Title: Contributions of function-altering variants in genes implicated in pubertal timing and body mass for self-limited delayed puberty

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Word count: 3684

Keywords: Puberty; delayed puberty; FTO; paediatric endocrinology, constitutional delay

Short title: Role of GWAS genes in self-limited delayed puberty

Declaration of interest:

The authors declare no conflict of interest

Funding:

SRH is funded by The Wellcome Trust (102745), Rosetrees Trust (M222) and the Barts and the London Charity (417/1551). AP is funded by Bart's & The London Charity Strategic Research Grant (MEAG1l6R). LG is funded by the Biotechnology and Biological Sciences Research Council (BB/L002671/1). LD is partly supported by the Academy of Finland (14135). MRB and CPC are funded by the National Institutes for Health Research (NIHR), and this work forms part of the portfolio of translational research of the NIHR Biomedical Research Unit at Barts. AD is funded by the MRC (MR/K021613/1). DR, GSHY, SOR and APC are funded by the Medical Research Council (MRC) Metabolic Disease Unit (MRC_MC_UU_12012/1) and animal work was carried out with the assistance of MRC Disease Model Core of the Wellcome Trust MRC Institute of Metabolic Sciences (MRC_MC_UU_12012/5.)

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Contributions of function-altering variants in genes implicated in pubertal timing and body mass for self-limited delayed puberty

Abstract

Context: Self-limited delayed puberty (DP) is often associated with delay in physical maturation, but whilst highly heritable the causal genetic factors remain elusive. Genome-wide association studies of the timing of puberty have identified multiple loci for age of menarche (AAM) in females and voice break in males, particularly in pathways controlling energy balance.

Objective/Main outcome measures: We aimed to assess the contribution of rare variants in such genes to the phenotype of familial DP.

Design/Patients: We performed whole exome sequencing (WES) in 67 pedigrees (125 individuals with DP and 35 unaffected controls) from our unique cohort of familial self-limited DP. Using a WES filtering pipeline one candidate gene (*FTO*) was identified. *In silico*, *in vitro* and mouse model studies were performed to investigate the pathogenicity of *FTO* variants and timing of puberty in $FTO^{+/-}$ mice.

Results: We identified potentially pathogenic, rare variants in genes in linkage disequilibrium with GWAS of AAM loci in 283 genes. Of these, 5 genes were implicated in the control of body mass. After filtering for segregation with trait one candidate, *FTO*, was retained. Two *FTO* variants, found in 14 affected individuals from 3 families, were also associated with leanness in these DP patients. One variant (p.Leu44Val) demonstrated altered demethylation activity of the mutant protein *in vitro*. $Fto^{+/-}$ mice displayed a significantly delayed timing of pubertal onset ($p < 0.05$).

Conclusions: Mutations in genes implicated in body mass and timing of puberty in the general population may contribute to the pathogenesis of self-limited DP.

Introduction

Puberty is the maturational process of the reproductive endocrine system that results in adult height and body proportion, in addition to the capacity to reproduce. A minimum level of energy availability is required for the onset of puberty, whilst increased fat mass has been shown to be associated with precocious onset of puberty[1,2]. However, a role for genes connected with regulation of body mass have not been clearly demonstrated in pubertal timing.

The existence of genetic heterogeneity in pubertal timing is supported by several large genome wide association studies (GWAS) of the age of menarche (AAM)[3-5]. Evidence ($P< 5 \times 10^{-8}$) for 123 signals at 106 genomic loci has been identified. Many of these loci were associated with Tanner staging in both sexes, suggesting this data is applicable to both men and women(6,7).

The first of many GWAS loci associated with AAM was the developmental gene *LIN28B*[3,8]. Additional signals in genes involved in energy homeostasis and growth have been found near *LEPR-LEPROT*, which encodes the leptin receptor. Leptin (a key regulator of body mass) is an important permissive signal for the onset of puberty[9]. In addition to leptin signaling, overlap with several genes implicated in body mass index was found, including *FTO*, *SEC16B*, *TMEM18*, and *NEGR1* (Supplementary Table 1) (5). Whether such

genes may regulate pubertal timing exclusively via impact on fat mass or via other BMI-independent mechanisms is unknown [10].

Disordered pubertal timing affects up to 5% of adolescents and is associated with adverse health and psychosocial outcomes [11-14]. Self-limited delayed puberty (DP) represents the extreme end of normal pubertal timing, and is defined as the absence of testicular enlargement in boys or breast development in girls at an age that is 2 to 2.5 standard deviations (SD) later than the population mean [3]. DP may be an isolated feature of the condition or be associated with constitutional delay in growth that can manifest from early childhood.

DP segregates within families, usually with an autosomal dominant pattern of inheritance [15,16]. Despite strong heritability in most cases the genetic basis of DP remains elusive (17) [18]. Moreover, the relevance of genetic factors influencing timing of puberty in the general population to patients with extreme pubertal delay has not been explored. Given the importance of energy balance for reproductive health, genes identified by AAM GWAS that relate to energy homeostasis are of particular interest. Our multi-generational DP families provide a highly valuable resource to investigate these candidate genes in familial DP.

Materials and Methods

Patients

The patients selected for this study are taken from a previously described, accurately phenotyped and characterized, Finnish DP patient cohort [19]. Diagnosis is based on objective evidence of a delayed pubertal growth spurt

148 rather than self-recall. Patients referred with DP to specialist paediatric care in
149 central and southern Finland (1982-2004) were identified. All patients (n=492)
150 met the diagnostic criteria for self-limited DP, defined as the onset of Tanner
151 genital stage II (testicular volume >3 ml) >13.5yr in boys or Tanner breast
152 stage II >13.0yr in girls (i.e. two SD later than average pubertal development)
153 [18,20]. Pubertal growth spurt in probands was more than 2 SD later than
154 average: age at acceleration of pubertal growth (take-off) beyond 13.8 and
155 12.2 yr and age at peak height velocity (PHV) later than 15.6 and 13.7 yr in
156 males and females, respectively (21).
157 Chronic illness and undernutrition was excluded by medical history, clinical
158 examination, and routine laboratory tests. HH, if suspected, was excluded by
159 spontaneous pubertal development at follow-up. In the 50% of patients who
160 choose to have pubertal induction via the use of exogenous sex steroids, all
161 patients were followed up until the point of full pubertal development (Tanner
162 stage G4+ or B4+) to ensure development did not arrest off treatment.
163 Families of the DP patients were invited to participate, with information about
164 medical history and pubertal timing obtained by structured interviews and from
165 archived height records. The criteria for DP in probands' family members were
166 one or more of: 1) age at takeoff or 2) PHV occurring 1.5 SD beyond the
167 mean, i.e. age at takeoff exceeding 12.9 and 11.3 yr, or age
168 at PHV exceeding 14.8 and 12.8 yr in males and females, or 3) age at
169 attaining adult height more than 18 or 16 yr, in males and females,
170 respectively [19]. Previous linkage analysis from this cohort did not find
171 evidence for linked families sharing chromosomal segments identical by

descent, suggesting a founder effect is unlikely to be responsible for this phenotype (19).

Written informed consent was obtained from all participants. The study protocol was approved by the Ethics Committee for Pediatrics, Adolescent Medicine and Psychiatry, Hospital District of Helsinki and Uusimaa (extended to encompass Kuopio, Tampere and Turku University Hospitals) (570/E7/2003). UK ethical approval was granted by the London-Chelsea NRES committee (13/LO/0257). The study was conducted in accordance with the guidelines of The Declaration of Helsinki.

Genetic Analysis

Genetic analysis was performed in 160 individuals from the 67 most extensive families from our cohort with DP. These included 67 probands (male n=57, female n=10), 58 affected family members (male n=36, female n=22) and 35 unaffected family members (male, n=13, female n=22). Whole exome sequencing (WES) was performed on DNA extracted from peripheral blood leukocytes. Variants were analyzed and filtered for potential causal variants in Ingenuity Variant Analysis (Qiagen) using filters for quality control, predicted functional annotation, minor allele frequency (MAF), and GWAS relevance (Figure 1). GWAS relevance filtering allowed identification of those remaining variants that lay within genes in linkage disequilibrium with 106 GWAS loci associated with AAM (n=760) (5). Filters for genes implicated in body mass regulation were applied using a biological context filter with pathway analysis. Variants were filtered for segregation with trait in family members using conventional Sanger sequencing.

196 Targeted exome sequencing using a Fluidigm array of the remaining
197 candidate gene identified post-filtering was then performed in a further 42
198 cohort families (288 individuals, 178 with DP; male=106, female=69 and 110
199 controls; male=55, female=58, Figure 1). Whole gene rare variant burden
200 testing was performed post sequencing.
201 Growth Pattern Analysis
202 The pattern of prepubertal growth in the individuals carrying *FTO* variants was
203 analyzed by using five screening parameters: 1) height for age standard
204 deviation score (HSDS); 2) body mass index (BMI; calculated as weight in
205 kilograms divided by height in meters squared) for age SDS (BMI SDS); 3)
206 HSDS distance from target height (TH) (TH formula = 0.791 × mean parental
207 height SDS − 0.147 for girls and 0.886 × mean parental height SDS – 0.071 for
208 boys; 4) change in height SDS (ΔHSDS); 5) change in BMI SDS (ΔBMI SDS)
209 across time with free age intervals. The calculations of the age-specific and
210 sex-specific normal values for ΔHSDS and ΔBMI SDS were based on
211 longitudinal reference measurements (22). Normality of linear growth was
212 tested by using auxological screening rules based on data from >70,000
213 healthy Finnish children[23].
214 *In silico* Analysis
215 The FTO experimentally solved structure (PDB identifier: 4cxx) was used to
216 study the structural effect of FTO variants. The following interactions involved
217 in protein stability were considered: i) salt bridges; ii) hydrogen bonds (H-
218 bond); and iii) disulphide bridge (S-S bridge). N-glycosylation sites were
219 determined based on the consensus sequence Asn-X-Thr/Ser (X= any amino

acid, except proline). The DSSP program was used to calculate surface accessibility and Disopred3[24] to predict disordered protein regions.

Functional Annotation of FTO mutant proteins

Cloning of wild-type human FTO cDNA into pET302/NT-His has been described previously[25]. The p.Leu44Val and p.Ala163Thr point mutations were introduced using PCR-mediated mutagenesis (Quickchange II, Agilent Technoligies) using primers FTO_L44V FOR: 5'-GAATTCTATCAGCAGTGGCAGGTGAAATATCCTAAACTAATTCT-3', REV: 5'-AGAATTAGTTTAGGATATTTCACCTGCCACTGCTGATAGAATTC-3' and FTO_A163T FOR: 5'-CACAGCATCCTCATTAGTCTTCTCTTTGGCAGCAA-3', REV: 5'-TTGCTGCCAAAGAGAAGACTAATGAGGATGCTGTG-3' and verified by sequencing. An RNase-cleavage assay[26] was used to measure the demethylation activity of FTO on 3-methyl-uridine (3-meU). Recombinant wild-type and mutant FTO expression plasmids were transformed into *Escherichia coli* BL21-Gold (DE3) (Stratagene) and cultured in LB broth and 50 μg/ml carbenicillin. Expression of the cloned gene was induced by the addition of IPTG (isopropyl-β-D-1-thiogalactopyranoside) at 1 mM final concentration at 15◦C for 4 h. The cells were harvested and pellets resuspended in lysis buffer [50 mM HEPES-KOH (pH 8.0), 2 mM 2-mercaptoethanol, 5% glycerol and 300mM NaCl] before digestion with lysozyme (1 mg/ml). The cleared lysate was supplemented with imidazole (final concentration 10 mM) before mixing with 1 ml of pre- washed Ni-NTA (Ni2+-nitrilotriacetate) beads (Qiagen). After binding for 1 h in the cold, the mixture was washed with lysis buffer supplemented with increasing concentrations of imidazole. FTO was eluted with 2 ml of lysis buffer

245 containing 250 mM imidazole. The eluate was concentrated with a 30 kDa
246 molecular-mass cut-off concentrator (Sartorius Stedim) with buffer changing
247 to 20 mM HEPES-KOH (pH 8), 5 % glycerol and 50 mM NaCl. Purified
248 proteins were snap-frozen and stored at −80◦C. Protein purity was estimated
249 by Commassie Blue staining after resolving by SDS/PAGE (4–12 % gradient
250 gels; Invitrogen).
251 Dose response of FTO on 3-meU demethylation: Recombinant FTO proteins
252 were assayed as previously described[26]. Each protein, at different protein
253 concentrations from 0 -1000 nM, was assayed in a reaction containing 100
254 nM substrate, 75 μM Fe(NH4)2(SO4)2, 300 μM 2-OG, 2 mM ascorbate,
255 50 μg/ml BSA and 62.5 μg/μl of RNase A in 50 mM Tris/HCl buffer at pH 7.0.
256 Samples were prepared in duplicate in a dark flat-bottomed 96-well plate and
257 the FAM (6-carboxyfluorescein) emission was measured for 30 min at a
258 wavelength of 520 nm with excitation at 485 nm. The measurement was
259 performed at room temperature (25°C) using a microplate reader [Infinite
260 M1000, Tecan]. Wild type (WT) FTO protein and catalytically inactive mutant
261 p.Arg316Gln (R316Q) served as positive and negative controls respectively.
262 Mouse experiments
263 Fto deficient mice were a generous gift from Prof. Roger Cox (MRC Harwell,
264 Oxford) and were genotyped as previously described (27). This research is
265 regulated under the Animals (Scientific Procedures) Act 1986 Amendment
266 Regulations 2012 following ethical review by the University of Cambridge
267 Animal Welfare and Ethical Review Body (AWERB). Animals were kept under
268 controlled temperature (22°C) and a 12-h light, 12-h dark schedule (lights on

07:00–19:00). Standard chow (Special Diet Services) and water were available *ad libitum*.

For the vaginal opening study female *Fto* heterozygous mice ($Fto^{+/-}$) (n=45) and their WT littermates (n=24) were taken from either a male *Fto* WT x female $Fto^{+/-}$ cross or a male $Fto^{+/-}$ x female *Fto* WT cross. From P21 (day of weaning) all female mice were weighed and visual examination of the vagina was carried out by placing the mouse on top of a cage lid and lifting the tail vertically away from the body. No excessive force was involved. First day of vaginal opening was recorded when a complete opening was observed.

For all experiments, data are expressed as the mean ± SEM. To determine statistical significance, we used the unpaired t test (2-tailed) using SPSS Software (version 24). A p value of <0.05 was considered statistically significant.

Results

Variants in GWAS genes implicated in body mass were identified following exome sequencing in families with self-limited delayed puberty

WES performed in the 67 largest and best phenotyped families from our cohort (160 individuals: a total of 125 individuals with DP, male=93, female=32; and 35 controls, male=13, female=22), identified 6,952,773 variants after quality control (Figure 1). Filtering to identify high quality, rare, predicted deleterious variants not present in control subjects selected 12,371 variants in 7,470 genes. Of these 7,470 genes, 238 were found to be in linkage disequilibrium with a GWAS locus for timing of puberty, and 5 of these

238 were genes implicated in body mass regulation or growth by pathway analysis. Of these 5 genes, 4 (*GPD2*, *GHR*, *ESR1* and *VDR*) were found to have only variants that did not segregate with the DP trait in family members. The remaining candidate gene, *FTO* (Fat mass and obesity-associated protein, ENSG00000140718, gene identification number 79068), has been previously described in the literature as involved in pathways of energy homeostasis and growth[5], and is known to act as an Fe(II) 2-OG (2-oxoglutarate) -dependent dioxygenase to repair alkylated DNA and RNA by demethylation[26]. *FTO* contributes to the regulation of energy balance, and thus to the regulation of body size and fat accumulation.

Two variants in *FTO* (NM_001080432.2: c.130C>G p.Leu44Val and NM_001080432.2: c.487G>A (rs145884431) p.Ala163Thr) were identified in three families from our cohort and found in one or fewer control subjects (rare variant burden testing adjusted p = 0.058). Both variants are rare (MAF < 0.2%) heterozygous missense variants and predicted benign or tolerated by >2/5 prediction software tools.

Families with potentially pathogenic *FTO* variants display autosomal dominant inheritance of DP phenotype and low body mass.

The family identified with the p.Ala163Thr variant (family 1) and both of the families with the p.Leu44Val variant (families 2 and 3) displayed the typical autosomal inheritance pattern of the DP trait, with perfect segregation (Figure 2, panel A). Affected individuals from family 1 with the p.Ala163Thr variant and from family 3 with the p.Leu44Val variant were particularly underweight in childhood, with the two probands from these families (individuals 1.III.2 and 3.III.2) falling into the thinness grade 2 category(28) before puberty (Figure 2,

panels B and D). Although there was some variability in this phenotype, all family members carrying *FTO* variants had ISO-BMI values in the lower range (<23) (Figure 2 and Supplementary Fig. 1-3, Table 1). In addition, both of the probands from families 2 and 3 who carry the p.Leu44Val displayed faltering growth in early childhood. Both displayed significant deflection from previous height measurements in the 2 years following birth, as well as height significantly below target height in later adolescence associated with delayed pubertal growth (Figure 2, panels C and D)[22].

In silico analysis of potential mutations

We carried out *in silico* analysis using the solved structure of FTO (PDB identifier: 3lfm) to determine the possible pathogenicity of the identified variants. The hydrophobic residue Leucine 44 is part of a solvent-exposed alpha helix on the surface. Substitution with Valine is not predicted to alter the structure of FTO or interaction with iron molecules or DNA. However, L44 and other residues in the same solvent-exposed alpha helix form a motif (Supplementary Fig. 4 and 5), which is highly conserved across placental mammals but not reptiles, birds or fish (Supplementary Fig. 6). This motif (residues 36-48) forms a patch on the FTO protein surface (Supplementary Fig. 7). This may act as a mammal-specific interaction site (between FTO and another protein), required for FTO function for example in reproductive development. In this scenario, a small change in side chain volume, such as Leucine-to-Valine, may have a subtle effect in protein-protein interaction and lead to a change in FTO activity *in vivo*.

Alanine 163 is a hydrophobic, not highly conserved residue (Supplementary Fig. 8, panel A). Alanine 163 is at the end of the H4 alpha helix and the

beginning of a long, disordered region (Supplementary Fig. 8, panel B), which connects helices H4 and H5 (Supplementary Fig. 8, panel C).

FTO p.Leu44Val mutant protein displays reduced demethylase activity *in vitro*

We carried out functional characterization of the identified mutant FTO proteins (p.Leu44Val and p.Ala163Thr) as compared to WT protein. A previously verified RNase-cleavage assay was used to measure the demethylation activity of FTO on 3-meU (26). Although kinetic activity of the mutant protein p.Ala163Thr did not vary from WT using this assay, mutant protein p.Leu44Val showed an approximately 20% lower kinetic activity than WT activity (Figure 3).

***FTO* deficiency *in vivo* results in delayed vaginal opening in mice**

In order to examine the influence of FTO activity on pubertal timing in an *in vivo* model, we examined timing of puberty in mice deficient for FTO in the heterozygous state (*Fto*$^{+/-}$), in keeping with the human genotype identified. *Fto*$^{-/-}$ mice were not selected for these experiments because of their poor postnatal health (29). *Fto*$^{+/-}$ mice had significantly delayed timing of vaginal opening (VO) (mean postnatal day +/- SEM: 27.20 +/- 0.44 in wild-type (n=24) vs 28.56 +/- 0.48 in *Fto*$^{+/}$ mice (n=45), p =0.047), an event which reflects the pubertal rise in estradiol [30] (Figure 4). Mean body weight of the *Fto*$^{+/}$ group was not significantly different to the WT mice (mean body weight (in g) +/- SEM: 11.64 +/- 0.21 in wild-type vs 11.45 +/- 0.14 in *Fto*$^{+/}$ mice, p=0.467) (Figure 5).

Using simple linear modelling, *Fto* genotype of the pup (Het vs WT) explained approximately 3% of the total variation in timing of VO. Consideration of an

369 additional factor, maternal genotype, improved the model by increasing the
370 significance of the association between pup genotype and timing of VO
371 slightly (p=0.04), and accounted for 6% of the total variation in timing of VO.
372 In contrast, paternal genotype decreased the significance and total variation
373 accounted for by the model.
374
375 **Discussion**
376 Genome wide association studies of AAM in the general population have
377 attempted to unravel the complex conundrum of which genetic
378 factors influence the timing of puberty. Despite many loci being identified,
379 clear evidence for the role of particular genes and pathways is for the most
380 part lacking. Those genes lying within pathways of energy metabolism and
381 growth appear promising, with the discovery of the role of *Lin28B* in C.elegans
382 development[3] and the importance of leptin as a permissive signal in triggering
383 the onset of puberty(9,31).
384 The inheritance of DP is known to be under strong genetic influence with
385 commonly an autosomal dominant inheritance pattern, and thus represents a
386 useful basis for the investigation of puberty genetics. Notably, self-limited or
387 constitutional DP is often associated with slow maturation throughout
388 childhood, implicating growth and energy metabolism pathways in its
389 pathogenesis. Previously, genes in such pathways identified through GWAS
390 have not been screened in patients with DP.
391 Our results have identified variants in *FTO* as a potential contributory factor in
392 the development of self-limited DP in three pedigrees from our large cohort of
393 patients with familial DP. *FTO* (fat mass and obesity associated gene) was the

394 first obesity-susceptibility gene identified through GWAS and continues to be
395 the locus with the largest effect on body mass index (BMI) and obesity risk[10].
396 Those DP patients identified with *FTO* variants from our study showed
397 reductions in body mass. The *FTO* variants carried by our DP patients may
398 result in reduced fat mass, which would in turn contribute to a delay in the
399 timing of pubertal onset. This delay may be mediated directly through reduced
400 leptin levels. Although we do not routinely measure leptin levels in DP
401 patients, leptin levels have been shown to be significantly lower in pubertal-
402 age patients with self-limited DP(32).
403 Notably, in an *in vivo* model $Fto^{+/-}$ mice had a significantly delayed onset of
404 puberty as compared to WT mice. In the 7 days preceding puberty onset,
405 however, body weight was not significantly different between the two pup
406 genotype groups. Previous studies have demonstrated that $Fto^{-/-}$ mice show a
407 30-40% reduction in body weight by 6 weeks of age[29] and that transgenic
408 mice with additional copies of *Fto* show a dose-dependent increase in body
409 and fat mass[33]. However, the relationship between *FTO* genotype, fat mass
410 and leptin levels remains somewhat unclear. *Fto* deficient mice do become
411 obese when subjected to a high fat diet, although they remain sensitive to the
412 anorexigenic effects of leptin (29,34).
413 Moreover, it is possible that *FTO* gene dosage may have an effect on energy
414 homeostasis independent of effects on fat mass[33], including on the balance
415 between catabolic and anabolic pathways (35). *FTO* has been identified as an
416 amino acid sensor acting, via mTOR, to influence appropriate levels of
417 development and translation[36]. *FTO* is expressed within the hypothalamus in
418 several sites critical for energy balance, including in the arcuate nucleus

419 within proopiomelanocortin (POMC) neurons(37,38). In one study *Fto* levels in
420 the arcuate nuclei of fasted mice fell by up to 60%, and this was not rescued
421 by leptin administration. Other studies have shown conflicting results in the
422 effects on *Fto* mRNA levels of fasting, depending on whether whole
423 hypothalamus or arcuate nucleus were studied and on the length of fast (38).
424 However, *Fto*$^{-/-}$ mice display blunted starvation-induced Npy mRNA
425 induction[29]. More recent studies have suggested that Fto may influence the
426 metabolic outcomes of a high fat diet via hypothalamic signaling pathways
427 acting independently of body weight (34). Mutations in *FTO*, including those
428 with greatly reduced demethylase activity (e.g. pR316Q, Figure 3), have been
429 identified in human subjects associated with both lean and obese phenotypes
430 [25]. We were not able in our study to identify the mechanism by which the
431 p.Ala163Thr variant might affect protein function; although no reduction in
432 demethylation activity was demonstrated it is possible that this variant may
433 produce a deleterious effect by another route, for example defects in post-
434 translational modification or protein degradation.
435 Thus, FTO may be important for signaling energy sufficiency
436 and the 'healthy energy balance' required for pubertal onset. Our *in*
437 *silico* analysis suggests that the p.Leu44Val mutation we have identified may
438 represent a mammal-specific interaction site between FTO and another
439 protein (or DNA), important for FTO function in terms of reproductive
440 development. Moreover, maternal genotype may contribute to pubertal timing,
441 as demonstrated from our *Fto*$^{+/-}$ mice data. A reproductive phenotype present
442 in *Fto* heterozygote mothers could expose pups to a suboptimal environment
443 that could influence their puberty timing.

Finally, our finding of maturational delay in growth in early childhood in the two probands with p.Leu44Val mutation is of interest. Constitutional delay in growth is seen in a subset of patients with DP, and our findings implicate mutations in energy pathway genes in the pathogenesis of patients with such a phenotype.

Overall, our discovery of two rare variants in *FTO* associated with self-limited DP in our large familial cohort, and of delayed vaginal opening in *FTO*-deficient mice, provides evidence that perturbations in pathways of energy homeostasis and growth may potentially produce a phenotype of DP. We note that despite this extensive analysis, only three of 67 probands were identified with potentially pathogenic variants in such pathways, highlighting the high degree of heterogeneity in the genetic basis of self-limited DP. These findings merit further exploration in our own cohort and in other populations, including sub-group analysis of DP patients with low BMI from early childhood.

Acknowledgements

We are very grateful to the patients and families who contributed their time, medical information, and DNA samples to this study.

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Figure Legends:

Figure 1 – Flowchart of WES (whole exome sequencing) filtering strategy to identify candidate genes.

Whole exome sequencing was initially performed on DNA extracted from peripheral blood leukocytes of 160 individuals from the 67 most extensive families from our cohort (125 with DP and 35 controls), with exome capture on a Nimblegen V2 or Agilent V5 platform and sequencing on the Illumina Hiseq 2000. The exome sequences were aligned to the UCSC hg19 reference genome. Picard tools and the genome analysis toolkit were used to mark PCR duplicates, realign around indels, recalibrate quality scores and call variants. Variants were then analyzed further and filtered for potential causal variants using filters for quality control, predicted functional annotation, minor allele frequency (MAF), segregation with trait and GWAS relevance (See methods for further information on filtering criteria). Targeted exome sequencing using a Fluidigm array of a candidate gene identified post-filtering was then performed in a further 42 families from the same cohort (288 individuals, 178 with DP and 110 controls). Variants post targeted re-sequencing were filtered using the same criteria as the whole exome sequencing data. Functional annotation of the variants as described elsewhere in methods. DP – delayed puberty.

Figure 2 – Pedigrees and auxological data of the families with potentially pathogenic *FTO* variants

Panel A: Squares indicate male family members, circles female family members. Black symbols represent clinically affected, grey represent unknown phenotype, clear symbols represent unaffected individuals. The arrow with 'P' indicates the proband in each family and 'us' indicates un-sequenced due to lack of DNA from that individual. The mutation in each family is given next to the family number; a horizontal black line above an individual's symbol indicates they are heterozygous for the variant as confirmed by either whole exome sequencing or Fluidigm array, and verified by Sanger sequencing. A red dot indicates the individual was underweight (thinness grade 2 or more significant) and '?' indicates that BMI information for that individual is not available.

Panels B-D: BMI and height standard deviation score (SDS) charts for the probands of each of the three pedigrees (family 1.III.2, family 2.III.5 and family 3.III.2). Underweight values are shown in red, green dots indicate a significant deflection from previous height measurements and orange dots indicate significant deflection from target height. Normal values, based on data from >70,000 healthy Finnish children, have been previously published [22].

Figure 3 – Demethylation assay assessing kinetic activity of mutant versus wild type FTO proteins.

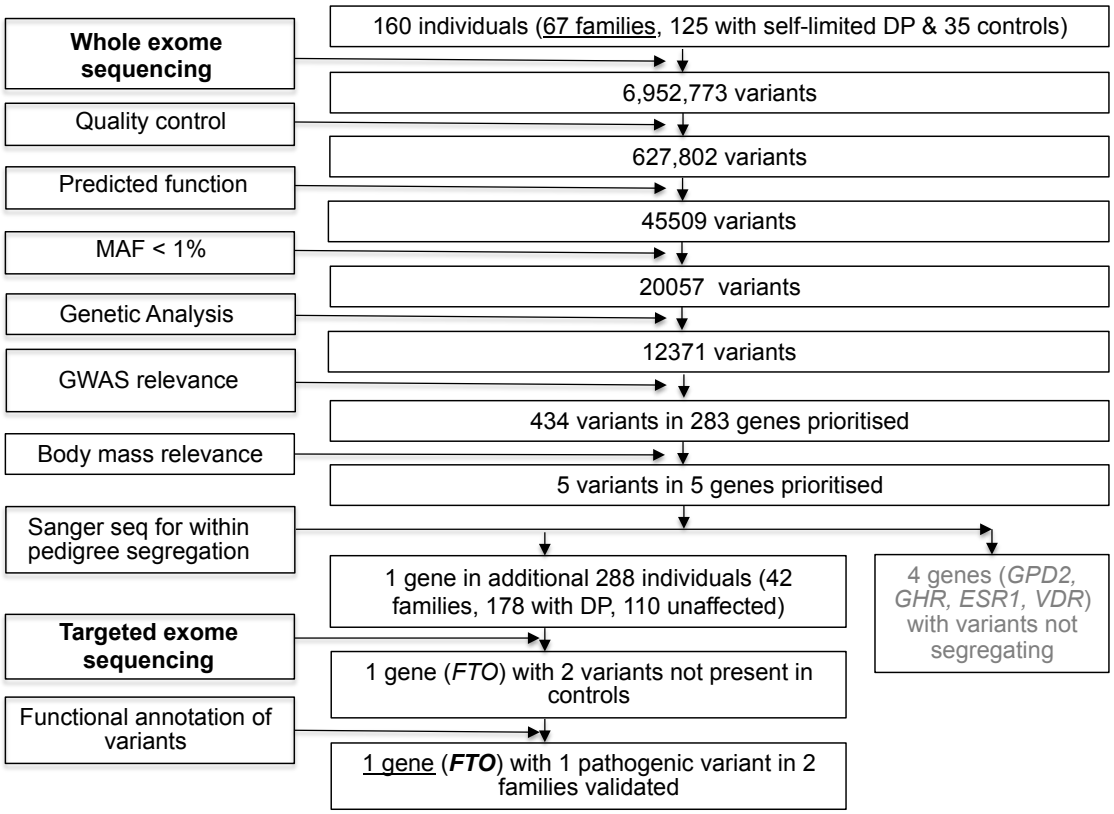
FTO activity is proportional to the concentration present in the reaction. Demethylase activity is likely to be related to the ability of FTO to function as a sensor for cellular metabolism (36). The R316Q mutant is enzymatically dead across all concentrations tested. The A163T and L44V mutants showed

725 demethylase activity towards methylated-uridine in a dose-dependent manner
726 but with different affinities. WT – wild-type
727
728 **Figure 4 – Timing of vaginal opening in wild-type (WT) and *FTO*$^{+/-}$**
729 **heterozygous (Het) mice.**
730 Cumulative percentages of mice displaying vaginal opening by postnatal day
731 are shown for WT and *FTO*$^{+/-}$ mice. WT mice n=24, *FTO*$^{+/-}$ n=45; p <0.05 by
732 un-paired t test.
733
734 **Figure 5 – Mean body weight (g) for wild type (WT) and *Fto*$^{+/-}$ (Het) mice**
735 **in 7 days prior to vaginal opening**
736 Mean body weight (g) +/- SEM: 11.64 +/- 0.21 in wild-type (n=24) vs 11.45 +/-
737 0.14 in *Fto*$^{+/}$ mice (n=45), p=0.467 by un-paired t test. Error bars show SEM
738 for each group each day.
739
740

Case	Sex	Amino acid alteration	Height SDS at age 4 yrs	Height SDS at age 8/9 yrs	Height SDS at age 18 yrs	ISO-BMI at 18 yrs
1.II.1	M	p.Ala163Thr	-	1.1	1.7	16.9
1.III.2 (P)	M	p.Ala163Thr	1.1	0.5	1.1	17.1
1.III.1	F	p.Ala163Thr	0.9	1.0	1.1	17.3
1.II.5	M	p.Ala163Thr	-1.0	-1.0	-0.4	-
2.III.5 (P)	M	p.Leu44Val	-0.9	-1.4	-1.5	18.8
2.III.6	M	p.Leu44Val	-1.1	-1.3	-	-
2.II.2	M	p.Leu44Val	-	-0.8	-0.8	20.5
2.III.1	M	p.Leu44Val	0	-1.4	-	-
3.II.2	M	p.Leu44Val	-	-1.0	-0.9	18.6
3.III.2 (P)	M	p.Leu44Val	-0.9	-1.1	-1.3	18.7
3.II.3	M	p.Leu44Val		-0.4	-0.1	22.7
3.III.3	M	p.Leu44Val	-0.1	0.2	0.5	17.8

Table 1 – Clinical data of probands with FTO variants

Height is expressed in s.d. score (SDS) for national reference data for Finland at 4 years of age and at either 8 years for girls or 9 years for boys. Normal limits: delta HSDS <1.21, distance to target height at 4 yrs <1.76, distance to target height at 8/9 yrs <1.72(22). P – proband.



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750 **Figure 1 – Flowchart of WES (whole exome sequencing) filtering**

751 **strategy to identify candidate genes.**

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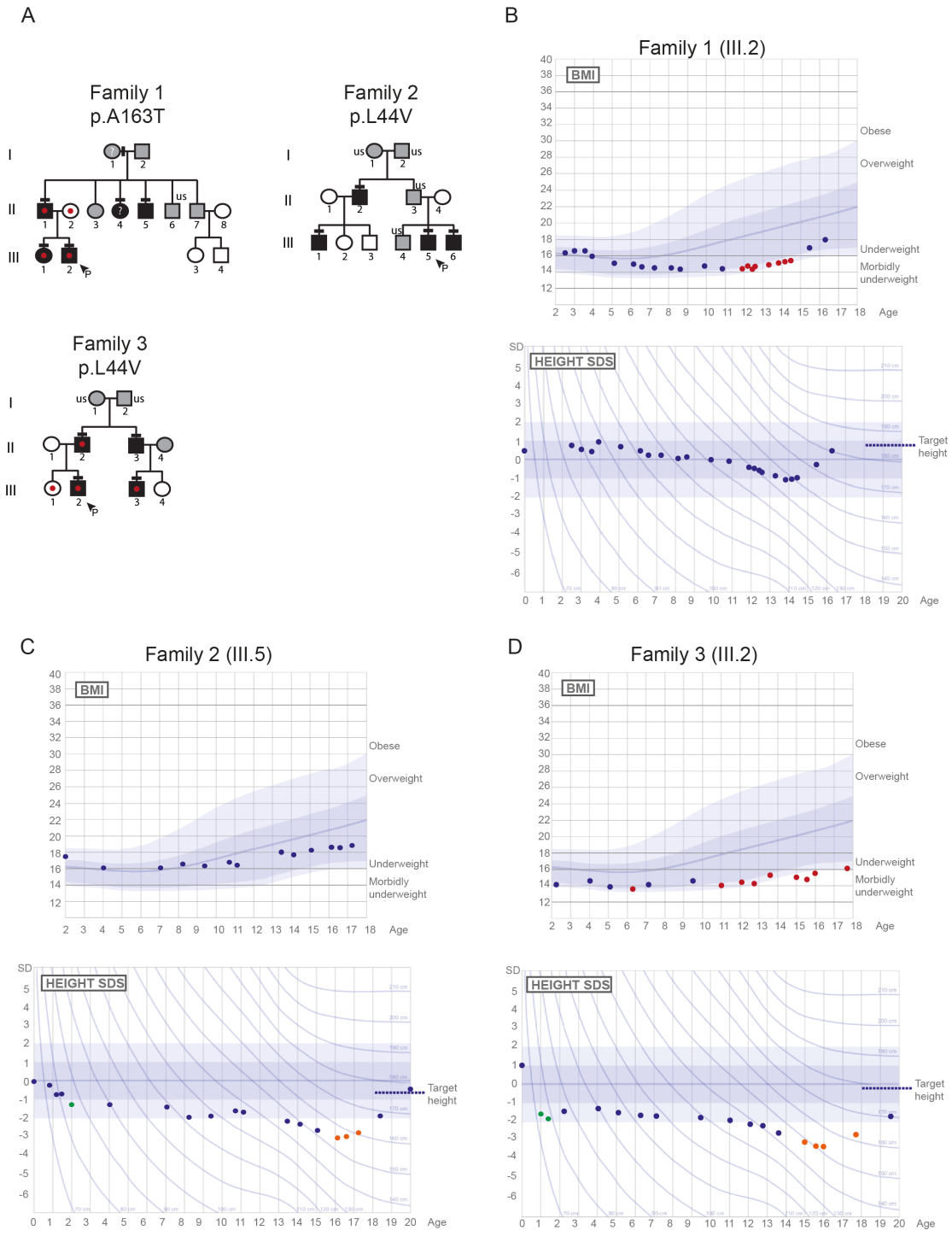
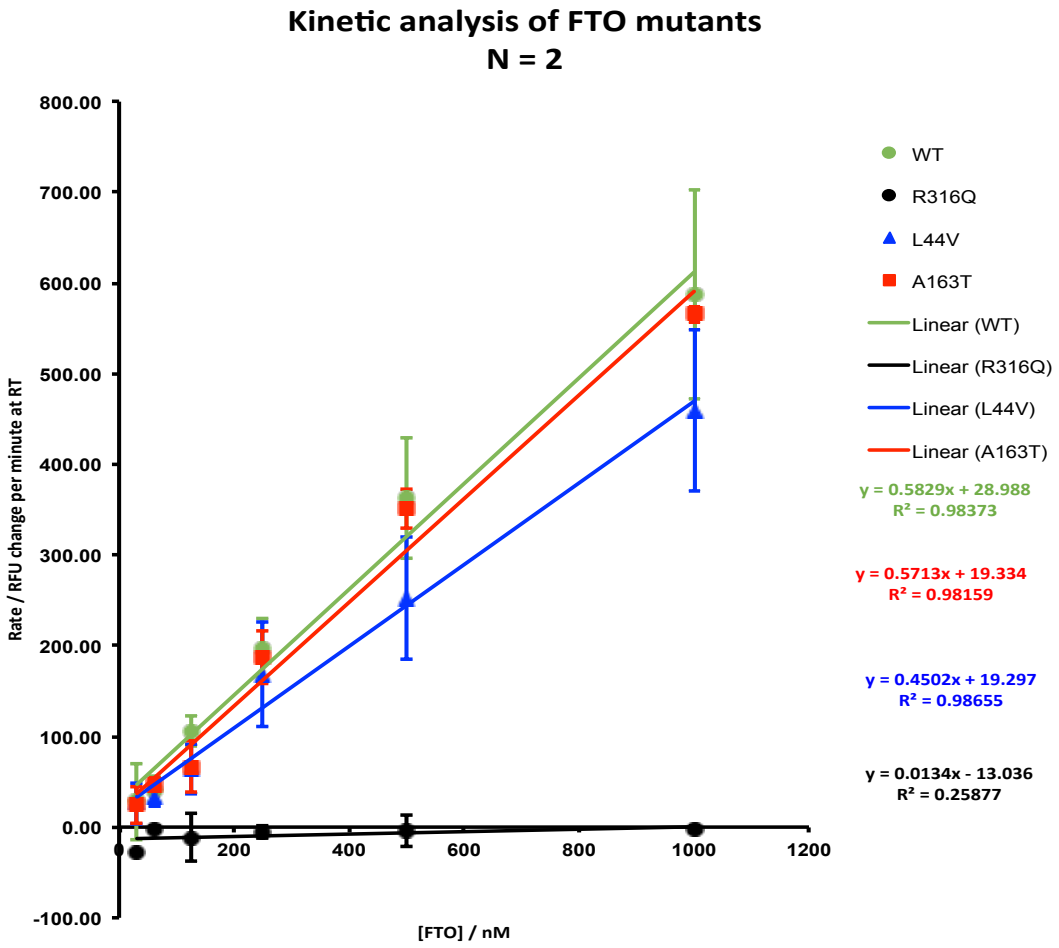


Figure 2 – Pedigrees and auxological data of the families with potentially pathogenic *FTO* variants



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763 **Figure 3 – Demethylation assay assessing kinetic activity of mutant**

764 **versus wild type FTO proteins.**

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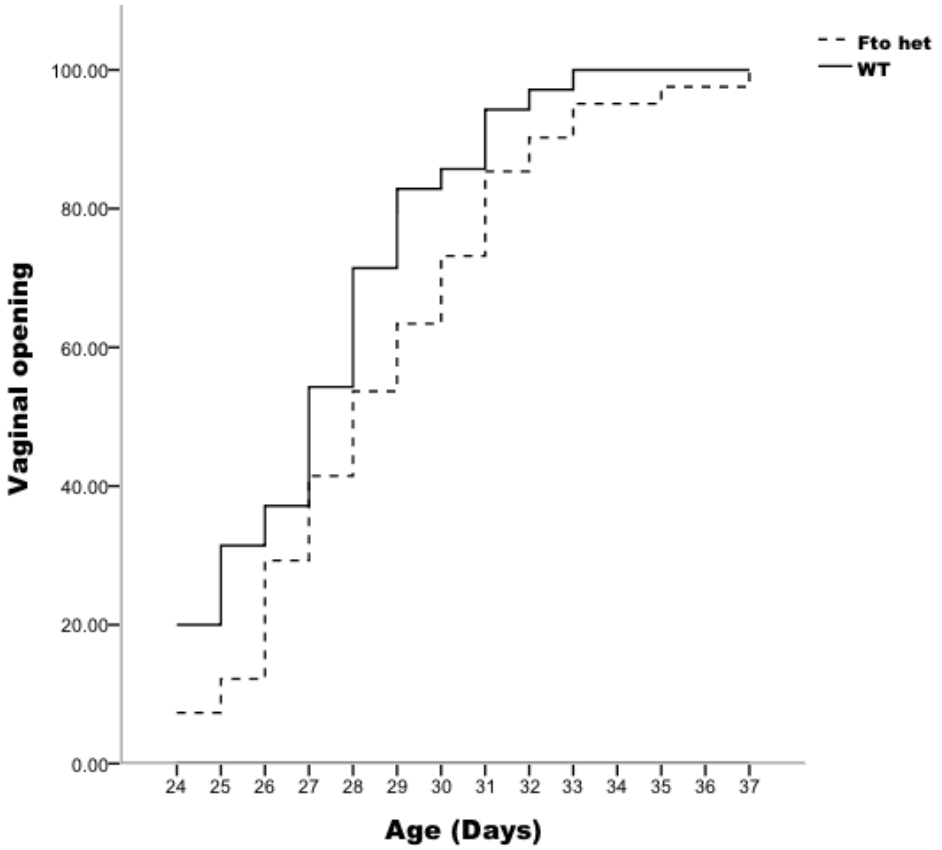

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769 **Figure 4 – Timing of vaginal opening in wild-type (WT) and *FTO*$^{+/-}$**

770 **heterozygous (Het) mice.**

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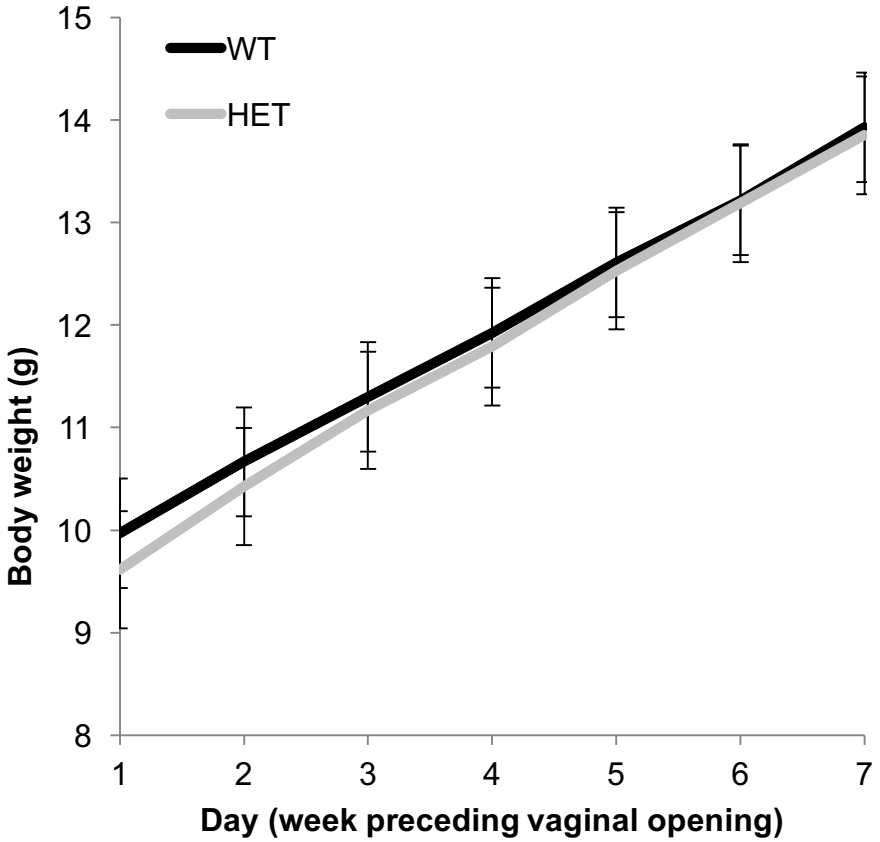

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777 **Figure 5 – Mean body weight (g) for wild type (WT) and *Fto*$^{+/-}$ (Het) mice in**

778 **7 days prior to vaginal opening**

779