of twins in the sequenced set, there were 35 sets of twins where one twin was in the sequenced set and the other twin was not. To avoid removing as many individuals from the sequenced set as possible, the twin not in the sequenced set was removed in these instances ($n = 35$).

A total of 216 individuals were excluded from the total sample to remove siblings (note that, at this point, none of these were from the sequenced set), which included 35 individuals (1 of a pair of twins not in the sequenced set), 3 individuals from a quadruplet, 2 individuals each from three triplets ($n = 6$) and 172 individuals from twin sets. Then, siblings in the sequenced set were removed, which included the two individuals from the one triplet set and one individual from each of the 48 twins ($n = 50$). After all exclusions, there were 5,724 individuals left in the sequenced set for all analyses.

To identify and estimate prevalence of carriers of LoF mutations, tabulations were used, separated by GoF, WT-like, pLoF and cLoF mutations in the sequenced set of ALSPAC participants.

Statistical analyses. For this paper, we focused on *MC4R* LoF of cAMP production as our main analysis, with comparison to impairment of β-arrestin-2 coupling and to a genome-wide PRS comprising over 2 million common genetic variants as sensitivity analyses. There was little evidence for a difference in the phenotypic effect of *MC4R* LoF mutations across ethnicities (all estimates and CIs overlapped) and no evidence of overt relatedness (maximum values were ten-times less than first cousins) across mutation carriers. All analyses were conducted using Stata (v.15 and 16) and MLwiN v.3.04 called from Stata using the runmlwin command[43].

Representation. To explore how representative the participants of the sequenced set were of the wider ALSPAC cohort, measures of education, socioeconomic status and parental factors were compared between individuals within the sequenced set and those not in the sequenced set. These variables were selected based on those used in previous papers comparing the ALSPAC cohort with statistics from a national sample of the United Kingdom[23,24]. These measures included academic attainment, sex, ethnicity, household income, maternal age at birth of first child, maternal pre-pregnancy BMI, maternal weight gain during pregnancy, highest household social class, parental education and parity (see above for details on how these were measured).

Means and s.d. of all continuous variables and percentages of binary or categorical variables were calculated and Student's *t*-tests were used to test whether summary statistics were different between participants included in the sequenced set and those in the wider ALSPAC cohort.

Age-specific associations with anthropometric traits. Age-specific analyses were conducted using linear regression across all available measures of BMI, height,

weight, WHR, fat mass and lean mass between birth and 24 years. All individuals with data on the *MC4R* mutations, anthropometric trait and sex were included in each model (complete case analysis); therefore, sample sizes differ across ages and anthropometric trait. For interpretability, we present estimates on all measured units (for example, kg m^{-2} and kg for BMI and weight, respectively); however, for the purposes of reference, we also present results for BMI on a standardized scale.

MC4R LoF produced by the mutations was analyzed by comparing carriers of LoF mutations (all individuals with pLoF and cLoF mutations) to 'non-LoF carriers' (all individuals with synonymous or common variants and no LoF mutation and those carrying WT-like or GoF mutations) as the reference group. Effect estimates therefore represent the mean difference in each anthropometric trait in carriers versus noncarriers of *MC4R* LoF mutations. Associations were adjusted only for participant sex. We also assessed the effect of carriage of WT-like mutations on BMI, weight and height compared to those with no detected LoF variant.

As *MC4R* mutation carriers have previously been reported to have lower BP than equivalently obese WT individuals, we additionally examined the association between *MC4R* LoF (defined in the same way as above) on clinically relevant obesity-related traits. Age-specific analyses were therefore conducted with measures of arterial and central BP and LVMI across the lifecourse, as proxies for cardiovascular health, both in a sex-adjusted model and a model additionally adjusted for BMI measured at the same occasion.

We also plotted the mean anthropometric trait at each time point, separating out carriers to the component mutational parts (comparing the reference 'non-LoF carrier' group to pLoF and cLoF separately). Not all individuals with *MC4R* mutations had anthropometric/cardiovascular measurements available at all time points between birth and 24 years and, on some occasions, all LoF groups were not represented (for example, no individuals carrying a cLoF mutation had anthropometric data at age 24 years and many time points before the age of 5 years had such data). In these instances, and for transparency, tables show results at all time points with all contributing individuals (and comment on the effect sizes and precision of these estimates) and figures only show results where all LoF mutational groups (pLoF and cLoF mutations) were represented by at least one individual (for example, from 18 months to 18 years for BMI).

Sensitivity analyses were conducted to assess the effect of carrying versus not carrying GoF mutations. While there was an absence of a detectable effect, there was insufficient analytical power to assess these groups across a meaningful number of time points (results available on request). We also assessed the effect of excluding individuals carrying GoF mutations in the main analyses, leaving only individuals with no LoF mutations and those carrying WT-like mutations in the 'non-LoF carriers' reference group, but this made very little difference to findings (results available on request).

Longitudinal associations with anthropometric traits. Longitudinal analyses using linear-spline multilevel models were conducted to examine the association between *MC4R* mutations and change in each anthropometric trait across the lifecourse. Given the limited number of WHR, fat mass and lean mass observations between birth and 24 years, longitudinal analyses focused on characterizing the association between *MC4R* mutations and BMI, weight and height only. Additionally, given the lack of individuals carrying a cLoF mutation and anthropometric traits at age 24 years, longitudinal analyses were restricted to capture *MC4R*-driven anthropometric variation from the first instance that all LoF mutational groups were represented (18 months for BMI and height and birth weight) to 18 years of age. Multilevel models estimate the mean trajectories of each anthropometric trait, while accounting for nonindependence of repeated measures within individuals, change in scale and variance of measures over time and differences in number and timing of measurements between individuals (using all available data from all eligible participants under a missing-at-random assumption). Linear splines allow knot points to be fitted at different ages to derive periods of change that are approximately linear. All participants with at least one measure of anthropometric traits were included under a missing-at-random assumption to minimize selection bias in trajectories estimated using linear-spline multilevel models (with two levels of measurement occasion and individual).

Knot points were placed as follows for each anthropometric trait based on distribution and longitudinal pattern of measures between the earliest measure and 18 years: at ages 3.5, 5, 8 and 15 years for BMI; at ages 5 and 15 years for height; and at ages 1, 8 and 15 years for weight. Interaction terms between the variable indicating *MC4R* LoF (comprising the 'non-LoF carrier' reference group (individuals with synonymous, common variations or no LoF mutation and individuals with GoF mutations) and carriers of LoF mutations) and each spline were included in the models to estimate the difference in intercepts (earliest anthropometric trait measurement) and slopes (change in anthropometric trait from the earliest measure to 18 years across splines) between *MC4R* LoF. Additionally, interaction terms between sex and each spline were included to estimate the difference in intercepts and slopes between males and females; therefore, models were adjusted for sex.

Sensitivity analyses. Comparison with β-arrestin-2 coupling. To understand the impact of β-arrestin-2-based *MC4R* functional classification on adiposity relative to those acting through cAMP signaling, we examined age-specific associations between β-arrestin-2 LoF mutations with the same anthropometric traits as described above. Functional impairment of β-arrestin-2 coupling was coded in the same way as the main analyses, comparing carriers (those carrying pLoF and cLoF mutations) to non-LoF carriers (individuals with synonymous, common variations or no LoF mutation and those with either WT-like or GoF mutations).

Comparison between rare and common variation. There has been growing interest in the use of the PRSs as predictors of phenotypes such as obesity and related metabolic fluctuations[28]. We assessed the comparative variation in anthropometric traits (namely, BMI) between carriage of *MC4R* LoF mutations with that of a genome-wide PRS comprising over 2 million common genetic variants weighted according to their effect sizes on BMI.

The weighted genome-wide PRS was generated in the same way as that used by Khera et al.[28] and categorized as a binary variable reflecting individuals in the lower 90th and upper 10th percentiles of PRS distribution. Age-specific analyses were conducted in the same way as above, with all available measures of BMI, and longitudinal models were performed on data capturing BMI from 18 months to 18 years of age, where groups of PRS were the independent variable. For direct comparison with effect sizes of the *MC4R* rare variation, these analyses were restricted to individuals who were in the original sequenced set ($n=5{,}724$).

To assess whether the genetic impact of *MC4R* LoF mutations and PRS were interconnected, we adjusted the main age-specific analyses of the association between *MC4R* LoF mutations and BMI for genome-wide PRS at all available ages. We also calculated variance in BMI explained by PRS as compared to *MC4R* LoF mutations using measurement at age 18 years as an exemplar. We took the subset of participants who had both BMI measured at age 18 years and a derived PRS ($n=3{,}164$, including 7 carriers) and ran a linear regression, first with LoF mutation carrier status as the independent variable and second, with PRS as the independent variable (with BMI at age 18 years as the dependent variable in both cases). The R^2 from these models was taken as an estimate of the variance explained in each case.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Full details of the cohort and study design have been described previously and are available at http://www.alspac.bris.ac.uk. Please note that the study website contains details of all the data that are available through a fully searchable data dictionary and variable search tool (http://www.bristol.ac.uk/alspac/researchers/our-data/). ALSPAC data are available through a system of managed open access. Data for this project were accessed under the project number B2891. The application steps for ALSPAC data access are below.

(1) Please read the ALSPAC access policy, which describes the process of accessing the data in detail and outlines the costs associated with doing so.

(2) You may also find it useful to browse the fully searchable research proposals database, which lists all research projects that have been approved since April 2011.

(3) Please submit your research proposal for consideration by the ALSPAC Executive Committee.

You will receive a response within 10 working days to advise you whether your proposal has been approved. If you have any questions about accessing data, please email alspac-data@bristol.ac.uk.

Code availability

Code for data analyses is freely available on request.

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Author contributions

K.H.W., B.Y.H.L., A. Melvin, G.S.H.Y., N.J.T. and S.O.R. designed the study. C.Z., K.R. and K.D. conducted the genomic sequencing. J.M. and I.S.F. contributed to the design of in vitro assays. W.P., J.H.C., K.L., K.D., J.M. and A.W. planned and performed the in vitro studies. B.Y.H.L., A. Melvin and A. Mörseburg conducted bioinformatic analysis and analyzed the data from in vitro experiments. K.H.W. conducted the analysis of phenotype data in the ALSPAC cohort. L.J.C. critically reviewed the analysis in ALSPAC and the manuscript. D.A.H., K.N. and S.N. were involved in early conversations on the project and provided access to phenotypic, genotypic and exome data. K.H.W., B.Y.H.L., A. Melvin, G.S.H.Y., N.J.T. and S.O.R. wrote the manuscript and it was reviewed by all authors.

Competing interests

S.O.R. has undertaken remunerated consultancy work for Pfizer, AstraZeneca, GSK and ERX Pharmaceuticals. The remaining authors declare no competing interests.

Additional information

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Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41591-021-01349-y.

Correspondence and requests for materials should be addressed to N.J.T. or S.O.

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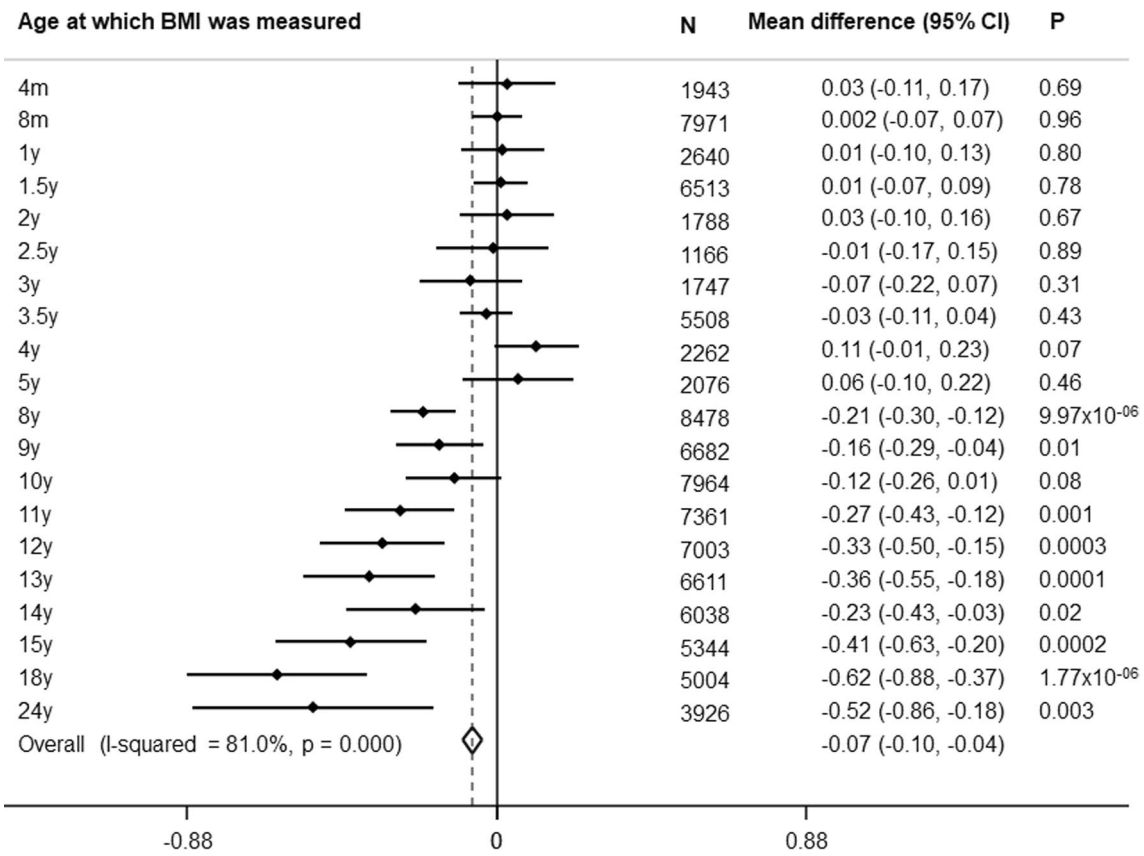
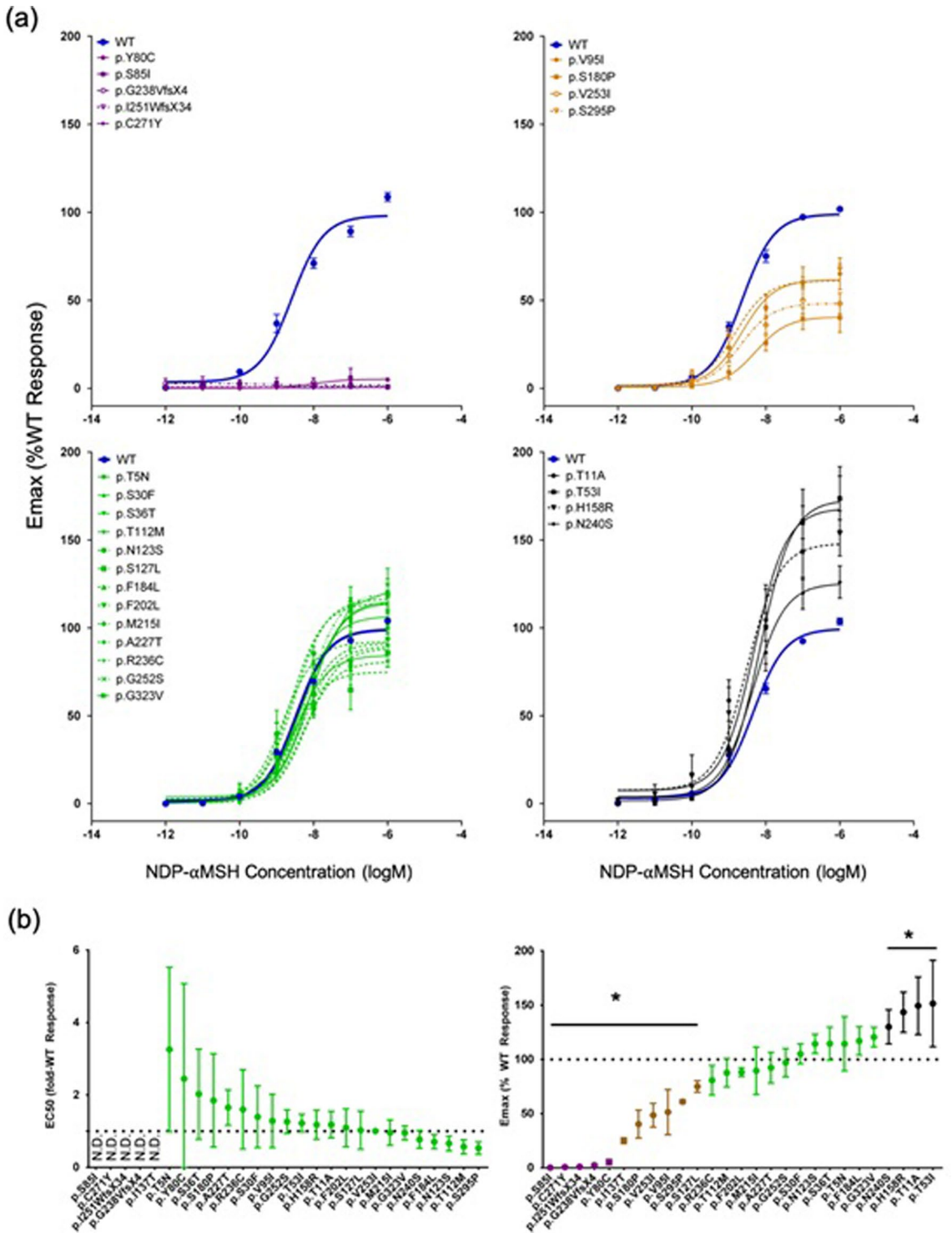
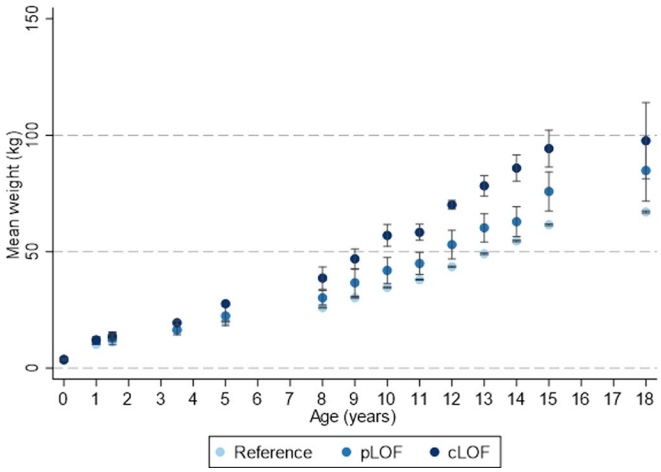


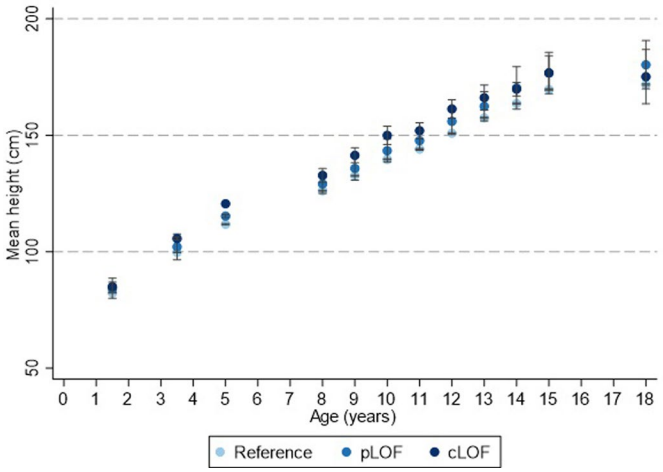
Extended Data Fig. 1 | Differences in body mass index in those sequenced for *MC4R* LoF mutations and those not sequenced from the rest of ALSPAC. Estimates reflect the mean difference (95% CI) in BMI (kg/m^2) comparing those sequenced for *MC4R* LoF mutations to those not sequenced, obtained from two-sided t-tests (p-values were not corrected for multiple comparisons). ALSPAC = Avon Longitudinal Study of Parents and Children; BMI = body mass index; CI = confidence interval; LoF = loss of function.



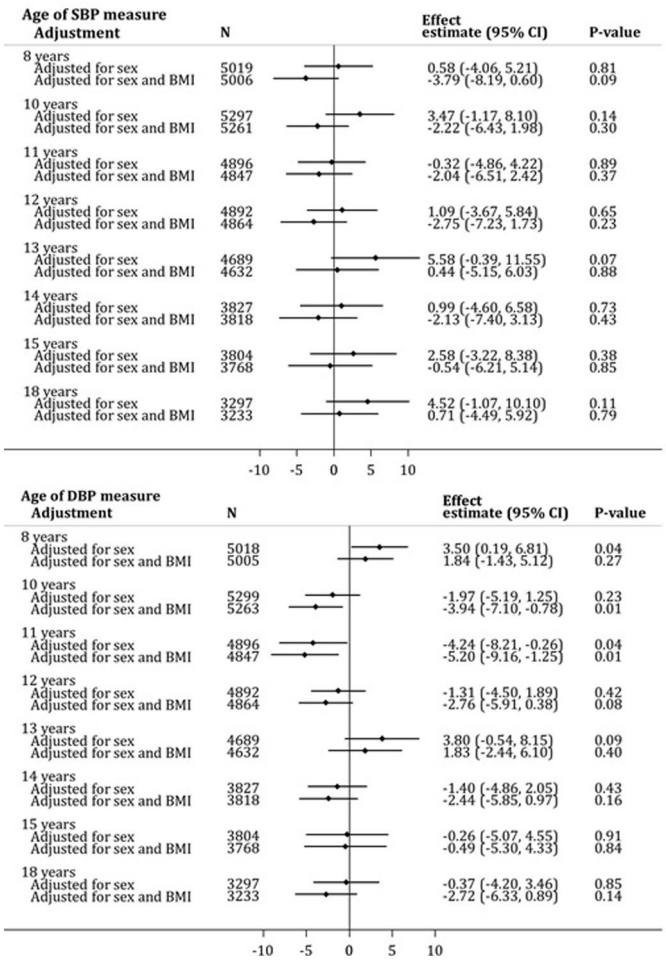
Extended Data Fig. 2 | β-arrestin-2 functional classification of *MC4R* variants. Colors represent cLoF (purple), pLoF (light brown), WT-like (green) and GoF (black). β-arrestin-2 coupling activity of *MC4R* mutations were characterised using a protein-protein interaction assay. **a**, Dose response curves of *MC4R* mutants upon activation by NDP-MSH grouped by cLoF, pLoF, WT-like and GoF compared wild-type *MC4R*. Means + /- S.E.M. are shown (N = 3–8 independent experiments); and **b**, The relative EC50 (fold WT response, left panel) and Emax (% WT response right panel) were determined for each mutants and are presented in mean and 95% CI. * indicates $p < 0.05$ by paired t-test (p-values were two-sided and uncorrected for multiple comparisons). CI = confidence interval; cLoF = complete loss of function; GoF = gain of function; N.D. = not determined; NDP-αMSH = [Nle4,D-Phe7]-α-melanocyte-stimulating hormone; pLoF = partial loss of function; WT-like = wild-type like.



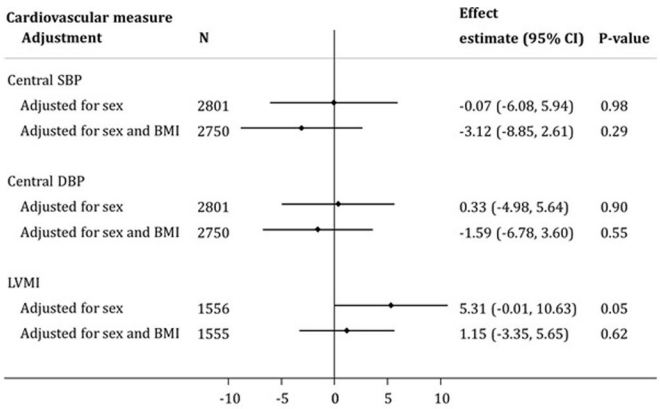
Extended Data Fig. 3 | Mean weight across time with *MC4R* LoF of cAMP accumulation. Mean weight +/− 95% CI at different ages (sample sizes across all ages presented in Supplementary Table 4) with *MC4R* LoF of cAMP (carriers of pLoF and cLoF) and the reference group (that is, non-LoF carriers – combining individuals with synonymous, common variations or no LoF mutations and individuals with WT-like and GoF mutations). Figures only show results where all mutational groups (that is, WT-like, GoF, pLoF and cLoF mutations) were represented by at least one individual at all time points between birth and 24 years. CI = confidence interval; cLoF = complete loss of function; GoF = gain of function; LoF = loss of function; pLoF = partial loss of function; WT = wild-type.



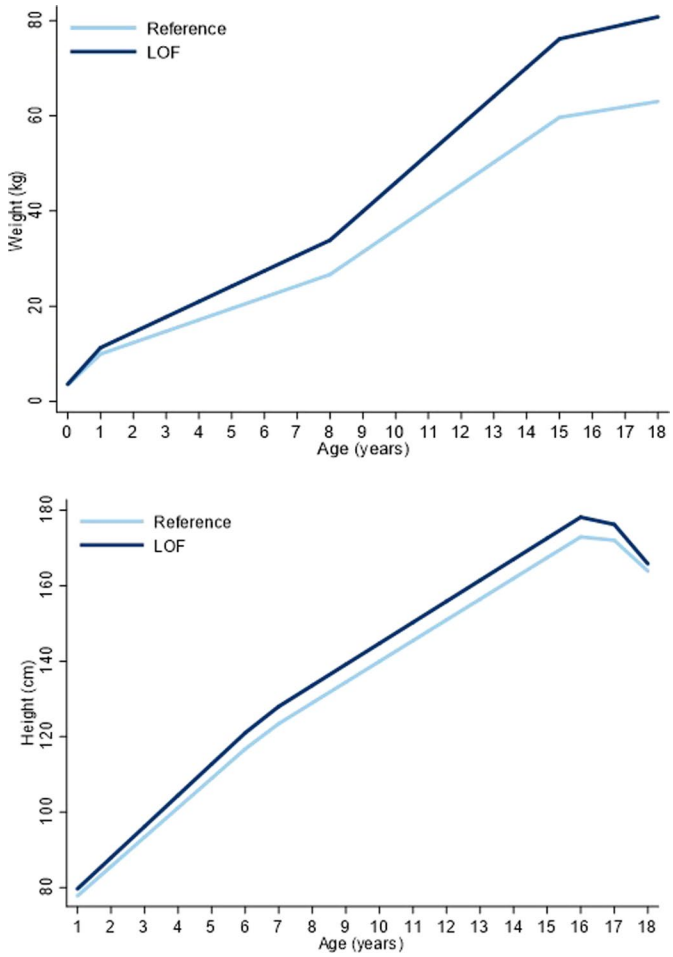
Extended Data Fig. 4 | Mean height across time with *MC4R* LoF of cAMP accumulation. Mean height +/− 95% CI at different ages (sample sizes across ages presented in Supplementary Table 4) with *MC4R* LoF of cAMP (carriers of pLoF and cLoF) and the reference group (that is, non-LoF carriers – combining individuals with synonymous, common variations or no LoF mutations and individuals with WT-like and GoF mutations). Figures only show results where all mutational groups (that is, WT-like, GoF, pLoF and cLoF mutations) were represented by at least one individual at all time points between birth and 24 years. CI = confidence interval; cLoF = complete loss of function; GoF = gain of function; LoF = loss of function; pLoF = partial loss of function; WT = wild-type.



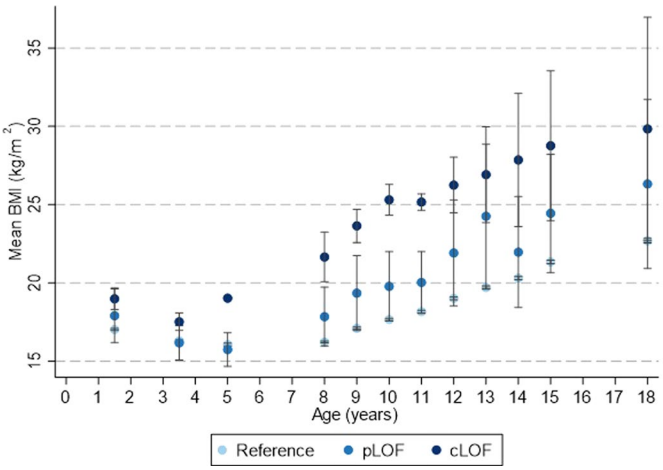
Extended Data Fig. 5 | Association between *MC4R* LoF of cAMP accumulation and arterial BP across age in a model adjusting only for sex and a model adjusted for sex and BMI at the same age. Estimates represent the change (mmHg) in SBP or DBP (top and bottom panel, respectively) in carriers (that is, pLoF or cLoF) vs. non-LoF carriers (that is, individuals with synonymous, common variations or no LoF mutations and individuals with WT-like and GoF mutations) of *MC4R* LoF mutations, obtained from linear regression (p-values presented are two-sided and not corrected for multiple comparisons). BMI = body mass index; BP = blood pressure; CI = confidence interval; cLoF = complete loss of function; DBP = diastolic blood pressure; GoF = gain of function; LoF = loss of function; pLoF = partial loss of function; SBP = systolic blood pressure; WT-like = wild-type like.



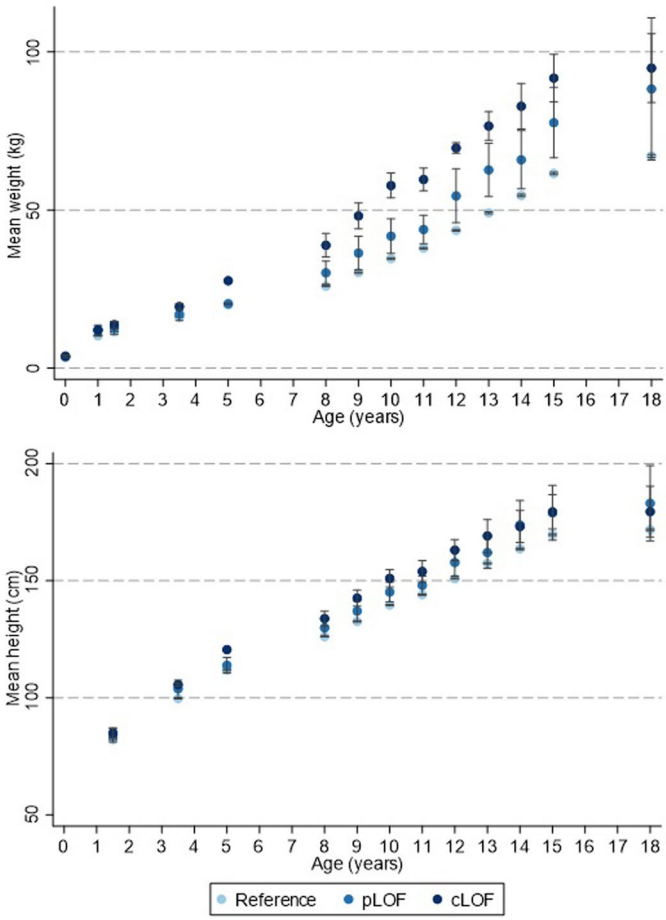
Extended Data Fig. 6 | Association of *MC4R* LoF of cAMP accumulation with both central BP and LVMI at age 18 years in a model adjusting only for sex and a model adjusted for sex and BMI at the same age. Estimates represent the change in central DBP (mmHg), central SBP (mmHg) and LVMI ($g/m^{2.7}$) in carriers (that is, pLoF or cLoF) vs. non-LoF carriers (that is, individuals with synonymous, common variations or no LoF mutations and individuals with WT-like and GoF mutations) of *MC4R* LoF mutations, obtained from linear regression (p-values presented are two-sided and not corrected for multiple comparisons). BMI = body mass index; CI = confidence interval; cLoF = complete loss of function; DBP = diastolic blood pressure; GoF = gain of function; LoF = loss of function; LVMI = left ventricular mass index; pLoF = partial loss of function; SBP = systolic blood pressure; WT-like = wild-type like.



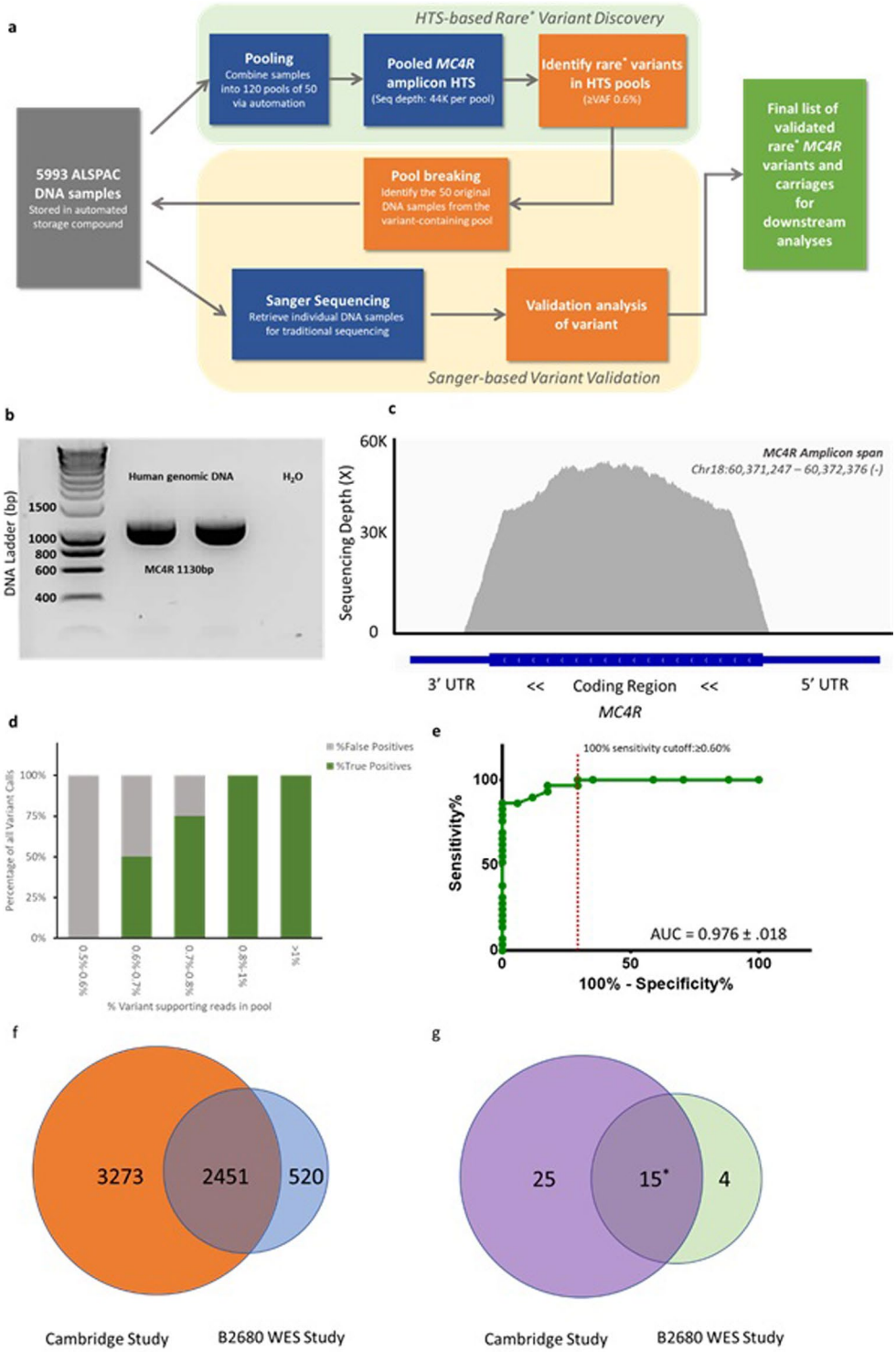
Extended Data Fig. 7 | Association between *MC4R* LoF of cAMP accumulation with weight and height trajectories between birth and 18 years using linear spline multi-level models. Values for the reference group (that is, all individuals with synonymous, common variations or no LoF mutations and individuals with WT-like and GoF mutations) and LoF mutations (that is, combining pLoF and cLoF mutations) are depicted in light and dark blue, respectively (N = 5716). Effect estimates and confidence intervals of these associations are presented in Supplementary Table 11 and Supplementary Table 13 for weight and height, respectively, and were generated using linear spline multi-level models. cLoF = complete loss of function; GoF = gain of function; LoF = loss of function; pLoF = partial loss of function; WT-like = wild-type like.



Extended Data Fig. 8 | Mean BMI across time with *MC4R* LoF of β-arrestin-2 coupling. Mean BMI +/− 95% CI at different ages (sample sizes across all ages presented in Supplementary Table 14) with *MC4R* LoF of β-arrestin-2 (carriers of pLoF and cLoF) and the reference group (that is, non-LoF carriers – combining individuals with synonymous, common variations or no LoF mutations and individuals with WT-like and GoF mutations). Figures only show results where all mutational groups (that is, WT-like, GoF, pLoF and cLoF mutations) were represented by at least one individual at all time points between birth and 24 years. CI = confidence interval; cLoF = complete loss of function; GoF = gain of function; LoF = loss of function; pLoF = partial loss of function; WT = wild-type.



Extended Data Fig. 9 | Mean weight and height across time with *MC4R* LoF of β-arrestin-2 coupling. Mean weight and height +/− 95% CI at different ages (sample sizes across all ages presented in Supplementary Table 14) with *MC4R* LoF of β-arrestin-2 (carriers of pLoF and cLoF) and the reference group (that is, non-LoF carriers – combining individuals with synonymous, common variations or no LoF mutations and individuals with WT-like and GoF mutations). Figures only show results where all mutational groups (that is, WT-like, GoF, pLoF and cLoF mutations) were represented by at least one individual at all time points between birth and 24 years. CI = confidence interval; cLoF = complete loss of function; GoF = gain of function; LoF = loss of function; pLoF = partial loss of function; WT = wild-type.



Extended Data Fig. 10 | Identification of rare *MC4R* variants by pooled sequencing. Pooled *MC4R* sequencing workflow for DNA samples from the ALSPAC cohort; **b**, Agarose gel showing a 1130 bp PCR product using *MC4R* exon primers (Supplementary Table 1), PCR products from 2 independent DNA samples are shown; **c**, A plot showing the sequencing coverage of *MC4R* coding region from a representative pool. The average per-base sequencing depth for all pools was 43,654 ± 356-fold; **d**, The percentage of true positive and false positive calls binned by variant allele frequency (VAF) detected in pools; **e**, Receiver operating curve analysis of VAF and call accuracy; **f**, Comparison of participants included in the current study and another whole-exome sequencing (WES) study (with ALSPAC project number: B2680); **g**, Comparison of non-synonymous variant carriages between this study and WES study. Of 40 mutational carriages found in Cambridge, 15 were found in both studies. 25 carriers found in the Cambridge study were not part of the WES study and 4 carriers found only in the WES study were not part of the Cambridge study. *rare = minor allele frequency < 0.01%, both p.V103I and p.I251L were above 0.01% and excluded in the analyses. ALSPAC = Avon Longitudinal Study of Parents and Children; AUC = area under the curve; HTS = high-throughput sequencing; WES = whole-exome sequencing.

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used
Data analysis	Stata versions 15 and 16; MLwiN version 3.04; BWA MEM version 0.7.12; Picard version 1.127; GATK version 3.8; Varscan version 2.4.2; Sequencher version 4.8 build 3767; Graphpad Prism versions 6 and 7.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Full details of the cohort and study design have been described previously and are available at http://www.alspac.bris.ac.uk. Please note that the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool (http://www.bristol.ac.uk/alspac/researchers/our-data/). ALSPAC data are available through a system of managed open access. Data for this project was accessed under the project number B2891. The application steps for ALSPAC data access are:
1. Please read the ALSPAC access policy which describes the process of accessing the data in detail, and outlines the costs associated with doing so.
2. You may also find it useful to browse the fully searchable research proposals database, which lists all research projects that have been approved since April 2011.
3. Please submit your research proposal for consideration by the ALSPAC Executive Committee.

You will receive a response within 10 working days to advise you whether your proposal has been approved. If you have any questions about accessing data, please email alspac-data@bristol.ac.uk.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The purpose of this paper was to estimate the frequency of mutations across the MC4R gene region and downstream impact of a rare mutation within pre-existing data; therefore, all participants were included.
Data exclusions	Data were excluded if participants were duplicated, had missing ID information or were related.
Replication	The sequencing technique used to identify rare variants using NGS was verified with whole exome Sanger sequencing (see Extended Data 10) and all the rare, true positive hits were successfully validated. No other replication was performed. A comparative analysis with Broad's WES dataset was further performed, we found all rare variants called via the two sequencing pipelines were 100% concordant. Functional characterisation of variants were validated at least 3 times.
Randomization	This is not relevant to the study as the analyses focused on calculating the frequency and downstream impact of a rare mutation within pre-existing data from a cohort study - this was an observational study.
Blinding	This is not relevant to the study as the analyses focused on calculating the frequency and downstream impact of a rare mutation within pre-existing data from a cohort study - this was an observational study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	We used COS7 and HEK293 cells, both from the lab stock and screened in the past year for mycoplasma.
Authentication	None of the cell lines were authenticated.
Mycoplasma contamination	The cell lines tested negative for contamination in the past year for mycoplasma.
Commonly misidentified lines (See ICLAC register)	N/A

Human research participants

Policy information about studies involving human research participants

Population characteristics	The Avon Longitudinal Study of Parents and Children (ALSPAC) is a large geographically-homogeneous prospective birth cohort from the southwest of England established to investigate environmental and genetic characteristics that influence

	health, development and growth of children and their parents. Full details of the cohort and study design have been described previously and are available at http://www.alspac.bris.ac.uk.
Recruitment	Full details of the cohort and study design have been described previously and are available at http://www.alspac.bris.ac.uk. This is an established cohort study where selection bias/representative nature of the study have already been described in detail (e.g., Boyd et al. International Journal of Epidemiology 42, 111-127 (2013); and Fraser et al. International Journal of Epidemiology 42, 97-110 (2012)). In addition, we have discussed the likely limitations of selection and representativeness in discussion of the current manuscript).
Ethics oversight	Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for biological samples has been collected in accordance with the Human Tissue Act (2004) and Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time. Written informed consent was obtained from mothers at recruitment, from the main carers (usually the mothers) for assessments on the children from ages 7 to 16 years and, from age 16 years onwards, the children gave written informed consent at all assessments.

Note that full information on the approval of the study protocol must also be provided in the manuscript.