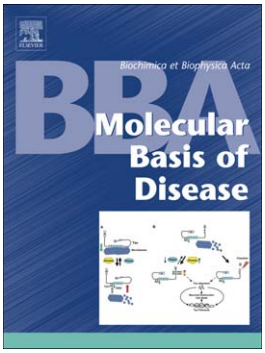

Polymorphisms and mutations in the melanocortin-3 receptor and their relation to human obesity

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Title: Polymorphisms and mutations in the melanocortin-3 receptor and their relation to human obesity

Running Title: MC3R polymorphisms in humans

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Abstract

Inactivating mutations in the melanocortin 3 receptor (*Mc3r*) have been described as causing obesity in mice, but the physiologic effects of *MC3R* mutations in humans have been less clear. Here we review the *MC3R* polymorphisms and mutations identified in humans, and the *in vitro*, murine, and human cohort studies examining their putative effects. Some, but not all, studies suggest that the common human *MC3R* variant *T6K+V81I*, as well as several other rare, function-altering mutations, are associated with greater adiposity and hyperleptinemia with altered energy partitioning. In vitro, the *T6K+V81I* variant appears to decrease MC3R expression and therefore cAMP generation in response to ligand binding. Knockin mouse studies confirm the *T6K+V81I* variant increases feeding efficiency and the avidity with which adipocytes derived from bone or adipose tissue stem cells store triglycerides. Other *MC3R* mutations occur too infrequently in the human population to make definitive conclusions regarding their clinical effects.

Introduction

Human body weight is a complex trait determined by the interactions between environmental influences and genetic risk factors [1]. Twin and adoption studies suggest heritable characteristics explain 60-80% of the observed variance of human body weight [2]. It is also well established that the hypothalamus has a critical role in maintaining energy balance by regulating appetite and metabolism, and perturbations of hypothalamic function can lead to obesity [3]. Therefore, significant research has recently focused on the genes and proteins involved in hypothalamic control of body weight, including the leptin-melanocortin pathway. One hypothalamic protein, the melanocortin 3 receptor (MC3R), is believed to be one of the many factors important for regulating energy homeostasis; however its role is not completely understood. Moreover, recent evidence suggests that MC3R may regulate metabolism not only via central but also via peripheral actions [4-6]. Herein we briefly review MC3R structure and function, discuss the polymorphisms and mutations identified in humans, and summarize the *in vitro*, murine, and human studies examining their putative effects.

MC3R and its downstream signaling pathways

MC3R is a member of the melanocortin receptor family. Other members of the family include MC1R, important for skin and hair pigmentation; MC2R or the adrenocorticotropin (ACTH) receptor, critical for proper cortisol regulation; MC4R, integral for proper energy homeostasis and metabolism; and MC5R, important for piloerection [7, 8]. The human *MC3R* was first cloned in 1993 as a single exon gene located in chromosome 20q13.2. A sequence with high homology was then later found in mice on chromosome 2q [9-11]. The *MC3R* gene is

highly conserved, with orthologs in 171 organisms including monkey, dog, mouse and rat (https://www.ncbi.nlm.nih.gov/gene/4159). MC3R and MC4R have 58% identity and 76% similarity [12].

Studies have evaluated the transcript structure of murine *Mc3r* and human *MC3R* by examining its untranslated regions (UTRs) [13, 14], which can play important roles in gene expression, mRNA stability, and translational efficiency. Research from our group [14] demonstrated that the murine *Mc3r* 5′ UTR has two transcription start sites: one 368 bases upstream of the translation start site and another 440 bases upstream, with putative initiator sequences associated with each start site. The murine *Mc3r* 3′ UTR terminates 1286 bases after the translational stop codon, but with a 787 base splice between consensus splice donor and acceptor sites from 171 bases to 958 bases downstream of the stop codon. The human *MC3R* 5′ UTR, on the other hand, has a transcription start site 527–544 bases upstream of the classical translation start site, with most transcripts starting 533 bases upstream, near a putative initiator sequence. There is also a 248 base splice from 140 to 388 bases upstream of the start codon between consensus splice donor and acceptor sites [13, 14]. The human *MC3R* 3′ UTR was found to end only 115–160 bases after the translational stop codon [13, 14]. The human *MC3R* has more than one potential start site for translation, with additional in-frame ATGs located 37 and 109 codons downstream. The first ATG is found only in humans and nonhuman primates, while the second ATG appears widely conserved among vertebrates [13]. Park *et al.* reported that the full-length 5' UTR directs utilization of the second in-frame ATG as the primary translation start site instead of the canonically-assumed first ATG [13]. This leads to a protein transcript which is 37 codons shorter than the classical *MC3R* transcript [15]; although

other research suggests that translation can occur from the first ATG site as well [16]. These studies suggested the possibility of transcript heterogeneity for both human *MC3R* and murine *Mc3r* that could potentially enable tissue-specific gene regulation.

MC3R is a G-protein-coupled receptor (GPCR), consisting of seven transmembrane helices with and extracellular N-terminus and an intracellular C-terminus [Figure 1]. While some GPCRs, such as MC4R, demonstrate substantial basal activity, MC3R likely has little or no basal activity [17]. Upon ligand binding, MC3R's effects are believed primarily to be mediated via the Gsα protein, which activates adenylyl cyclase and produces cAMP. This secondary messenger, in turn, initiates further downstream signaling. MC3R has also been shown to activate ERK1/2 signaling, although somewhat inconsistent data have been reported [18-20]. Further studies are needed to elucidate how the two signaling pathways behave in different tissues.

MC3R and metabolic function

MC3R was initially found to be expressed in areas of the central nervous system associated with complex behaviors related to food ingestion and autonomic function [21]. Relatively high levels of expression are observed in hypothalamic and limbic structures; however expression has also been reported in peripheral tissues including kidney, heart, liver, gut, leukocytes, and adipose tissue [9, 22-24].

MC3R's effects are governed by the binding of endogenous melanocortin ligands, which in turn are regulated and released by signals reflecting metabolic conditions. The endogenous agonists α-melanocyte-stimulating hormone (α-MSH) and γ-MSH are neuropeptides, cleaved from the pro-hormone proopiomelanocortin (POMC) that can activate MC3R. α-MSH is a non-

selective melanocortin receptors agonist, capable of binding to MC1R, MC3R, MC4R, and MC5R [25]. γ-MSH, on the other hand, is more selective for MC3R [26, 27], suggesting that MC3R-specific regulatory pathways could exist.

Roles of MC3R in energy homeostasis

The MC3R appears to regulate metabolism in multiple distinct fashions in the CNS and at peripheral sites, such that its impact on energy homeostasis is not straightforward. Within the arcuate nucleus, the MC3R appears to function as an inhibitory autoreceptor for POMC neurons. Peripheral injection of D-Trp^{8}-γ-MSH, a very selective and potent MC3R agonist [28], stimulates short-term feeding behavior, providing physiological evidence that, at least acutely, MC3R activation is orexigenic [26]. In the rostral arcuate nucleus, MC3R mRNA is expressed in about 40% of POMC neurons and >50% of NPY/AGRP neurons [29, 30]. Arcuate MC3R activation is believed to lead to release of GABA from NPY/AGRP neurons and thus inhibition of POMC neuronal activity. In rat arcuate brain slices, D-Trp^{8}-γ-MSH increases the frequency of GABA-mediated mini-inhibitory postsynaptic currents in POMC neurons, causes hyperpolarization, and decreases action potential generation [31]. Roger Cone's group has reported that mice with inactivation of MC3R have greater anorexia and loss of lean tissues in response to lipopolysaccharide injection or tumor growth [32], as well as impaired feeding response to fasting [33], all consistent with a role for MC3R activation as stimulatory for energy intake and gain of lean mass. Data also suggest activation of MC3R is essential for proper alignment of metabolic responses during entrainment to restricted feeding regimens [34] and that MC3R in the limbic system is important for motivating feeding after energy restriction [35].

Nevertheless, inactivation of *Mc3r* in mice is associated with an obesity phenotype that becomes more evident with high-fat diet feeding [36-38]. The obesity is moderate compared to *Mc4r* deficiency and total body mass is less affected than adipose tissue triglyceride content. *Mc3r*$^{-/-}$ mice exhibit increased fat mass but reduced fat-free mass, decreased linear growth and femur length [36, 37], and reduced adipose tissue inflammation [39] along with some evidence of reduced effects of leptin administration on food intake [40]. More consistently, however, *Mc3r*$^{-/-}$ mice show greater feeding efficiency (the ratio of weight gain to energy intake) [36]. They have also been reported to have decreased locomotor activity particularly during the dark phase [41] and altered circadian rhythmicity in constant dark conditions [42]. They are, under most conditions hypophagic or normophagic relative to controls and appear to maintain normal metabolic rate and metabolic profiles despite their obese phenotype. Thus, the obesity phenotype of *Mc3r* deficiency differs dramatically from that of murine *Mc4r* deficiency, where marked hyperphagia, increased lean mass, substantial insulin resistance, and adipose tissue inflammation are seen. It is clear that MC3R and MC4R play distinct roles in body weight and body composition regulation that are not redundant, because mice with simultaneous knockout of the *Mc3r* and *Mc4r* are more obese than mice with either knockout alone [37]. MC3R does not appear to act solely by altering the activation state of POMC neurons or of MC4R; it also regulates body weight at other central nervous system and peripheral sites.

The precise mechanisms through which MC3R alters metabolism to cause an obese state have not been fully identified. Several lines of evidence point to a role for MC3R in nutrient partitioning, namely towards adipose tissue and away from lean tissues [33]. *Mc3r* deletion has been found to result in slightly increased nadir corticosterone, but a somewhat

suppressed glucocorticoid response to fasting. Several phenotypic features of *Mc3r* deficiency are reminiscent of a Cushing-like syndrome, including hypercorticosteronemia and increased visceral adiposity [33], although lipolysis and hormone-sensitive lipase expression in response to fasting is reported to be reduced in mice with *Mc3r* deficiency [33] and is usually well maintained in states of glucocorticoid excess [43]. Hypercorticosteronemia is also known to result in increased bone resorption and decreased bone deposition [44], and so the elevated corticosterone could potentially explain decreased linear growth of *Mc3r*-deficient mouse models. A study from Moller *et al* demonstrated mRNA expression of *MC3R* in human subcutaneous adipocytes, albiet to a lesser extent than *MC1R, MC2R,* or *MC5R* [23]. Additionally You *et al* showed mRNA expression of *Mc3r* in rat brain, liver and adipose tissue [45]. Taken together, the reduced capacity for lipolysis described in *Mc3r*$^{-/-}$ mice [33] along with the evidence above suggest adipose tissue might be an important site for MC3R-related body weight regulation.

Begriche *et al.*, investigated the central versus peripheral roles of MC3R using a lox-stop-lox (LoxTB) sequence that globally blocked *Mc3r* transcription and could be reactivated in tissues of interest with tissue-specific Cre recombinase [6]. Homozygous carriers of the inactivating LoxTB *Mc3r* alleles (*Mc3rTB/TB*) displayed an obese phenotype similar to that observed in *Mc3r*$^{-/-}$ mice. *Mc3rTB/TB* mice displayed reduced lean mass, increased fat mass, and accelerated diet-induced obesity. However, after rescuing *Mc3r* expression only in the nervous system using a Nestin-Cre transgene, there was only a partial reduction of obesity in chow-fed conditions and little effect on high fat diet-induced obesity [6]. These data suggest that MC3R does not solely act in the CNS; rather, peripheral MC3R has a distinct pathway for

body weight regulation that may be of importance for whole body homeostasis beyond the effects of the central receptors that have been the subject of much prior investigation.

To this point, liver microarray data suggest that *Mc3r*$^{-/-}$ mice exhibit increased expression of enzymes involved in hepatic lipogenesis and triglyceride synthesis with suppression of gluconeogenic enzymes [5]. There is also evidence for activation of a "cellular stress response" in the liver of *Mc3r*$^{-/-}$ mice, with increased expression of heat shock proteins and autophagy-related genes [5]. Further research is needed to investigate MC3R's peripheral pathways of action.

Human Studies

Epidemiology of MC3R variants in Humans

In order to elucidate the molecular underpinnings of obesity in humans, linkage studies examining genotype-obesity phenotype correlations in different populations have been performed. A linkage analysis in French Canadians discovered a locus in 20q, homologous to an obesity quantitative trait locus found in mice, which was associated with increased adiposity and fasting insulin among humans [11]. A subsequent study described that obesity and fat mass percentage could be linked to markers in 20q13, within which is located the *MC3R* gene [46]. This locus does not seem to be represented, however, as a site with genome-wide significance in large genome-wide association studies. Based on the early linkage analyses and the *Mc3r* knockout obesity phenotype, sequencing of the *MC3R* gene and association studies with body weight in human cohorts have been performed in numerous populations.

The most common human variants encountered are C17A and G241A, which respectively change two amino acids in MC3R: Thr6Lys and Val81Ile (T6K+V81I). These variants occur in almost complete linkage disequilibrium. This common variant (T6K+V81I) has significant racial/ethnic differences in haplotype frequency. Results from published cohort studies show that homozygosity for this haplotype is rare in most races (<5%), except for non-Hispanic Blacks [Table 1] [47, 48]. Data from the 1000 Genomes Project confirm these findings, with the frequency of the minor allele varying greatly among African (44%), Asian (23%), Kuwaiti (14%), American (11%), and European (8%) samples [49]. The substitution of lysine for threonine is in the extracellular domain of the protein and potentially creates a novel site for ubiquitination. The substitution of isoleucine for valine (each a hydrophobic amino acid) is in the first transmembrane domain.

Certain variants found in the 5' flanking region also demonstrate different allelic frequencies among races, with -762 (T/C) and -239 (A/G) occurring more frequently in African Americans and Hispanics and -201 (C/G) more commonly in Caucasians [50, 51]. Most variants, however, occur too rarely to make firm conclusions regarding racial associations [Tables 2 and 3].

Functional Studies of MC3R variants

Functional studies of human *MC3R* variants shed light on the mechanisms behind the purported phenotypic differences. Constructs transfected into HEK293 cells demonstrated that the T6K+V81I MC3R has decreased maximal cAMP generation from α-MSH stimulation as compared to the wildtype construct, as well as decreased ligand binding capacity despite

preserved binding affinity. This is in part due to decreased MC3R protein expression, despite normal mRNA transcription, suggesting that the variant may either have decreased translation or increased degradation. Despite this, membrane localization appears unaffected [47, 52]. When T6K and V81I constructs were examined separately, no significant differences were seen in cAMP generation, lending further credence to the notion that both T6K and V81I are likely necessary for phenotypic effects [47, 53].

The findings from the functional studies of rare *MC3R* variants are summarized in Table 2. Interestingly, except for a few mutations, the *in silico* prediction, functional studies, and phenotype of the affected individuals rarely coincide. Yang and Tao have proposed a classification system for the deleterious effects of MC3R mutations [54]. Certain variants, such as A260V, have decreased protein synthesis or accelerated protein degradation (Class I). Others, such as T280S, demonstrate significantly reduced cell surface expression, which expectedly results in decreased ligand binding, and nearly abolished signaling (Class II) [19, 55, 56]. Mutations such as D158Y have preserved translocation to the cell surface, but inherent defects in ligand binding lead to poor signaling (Class III) [19, 55]. Lastly, mutations that have normal cell surface expression and ligand binding but impaired signaling ability are considered Class IV, while putative benign polymorphisms are Class V. Few functional studies have been performed for synonymous variants, so their potential effects are unclear.

Surprisingly, several mutations (e.g. S69C, F82S, L299V, I335S) which had profoundly decreased cell surface expression and/or ligand binding, still retained normal pERK1/2 signaling capacity. Also, every mutation examined in one paper [56], demonstrated significantly decreased cell surface expression, contradicting earlier research in several cases (e.g. A70T,

M134I), whereas most other *MC3R* mutations examined in other publications by this same lab had normal cell surface expression [19, 52, 53, 56, 57]. Ultimately, these functional studies need to be replicated to ensure that accurate conclusions can be drawn.

Metabolic Effects of MC3R variants

T6K + V81I

There is much debate over whether the T6K+V81I variant has any phenotypic effects. Li et al first sequenced *MC3R* in a cohort of morbidly obese females (BMI ≥ 40 kg/m^2) and non-obese (BMI ≤ 27 kg/m^2) controls, and found that the missense mutation V81I did not vary in frequency between the two groups [50]. Similarly, in a cohort study of French Caucasian adults, the T6K+V81I variant was not associated with T2D or obesity. However, among the nondiabetic, nonobese healthy controls, T6K+V81I was associated with greater fasting plasma glucose and insulin, as well as greater insulin levels at the 30, 60, and 90 minute time points of an oral glucose tolerance test (OGTT) [58]. Several other cohort studies also found no metabolic differences in carriers of the common variant, leading some to conclude that the *MC3R* T6K+V81I haplotype has no discernable phenotypic effect [16, 59-63].

However, many of the negative studies involved populations with relatively few individuals homozygous for the variant haplotype. If, hypothetically, the phenotypic effect from T6K+V81I primarily occurs in the homozygous state (i.e. autosomal recessive), it would not be surprising to find a lack of genotype-phenotype association in cohorts with a low minor allele frequency.

Indeed, in a relatively large US pediatric cohort enriched for obesity and African Americans, where 8% of subjects and 15.8% of African Americans were homozygous for the double mutation, significant metabolic associations were seen. Subjects homozygous for T6K+V81I had higher BMI-z, fat mass (FM), percent fat mass (%FM), and waist circumference [47]. The anthropometric results (BMI, BMI-z, FM, %FM) were reproduced in a later study from our laboratory, including 262 children not studied previously. Additionally, decreased fat-free mass and fat-free mass percentage were seen among the homozygous individuals [64], consistent with the knockout *Mc3r* mouse models [37].

It has been suggested that the T6K+V81I variant may exert its effects more prominently earlier in life and have much more profound effects in the homozygous than heterozygous state. A recent study from Singapore [65] examined subjects from birth until 48 months of age. By 24 months of age homozygous carriers had greater BMI-z and surrogate markers of fat mass (triceps skinfold and subscapular skinfold) than did wildtype infants, and these differences continued to increase with age [65].

Although most other studies, primarily with few homozygous carriers [Table 1], have not replicated these anthropometric findings, a pediatric cohort from Singapore associated variant carriers with increased FM, while obese T6K+V81I carriers in a Belgian cohort demonstrated greater BMI and body weight [52, 66].

Consistent with possibly increased fat mass, multiple studies have demonstrated higher leptin concentrations among T6K+V81I carriers [47, 52, 67, 68]. T6K+V81I may also be associated with insulin resistance, although the results to date have been inconsistent. Among pediatric cohorts, US children homozygous for T6K+V81I were found to have higher fasting

insulin and insulin resistance (as measured by HOMA-IR) [47], whereas in a Singapore cohort, carriers were associated with lower insulin resistance [52]. Some adult cohorts have also suggested greater insulin resistance in carriers [58, 67, 68], whereas others have not [16, 59, 61, 69].

Associations with other markers of the metabolic syndrome have been mostly negative. Among a Chilean cohort of obese children, T6K+V81I carriers were at higher risk for Metabolic Syndrome, hypertriglyceridemia, and low HDL, but these results did not remain significant after correction for multiple comparisons [70]. Most other studies showed no differences among genotype with respect to triglycerides, cholesterol, or blood pressure [16, 47, 48, 61, 69], although a Kuwaiti study did show association with increased systolic blood pressure among variant carriers [49].

Determinants of possible causes of adiposity/obesity associated with the T6K+V81I haplotype have also been investigated. Consistent with knockout mouse models [36], glucose oxidation was increased and lipid oxidation decreased in T6K+V81I carriers [69, 71]. T6K+V81I variant carriers may also have greater difficulty losing weight, consistent with the "metabolically thrifty" phenotype [72, 73]. Two studies found no differences in energy intake between genotypes [52, 59], and in a third, the association no longer was significant when the data were examined separately by race [64]. Additionally, no significant differences were seen in resting energy expenditure [59, 64, 69], total energy expenditure [64, 71], respiratory quotient [59, 64], or physical activity [52, 64, 71, 72].

Eating behavior patterns have also been investigated, with negligible differences reported between genotypes. No differences were seen in an Asian pediatric cohort or Hispanic adult cohort on the Three-Factor Eating Questionnaire, a validated tool that examines eating restraint, disinhibition/cognitive restraint, emotional eating, and hunger [52, 74, 75]. Lower emotional eating was seen among boys (but not girls) carrying the minor alleles in a Hispanic cohort [51]. Aside from increased "slowness in eating" in infants, no differences were seen on any subscale of the Child Eating Behavior Questionnaire in infants or children [51, 65].

A mouse model for MC3R T6K+V81I

To clarify the role the *MC3R T6K+V81I* variant plays in obesity and metabolism, our group generated two novel homozygous knock-in mouse models [4] where we replaced the murine *Mc3r* with either human wildtype (hWT) *MC3R* (*MC3R*$^{hWT/hWT}$) or the human double-mutant (hDM) *T6K+V81I MC3R* (*MC3R*$^{hDM/hDM}$). The *MC3R*$^{hWT/hWT}$ mice were phenotypically normal, while the *MC3R*$^{hDM/hDM}$ mice exhibited greater fat mass (Figure 2) and feeding efficiency with reduced fat-free mass – results similar to those found in the *Mc3r* knockout mouse.

Despite their greater adiposity, *MC3R*$^{hDM/hDM}$ mice also showed reduced obesity-associated metabolic dysfunction [4]. *MC3R*$^{hDM/hDM}$ mice had well-maintained insulin sensitivity with similar serum glucose, triglycerides, cholesterol, free-fatty acid and corticosterone concentrations when compared to the non-obese *MC3R*$^{hWT/hWT}$ mice. *MC3R*$^{hDM/hDM}$ mice exhibited higher circulating leptin that appeared related to their increased adiposity rather than to the presence of leptin resistance, since suppression of food intake by leptin appeared normal [4]. Additionally, *MC3R*$^{hDM/hDM}$ mice demonstrated increased bone marrow fat, along with

decreased crown-rump length and femur length. This was at least partially due to an alteration of mesenchymal stem cell fate, which seemed favored towards differentiation to triglyceride-accumulating adipocytes instead of osteoblasts. Additionally, unlike most other obese mouse models, *MC3R*$^{hDM/hDM}$ mice had greater circulating adiponectin, which we then also observed in samples from *MC3R*$^{hDM/hDM}$ humans [4]. It is possible that increased serum adiponectin may stimulate mesenchymal stem cell mobilization into adipose tissue, resulting in less bone mass [76] with shorter femur and reduced trabecular bone mineral density of *MC3R*$^{hDM/hDM}$ mice.

As noted above, in vitro studies find decreased *MC3R* protein expression and therefore partial inactivation of the *MC3R* by *T6K+V81I* [47]. Since T6K lies between the first and second in-frame translational start site, it might not necessarily be part of the translated protein [13]. We have proposed that *T6K+V81I* MC3R might affect the proportion of protein translated from the first and second in-frame ATGs and thus potentially interfere with MC3R actions, for instance by altering its membrane localization [13]. *MC3R*$^{hDM/hDM}$ mice do not appear to be phenocopies of MC3R$^{-/-}$ mice. For example, when compared with wildtype mice, *MC3R*$^{hDM/hDM}$ mice do not exhibit hyperglycemia under chow-fed conditions or increased inflammation in WAT under high-fat diet conditions. More studies are needed to understand better how *T6K+V81I* may affect receptor expression, stability, intracellular trafficking and signaling *in vivo*.

In summary, the metabolic effects of the T6K+V81I haplotype are not entirely straightforward given the conflicting results between studies. In humans, the most dramatic associations - namely increased BMI and fat mass - seem to occur in individuals homozygous for T6K+V81I and primarily in youth. In the heterozygous state or in adulthood, the haplotype's effects are milder. Why this is the case needs further investigation. Studies of the *MC3R*$^{hDM/hDM}$

knockin mouse model, though, confirm this variant increases the predisposition for triglyceride accumulation, with what appear to be direct effects at peripheral sites including adipose tissue.

Other coding variants

To date, 33 rare coding variants have been identified [Table 2]. These mutations have been identified in too few individuals to make definitive conclusions regarding their phenotypic consequences, and no mouse models exist to date. However, examining the *in* silico prediction, functional studies, and characteristics of the affected subjects and their family members, we suggest that six are likely pathologic mutations, ten are likely benign polymorphisms, six are synonymous (insufficiently studied), and the effect is unclear for 11 other variants.

The variants D158Y, I183N, T280S, L299V, I335S, and c.397_726delins228 are likely pathologic. In all of these cases cAMP production is significantly decreased and all affected individuals are obese with no cases of lean subjects described. In most cases the obesity phenotype occurred early in age, which is to be expected from an inherited genetic condition. The D158 residue has been shown to be integral for ligand binding, while I335 is part of the highly-conserved N/DPxxY motif which plays important roles in ligand binding, G-protein coupling, and internalization [77-79].

Sequence variants S17T, S69C, A70T, F82S, I87T, V124L, N128S, M134I, V211I, L297V, and E342K, have been classified as “possibly pathologic”, because the physiologic consequences of these variants are unclear. For example, S17T was identified in obese cases only, but *in silico* prediction modelling defines this as a benign variant, and data suggest that cAMP and pERK1/2 signaling remain relatively preserved [19, 55]. In other cases, such as S69C, F82S, I87T, V124L,

and E342K, the variant was found in lean individuals, even though *in silico* prediction or functional studies suggest that the mutation may be deleterious. The reason for this discordance may be that any or all of these mutations may only cause a weak predisposition to obesity, or significant clinical effects may only be realized in the homozygous state.

The other identified sequence variants (V177I, L249F, V255I, R257S, A260V, M275T, L285V, A293T, G353D, X361S) we presume are likely benign. For most of these variants, lean individuals were found carrying the variant (e.g. V255I, G353D) and/or functional studies demonstrated normal ligand binding and signal transduction. Interestingly, most of these variants are located in the intracellular regions of the protein or in transmembrane domain 6, which classically are important areas for G-protein binding and therefore cAMP production. However, alanine scanning studies did not identify any of the aforementioned residues as critical for functioning [80].

Lastly, the observed synonymous mutations are assumed to be benign, although recent studies have suggested that even synonymous mutations can have functional, consequences [81, 82].

Non-coding variants

14 sequence variants in the 5' UTR and four in the 3' UTR for *MC3R* have also been identified [Table 3]. The -4 codon is located in the promoter region of *MC3R*, and cell transfection studies suggest that the T-to-C mutation at this site suppresses gene transcription. This variant has only been identified in two obese subjects and no lean individuals [55].

The rs6014649(G/A) polymorphisms is in high linkage disequilibrium with the *T6K+V81I* variant ($r^2 > 0.8$), and minor allele carriers were associated with higher fasting glucose oxidation and lower fasting FFA [69]. In the same cohort, rs6127698(G/T) was shown to be associated with higher lipid oxidation in the fasted state among individuals homozygous for the minor allele, but otherwise no anthropometric differences were seen [69].

Among 3'UTR variants, homozygosity for +2138InsCAGACC in normal weight subjects was reported to be associated with increased FM and %FM. Conversely, in overweight subjects with homozygosity for the insertion allele was associated with decreased FM and %FM, and in obese subjects lower total abdominal fat was seen [59]. However, in other cohorts, no anthropometric differences were seen between +2138InsCAGACC carriers and wildtype subjects [51, 64, 70]. Among obese girls, +2138InsCAGACC carriers were reported in one study to have significantly higher emotional eating than wildtype subjects, whereas obese boys had lower food enjoyment, although it is not clear if these measures would have retained statistical significance had the study corrected for their multiple-comparisons [51].

Conclusions

Except for the common variants T6K and V81I, most sequence variants in *MC3R* are rare in humans. Several rare *MC3R* mutations abolish MC3R function, apparently leading to an obese phenotype, but most mutations are partially inactivating with unclear consequences and mouse models are lacking. The T6K+V81I haplotype is by far the most well studied; its phenotypic effects are most prominent in the homozygous state, with milder clinical effects seen in heterozygous carriers. Studies also suggest that the haplotype's effects may be more pronounced in infancy and childhood. The ability of T6K+V81I to alter energy homeostasis is

demonstrated by knockin mouse studies. Further research is needed to elucidate MC3R's differential functions between tissue types as well as its activity and regulation during different stages of life.

Table 1 – Distribution of T6K+V81I Haplotypes among different cohorts

Year	Country	Cohort	No. of Subjects	HOM (%)	HET (%)	WT (%)	Reference
2000	US	Adult Females BMI≥40 or BMI<27	209	3.3	19.1	77.5	[50]
		Black	*51*	*11.8*	*43.1*	*45.1*	
2001	France	Adults	526	1.7	14.3	84.0	[58]
2002	Canada	Adults	222	0.9	19.8	79.3	[59]
2002	NZ	Maori	12	0	66.7	33.3	[60]
2003	Finland[1]	Adults BMI≥40	244	1.2	15.2	83.6	[67]
2004	Greece[2]	Women	116	1.7	19.8	78.4	[68]
2005	US	Children	355	8.2	34.9	56.9	[47]
		Black	*152*	*15.8*	*52.6*	*31.6*	
		Caucasian	*176*	*1.7*	*18.2*	*80.1*	
		Other Race	*27*	*7.4*	*44.4*	*48.2*	
2007	Singapore	Severely obese Asian children	198	3.5	35.4	61.1	[52]
		Chinese	*105*	*2.9*	*36.2*	*61.0*	
		Malay	*68*	*0*	*35.3*	*64.7*	
		Indian	*19*	*21.1*	*36.8*	*42.1*	
2007	Finland[1]	Adults without T2D	214	2.3	16.4	81.3	[69]
2007	Italy	Obese children	184	0	10.9	89.1	[72]
2009	North America	Adults BMI≥40 or BMI≤25	1035	2.3	19.9	77.8	
2009	US	Children	416	7.7	30.3	62.0	[64]
		Black	*176*	*15.3*	*50.0*	*34.7*	
2010	Chile	Obese children	229	0.4	10.9	88.6	[51]
2010	Belgium	Adults	1321	0.6	13.6	85.8	
2011	South Africa	Children	431	17.4	49.0	33.6	[48]
		Black	*209*	*23.0*	*56.5*	*20.6*	
2013	Poland	Obese children	257	1.2	21.4	77.4	[63]
		Never-obese adults	94	0	19	75	
2015	Thailand	Adults+children	188	5.3	40.0	61.7	[83]
2016	Singapore	Infants	1090	6.1	35.3	58.6	[65]
		Chinese	*617*	*5.3*	*32.3*	*62.4*	
		Malay	*276*	*6.5*	*34.1*	*54.3*	
		Indian	*197*	*7.6*	*39.6*	*52.8*	

Subgroups presented in italics. HOM = homozygous for the minor allele variant, HET = heterozygous at either T6K or V81I, WT = homozygous wildtype. In studies where only one codon was reported, complete linkage disequilibrium was assumed. [1]T6K and V81I reported separately; frequencies of T6K+V81I haplotype estimated from the data given. [2]Data for only one subgroup was given.

Table 2 – Rare *MC3R* coding variants

Variant/Mutation	Domain	No. of Cases		*In Silico* Prediction PolyPhen-2	cAMP	pERK1/2	Ligand Binding	Cell surface Expression	References
		Obese	Lean						
F12F	EC N-terminus	0*	0						[55]
S17T	EC N-terminus	3	0	-	+[a]	+	+	-	[19, 55]
L35L	EC N-terminus	1	0						[84]
S69C	EC N-terminus	0	1	++	+[a]	-	+	++	[19, 56, 62]
S69S	EC N-terminus	1*	0						[55, 84]
A70T	EC N-terminus	1	0	-	+[a]	-	-	+[a]	[19, 52, 56]
F82S	TM1	4	4	++	++	-	++	++	[19, 62, 85]
I87T	TM1	1	2	+	-	+	+	++	[19, 55, 56, 62]
L95L	TM1	2*	1						[55, 85]
V124L	TM2	0	1	++					[85]
N128S	TM2	2	0	++	+	+		-	[19, 84, 85]
M134I	TM2	1	0	+	+[a]	++	-	+[a]	[19, 52, 56]
D158Y	TM3	1	0	++	++	++	++	-	[19, 55]
V177I	TM3	1	0	-	-	-	-	-	[19, 55]
I183N	TM3	2	0	++	++	-	-	+[a]	[19, 52, 53, 86, 87]
V211I	TM3	1	0	-	+			-	[84]
I226I	TM4	3*	0						[55, 84, 85]
L249F	IC loop 3	2	1	++	-	-	+	-	[19, 55, 56, 85]
V255I	IC loop 3	0	1	-					[85]
R257S	IC loop 3	4	9	++	-	-	+	-	[19, 55, 62, 63, 85]
A260V	IC loop 3	1	0	-	-	-	-	++	[19, 56, 62]
Q270Q	IC loop 3	0*	0						[55]
M275T	TM6	1	0	+	-	-	-	++	[19, 56, 62]
T280S	TM6	2	0	++	++	++	++	++	[19, 55, 56, 62]
L285V	TM6	0	1	++	-	-	+	-	[19, 55]
A293T	TM6	2	0	++	-	-	-	-	[10, 17, 19, 55]

L297V	TM6	1	0	++	-	+	-	++	[19, 56, 62]
L299V	TM6	1	0	++	++	-	++	+[a]	[19, 84]
I335S	TM7	5	0	++	++	-	++	++	[10, 17, 55, 57, 63, 84]
E342K	IC C-terminus	0	1	++					[85]
G353D	IC C-terminus	0	1	-					[85]
X361S	IC C-terminus	2	2		-	-	-	-	[10, 17, 19, 55]
c.397_726delins228	TM3-TM4 (?)	1	0		++				[62]

*not described whether one of subjects was obese or lean, ++ probably deleterious, + possibly deleterious, - probably benign or not significantly different from wildtype, EC = extracellular, TM = transmembrane domain, IC = intracellular, [a] Conflicting results between studies.

Table 3 – Non-coding *MC3R* locus variants

Codon Variant/ Mutation	Country	HET No. (%)	HOM No. (%)	Putative Functional Effects	References
5' UTR					
-769(T/C)	US	28 (13.4)	0	None	[50]
-762(A/T)	US	44 (21.1)	0	None	[50]
-239(A/G) (rs11575886)	US	5 (2.4)	0	None	[50]
	Chile	19 (8.3)	1 (0.44)	Homozygous mutants associated with higher waist-to-hip ratio.	[51]
	Chile	(8.5)	(0.5)	None	[70]
-201(C/G)	US	31 (14.8)	0	None	[50]
-4(T/C)	Italy & France	2 (<0.01)	0	Decreases translation	[55]
rs4627642(A/T)	Finland	82 (38.0)	21 (9.7)	None	[69]
rs6024728	Europe			None	[73]
rs6024730(G/A)	Finland	59 (27.3)	12 (5.6)	None	[69]
	Europe			None	[73]
rs6024731	Europe			None	[73]
rs16979603(T/C)	Finland	69 (31.9)	19 (8.8)	None	[69]
rs6014646(A/T)	Europe			T carriers may have more difficulty losing weight	[73]
rs6014649(G/A)	Finland	29 (13.4)	5 (2.3)	Linkage Disequilibrium with T6K+V81I ($r^2 > 0.8$). A carriers associated with higher fasting glucose oxidation and lower fasting FFA.	[69]
rs6127698(G/T)	Finland	110 (50.9)	45 (20.8)	TT associated with higher lipid oxidation in fasted state.	[69]
	Belgium	510 (50.6)	240 (23.8)	None	[66]
	Europe			None	[73]
rs11697509	Europe			None	[73]
3' UTR					
+2138InsCAGACC	Canada	126 (38.9)	15 (4.6)	Associated with variations in fat mass, percent body	[59]

(rs74181042)				fat, and total abdominal fat.	
	US	167 (40.0)	25 (6.0)	None	[64]
	Chile	67 (29.0)	7 (3.0)	No anthropometric effects. Female carriers had higher emotional eating scores. Males had lower scores in the enjoyment to food subscale.	[51]
	Chile	(29.7)	(2.5)	None	[70]
rs2870730 (G/C)		60 (27.8)	11 (5.1)	None	[69]
rs1543873(T/G)	Europe			None	[73]
rs6099058	Europe			None	[73]

Subjects represented as *n* (%). HET = heterozygous for the minor allele, HOM = homozygous for the minor allele.

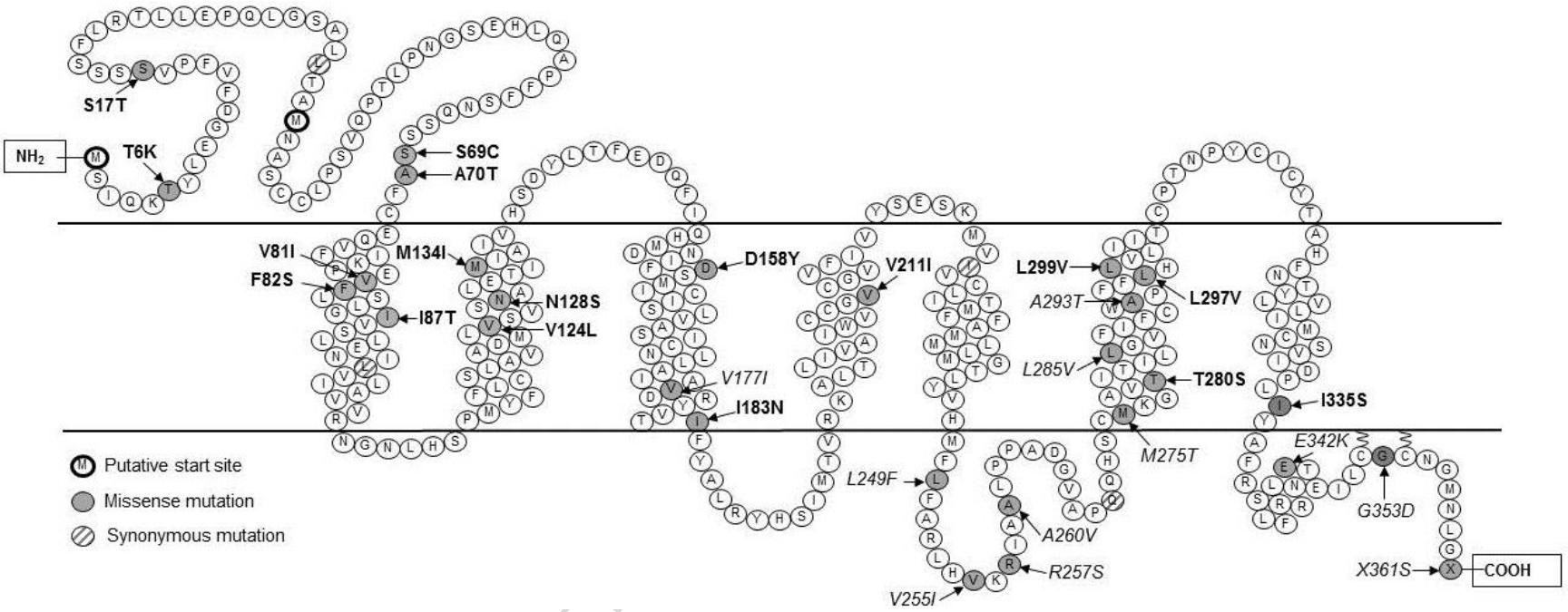

Figure 1. *MC3R* mutations and variants identified in humans. Mutations in bold font represent possibly deleterious mutations. Mutations in italics represent presumed benign sequence variants. Adapted from [54, 88].

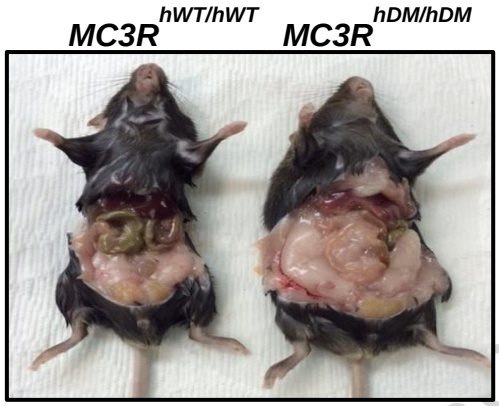

Figure 2. *hMC3R* knockin mice. The murine *Mc3r* was replaced with the human consensus "wildtype" sequence (hWT) or the C17A + G241A human "double-mutant" (hDM) variant, which changes two amino acids in MC3R: Thr6Lys and Val81Ile (T6K+V81I). Mice homozygous for *MC3R hDM* have greater adiposity than mice homozygous for *hWT*.

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Highlights

- Both central and peripheral actions of MC3R appear important for weight regulation.
- Certain human *MC3R* mutations are associated with greater adiposity and hyperleptinemia.
- MC3R function is altered by homozygosity for both rare alleles of the human variant *T6K+V81I*.
- *T6K+V81I* knockin mouse models suggest MC3R is involved in determining how mesenchymal stem cell differentiate.