Genome-Wide Association Study

of Fat intake in Adolescence

Seyed Amirreza Haghighi Kakhki

Department of Nutritional Sciences

University of Toronto

A thesis submitted in conformity with the requirements for the degree of

Masters of Sciences

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ABSTRACT

Dietary preference for fat, which increases risk for obesity, is a complex behavior regulated in part by the brain-reward system. We conducted a genome-wide association study (GWAS) to search for loci associated with relative fat intake in 598 adolescents (12-18 years) who were recruited from the French-Canadian founder population. The GWAS identified a locus in the \( \mu \)-opioid receptor gene (\( OPRM1 \), rs2281617, \( p=5.2\times10^{-6} \)), which encodes a receptor expressed in the brain-reward system and which has been shown previously to modulate fat preference in animals. The minor \( OPRM1 \) allele appeared to have a “protective” effect: it was associated with lower fat intake and lower body-fat mass, In addition, \( OPRM1 \) was associated with relative fat intake in an independent sample of 490 young adults. In summary, \( OPRM1 \) may modulate dietary
preference for fat and hence the risk for obesity. Further studies are required to confirm our findings.
This thesis is dedicated to my parents,

Hossein Haghighi and Nahid Rahimi,

for their unconditional love and support,

for cultivating me in the empathy and compassion for others,

and for inspiring me!
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## CONTENTS

**ABSTRACT** .......................................................................................................................... II

**DEDICATION** ........................................................................................................................ IV

**ACKNOWLEDGEMENT** .......................................................................................................... V

**LIST OF TABLES** .................................................................................................................... IX

**LIST OF FIGURES** .................................................................................................................. X

**ABBREVIATIONS AND ACRONYMS** ................................................................................ XI

**CHAPTER ONE (Introduction)** ............................................................................................ 1

**CHAPTER TWO (Literature Review)** .................................................................................... 5

2.1 Fat intake and obesity ......................................................................................................... 6

2.1.1 Animal studies ............................................................................................................... 7

2.1.2 Human studies ............................................................................................................... 8

2.2 Physiology of the regulation of food intake ..................................................................... 10

2.2.1 Homeostatic Mechanisms ........................................................................................... 10

2.2.2 Reward-related mechanisms ....................................................................................... 12

2.3 Physiology of the regulation of fat intake ....................................................................... 15

2.3.1 Homeostatic Mechanisms ........................................................................................... 17
2.3.2 Reward-related mechanisms ................................................................. 17

2.4 Amygdala and fat intake and preference .................................................. 18

2.5 Factors influencing fat intake and preference ............................................ 18

2.5.1 Environmental factors influencing fat preference ................................... 20

2.5.2 Genetic factors influencing fat preference ............................................. 23

2.5.2.1 Heritability of fat intake (Family and twin study) ................................. 24

2.5.2.2 Genetic approaches used to study complex traits ............................... 25

2.5.2.3 Genetic study of fat intake ................................................................. 29

CHAPTER THREE (Rationale, Hypothesis & Objective) ............................ 34

3.1 Rationale .................................................................................................... 35

3.2 Hypothesis .................................................................................................. 35

3.3 Objective ................................................................................................... 35

CHAPTER FOUR (Materials and Methods) ................................................. 36

4.1 Participants ................................................................................................ 37

4.2 Founder population .................................................................................. 39

4.3 Dietary assessment .................................................................................... 39

4.4 Magnetic resonance imaging of the amygdala ........................................ 44

4.5 Adiposity assessment ............................................................................... 44

4.6 Genotyping ............................................................................................... 45

4.7 Statistical analyses ................................................................................... 45

4.8 Replication study ...................................................................................... 46

CHAPTER FIVE (Manuscript - Opioid Receptor mu 1 Gene, Fat Intake and
Obesity in Adolescence) ............................................................................. 48
CHAPTER SIX (Discussion) .................................................................71

REFERENCES .....................................................................................78
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table 1:</th>
<th>SNPs most significantly associated with fat intake in the Saguenay Youth Study.</th>
<th>62</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2:</td>
<td><em>OPMR1</em> SNPs most significantly associated with fat intake in the Saguenay Youth Study (SYS) and Toronto Nutrigenomics Health Study (TNHS).</td>
<td>63</td>
</tr>
<tr>
<td>Supplementary Table 1:</td>
<td>Characteristics of the studied participants in the Saguenay Youth Study and Toronto Nutrigenomics Health Study.</td>
<td>64</td>
</tr>
<tr>
<td>Supplementary Table 2:</td>
<td>Chromosomal position and minor allele frequency of the top <em>OPMR1</em> SNPs associated with fat intake in the SYS and TNHS, respectively.</td>
<td>65</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure 1</th>
<th>OPRM1 locus of fat intake in the Saguenay Youth Study .............................................................66</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2</td>
<td>Association of the OPRM1 locus (rs2281617) with fat, carbohydrate, protein and energy intakes and adiposity measures in the Saguenay Youth Study .........................67</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Two possible scenarios for the role of OPRM1 in shaping the amygdala volume ................................68</td>
</tr>
<tr>
<td>Supplementary Figure 1:</td>
<td>SNPs most significantly associated with fat intake in the Saguenay Youth Study and Toronto Nutrigenomics Health Study .................................................................69</td>
</tr>
<tr>
<td>Figure 4</td>
<td>LD plot for the OPRM1 locus ...............................................................74</td>
</tr>
</tbody>
</table>
# ABBREVIATIONS & ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>Arc</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BP</td>
<td>Base pair</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine-amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>DAMGO</td>
<td>D-Ala2-N-Me-Phe4-gly5-ol-enkephalin</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygotic</td>
</tr>
<tr>
<td>EPIC</td>
<td>European Prospective Investigation into Cancer and Nutrition</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food-frequency questionnaires</td>
</tr>
<tr>
<td>FTO</td>
<td>Fat mass and obesity-associated</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HapMap</td>
<td>Haplotype map</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HTR2A</td>
<td>Serotonin type 2A receptor</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
</tbody>
</table>
LHA  Lateral hypothalamic area
MC4R  Melanocortin 4 receptor
MRI  Magnetic resonance imaging
MZ  Monozygotic
NPY  Neuropeptide Y
NTS  Nucleus tractus solitarius
OPEN  Observing Protein and Energy Nutrition
OPRM1  µ-opioid receptor gene
POMC  Pro-opiomelanocortin
PROP  6-n-propylthiouracil
PYY  Peptide tyrosine-tyrosine
SES  Socio-economic status
SH2B1  SH2B adaptor protein 1
SLSJ  Saguenay Lac St. Jean
SNP  Single nucleotide polymorphisms
SYS  Saguenay Youth Study
TAS2R38  Taste receptor, type 2, member 38
TNHS  Toronto Nutrigenomics and Health Study
TUB  Tubby gene
CHAPTER ONE

Introduction
The problem of obesity has become increasingly prominent and is now recognized as a critical target for public health interventions in many countries. Childhood obesity is linked to adult obesity and is accompanied by adverse health status already in childhood\(^1\). Overweight and obese children are being diagnosed more and more frequently with typically adult disorders, such as type 2 diabetes and hypertension\(^2\). Despite the growing understanding of the health risks that obesity carries, the prevalence of obesity is still high. In the US, for example, almost 17\% of children and adolescents were classified as obese in 2009-2010, and the prevalence did not improve compared with 2007-2008\(^3\). A study showed that eating patterns of young children who consumed energy-dense, low fiber, high-fat diets had a propensity towards having higher adiposity in their later years of childhood. This suggests that interventions to change diet should start at an early age\(^4\).

Obesity develops when energy intake chronically exceeds energy expenditure. This imbalance is driven by complex biological, psychosocial and environmental factors\(^5\). However, physiological mechanisms of eating behavior are still not well understood.

A large body of research in humans and experimental animals suggests that excessive intake of fat contributes to obesity\(^6\)-\(^8\). Fats are of higher energy density and higher energy efficiency when compared with other macronutrients, namely carbohydrates and proteins. Each gram of fat contains twice the energy of a gram of carbohydrates or proteins. Even when the same energy is
consumed, almost all calories eaten as fats are stored, whereas 5-10% and 20-30% of calories eaten as carbohydrates and proteins, respectively, is lost during their absorption, processing and storage\(^9\). A meta-analysis of 28 clinical trials in adults showed that a 10% reduction in the proportion of energy intake from fat was associated with a meaningful reduction in body weight\(^8\). In children, it has been shown that adiposity (percent of body weight as body fat) is positively correlated with dietary fat intake (percent of total energy), even after controlling for differences in physical activity and other potentially confounding factors, such as sex, total energy intake and parental body mass index (BMI)\(^{10-12}\). Some studies argue, however, that dietary fat is not a major determinant of body fat\(^{13-15}\). For example, they suggest that fat reduction induced weight loss is modest and may be insignificant in the longer term and that confounding factors such as fibre content or glucose load might underlie the weight loss\(^{13-15}\).

Dietary preference for fat is a complex behavior regulated in part by: (1) *homeostatic mechanisms*, involving brain structures, such as the brainstem and hypothalamus, which serve to maintain energy balance, and (2) *reward-related mechanisms* involving brain structures, such as the amygdala, which process the hedonic properties of food independently of energy status\(^{16}\). The amygdala structures also processes the hedonic properties of addictive drugs\(^{17-19}\).

Genes play a significant role in determining inter-individual differences in fat intake (assessed as a proportion of energy eaten as fat), as suggested by family and twin studies\(^{20-24}\). However, individual genes and hence the
mechanistic pathways involved are not very well known. Therefore, we performed a genome-wide association study (GWAS) of dietary intake of fat to search for genes and underlying pathways modulating inter-individual differences in fat intake and, then, we tested whether the identified loci were also associated with adiposity and the size of the amygdala. We performed these studies in a population-based sample of 598 adolescents who were recruited from a French-Canadian population with known genetic founder effects.

Our results suggest that OPRM1 is a gene that may impact fat intake and risk for obesity and these effects may be associated with subtle variations in the amygdala volume. In addition, we tested our primary findings in an independent sample of 490 young adults of European ancestry from the Toronto Nutrigenomics Health Study.
CHAPTER TWO

Literature Review
This chapter reviews the current literature on (1) the association between fat intake and obesity, (2) the mechanisms involved in the regulation of food and fat intake, and (3) environmental and genetic factors influencing fat intake.

2.1 Fat intake and obesity

Excess intake of fat may promote the development of obesity\textsuperscript{25}. Several mechanisms have been proposed to explain how high-fat intake may lead to greater body adiposity\textsuperscript{26 27}. First of all, dietary fat is the most energy-dense macronutrient; its energy value (9 kcal/g) is higher than that of carbohydrates (4 kcal/g) or proteins (4 kcal/g)\textsuperscript{28}. In addition, fats lend greater flavor and palatability to foods and have a lower satiating power than the other two macronutrients, which could lead to their greater consumption\textsuperscript{29}. In addition, when studied under careful metabolic conditions, fats produce a lower thermogenic effect than carbohydrates or proteins, suggesting that dietary fat may be utilized more efficiently and thus accumulates as body fat more readily\textsuperscript{30 31}. Finally, as a substrate for energy metabolism, fats are at the bottom of the oxidative hierarchy determining fuel selection\textsuperscript{32}. There is evidence that the body has a tendency to oxidize carbohydrates and proteins before fats\textsuperscript{33}. Several studies have shown that an increase in intake of fat does not stimulate its oxidation, even if that increase is in excess of energy requirements\textsuperscript{34-36}. For all these reasons, dietary intake of fat is considered to be “obesogenic”.
2.1.1 Animal Studies

Numerous animal studies have shown that higher intake of fat increases adiposity\textsuperscript{37-39}. Data from a series of experiments, involving over 500 mice studied for 12 months, showed that, when fed a high-fat diet (58% energy contributed by fat), the mice gained significantly more weight in comparison with those fed a normal diet (containing 11% fat)\textsuperscript{40}. In another study, Boozer et al\textsuperscript{41} showed that dietary fat impacts body weight and adiposity in rats. Their study included controls (rats fed a low-fat diet of 12% for 6 weeks) and three case groups (rats fed diets of 24, 36, or 48% fat for the same time period); the total energy intake of all groups were kept equal. The results revealed a clear positive relationship between the amount of dietary fat consumed and the level of adiposity\textsuperscript{41}.

2.1.2 Human Studies

A positive association between dietary fat intake and body adiposity has also been observed in several large-scale studies, including (1) cross-sectional\textsuperscript{42-45}, (2) longitudinal\textsuperscript{46-48} and (3) interventional studies\textsuperscript{49-51}. In most of these studies, the association was independent of sex, age, socio-economic status, cigarette smoking and physical activity.

(1) Cross-sectional studies: Several cross-sectional studies have shown the positive relationship between fat intake and adiposity\textsuperscript{42-45}. A Flemish study analyzed the dietary records of 485 adult men and 362 women and showed in both genders that fat intake (kcal/day) was significantly higher in
overweight/obese than normal weight individuals. In addition, energy from fat but not energy from the other two macronutrients was shown to be positively associated with obesity in a large cohort study of 15,266 American men (55-79 years of age). The authors estimated that, for every 500 kcal of fat and total energy consumed, BMI was increased by 0.53 kg/m² and 0.14 kg/m², respectively. A similar relationship was also observed in children. A study of 162 boys and 100 girls (9-10 years of age) showed that percent body fat was positively associated with percent of energy from fat (r=20, p=0.002). Three groups were defined based on body-fat percentage in this study (leanest, moderate and fattest) and the results showed that the fattest group consumed significantly higher amount of energy from fat than the other two groups (p=0.02).

(2) Longitudinal studies: A cohort study of 19,478 US male health professionals, aged 40-75 years, was followed prospectively. In this cohort, energy-adjusted fat intake was positively associated with weight gain (0.1-kg increase for every 10 g of fat/day). Similarly, a longitudinal survey in China found that higher fat intake is associated with higher body weight and BMI. The authors showed that a 100-kcal increase in daily fat intake was associated with a 0.04-kg/m² increase in BMI, while a 100-kcal increase in daily protein and carbohydrate intake combined was associated with an increase of only 0.02-kg/m² in BMI. These findings suggest that the energy from fat may have a greater effect on body adiposity than energy from the other two macronutrients. Longitudinal studies in children have also shown that dietary fat is positively
related to adiposity\textsuperscript{4, 12, 48}. A longitudinal study followed 70 children (37 boys and 33 girls) from the age of 2 to 8. They showed that the BMI of 8 year-olds was positively correlated with their mean fat intake (as percentage of energy intake) \((p=0.009)\) but was inversely correlated with their carbohydrate intake (as a percentage of energy intake) \((p=0.01)\)\textsuperscript{48}.

Not all studies, however, observed an association between fat intake and adiposity\textsuperscript{53, 54}. For example, a study examining the association of percent energy intake from fat with subsequent weight change over a 10 year period in 2,580 men and 4,567 women (25-74 years of age) reported weight changes for men and women of +2.1 kg and +2.5 kg; respectively, but these changes were not associated with percent energy intake from fat\textsuperscript{54}. Although, by excluding those with clinical conditions (such as diabetes, hypertension, etc.), the positive association was seen in men.

(3) Intervention studies: Clinical trials have studied the impact of high-fat or low-fat diets on adiposity but only a few intervention studies have assessed the effects of high fat diets. For example, Lissner et al. carried out a short intervention study that asked 24 women to alternate between 3 types of diets for 2-week periods, with the total regime lasting 6-weeks. The mean daily intakes on the low- (15-20%), medium- (30-35%), and high- (45-50%) fat diets were 2,087 kcal, 2,352 kcal and 2,714 kcal, respectively. Thus, dietary fat level had a significant effect on energy intake. As a result, the weight of the subjects increased by 0.32 kg on the high-fat diet, did not change significantly on the
moderate-fat diet (-0.03 kg), and decreased on the low-fat diet by 0.40 kg during the 2-week treatment period. This demonstrates that high-fat diet increases energy intake and a body weight significantly after 2-weeks of high-fat diet feeding\textsuperscript{55}. Westerterp et al\textsuperscript{56} did a long-term intervention that lasted over six months and reported similar conclusions. However, when looking at fat-reduction intervention studies, some have reported conflicting results which could be attributed to differences in trial design and duration \textsuperscript{57,58}. In turn, a well carried out meta-analysis of 28 clinical trials in adults showed that a 10\% reduction in the proportion of energy intake from fat was associated with a meaningful reduction in body weight\textsuperscript{8}.

\textbf{2.2 Physiology of the regulation of food intake}

Two main complementary pathways regulate food intake: homeostatic and hedonic/reward-related. The homeostatic pathway regulates food intake according to the body’s energy requirements. The hedonic/reward-based pathway controls the desire of consumption of highly palatable foods and food intake, irrespective of the energy requirements\textsuperscript{59,60}. Importantly, the reward mechanisms can override the homeostatic mechanisms, leading to excess energy intake and obesity\textsuperscript{61}.

\textbf{2.2.1 Homeostatic mechanisms}

Energy homeostasis is regulated by the interaction of the brain with peripheral tissues, such as the gastrointestinal (GI) tract and adipose tissue.
These peripheral tissues release satiation and adiposity signals. While the satiation signals reflect the caloric and nutrient content of food, the adiposity signals reflect the body's energy stores (i.e., mostly as body fat in adipose tissues).

The satiation signals include peptides secreted from the enteroendocrine cells in the wall of the GI tract in response to short-time changes, such as wall distension or GI motility. These peptides stimulate adjacent sensory nerves that send message to the hindbrain and make the person feel that the stomach is full, resulting in the decision to stop eating. It has been shown that the peripheral administration of satiation peptides, at the beginning of a meal, can result in eating less, whereas that of their antagonists can result in eating more. These anorexigenic peptides include enterostatin, cholecystokinin (CCK), glucagon-like peptide (GLP-1), apolipoprotein A-IV, glucagon, amylin, oxyntomodulin, peptide tyrosine-tyrosine (PYY) and bombesin.

The adiposity signals include most prominently leptin and ghrelin that are released from adipocytes and stomach, respectively. The release rate of these signals correlates with adipose tissue stores and thus with long-term energy stores. These hormones are released in the blood stream and act on the hypothalamus where they regulate food intake and metabolic rate. Thus, leptin decreases food intake and increases metabolic rate, whereas ghrelin shows the opposite effects. The most important site of action for ghrelin and leptin is the arcuate nucleus (Arc) of the hypothalamus. Leptin receptor signaling stimulates
specific anorexigenic neurons in the arcuate nucleus that express pro-opiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART)\textsuperscript{70,71}. This stimulation results in increasing metabolic rate and decreasing food intake. In addition, leptin inhibits a group of orexigenic neurons, which express neuropeptide Y (NPY) and agouti-related peptide (AgRP), two peptides that stimulate appetite and food intake\textsuperscript{70,72,73}. In contrast, ghrelin stimulates the NPY/AgRP neurons\textsuperscript{69} and levels of this hormone inversely correlates with energy expenditure, suggesting that it plays an important role in energy balance\textsuperscript{74-76}.

### 2.2.2 Reward-related mechanisms

The brain-reward processes involved in the regulation of food intake have two main components, “wanting” and “liking”, which involve, among others, the dopaminergic and opioidergic systems, respectively. There is significant evidence in humans and rodents that supports the role of reward-processing regions of the brain\textsuperscript{60}, such as the amygdala and striatum\textsuperscript{77,78}.

The “wanting” component of reward\textsuperscript{79} can be defined as the motivation to go for something for which pleasure was experienced in the past\textsuperscript{80}. Research has suggested that dopamine signalling within the nucleus accumbens plays an important role in this process\textsuperscript{81} and that the sensation of palatable foods can stimulate the release of dopamine in the nucleus accumbens. It has been suggested that dopamine release regulates different elements related to seeking food, such as wakefulness, psychomotor activation and conditioned learning (remembering food-associated stimuli)\textsuperscript{59}. A proposed mechanism for food-
triggered dopamine release is that feeding could stimulate the release of orexin from neurons, which then induces dopamine release from the ventral tegmental area.82

The “liking” component of reward can be defined as the hedonic response to a stimulus and the opioid system is thought to play a major role in this aspect of food reward.79 83-86 Endogenous opioid peptides are produced in the brain and their role is to enhance pleasure. It is known that opioid signalling increases food intake and alters sensory preferences for macronutrients.87-89 The effect of opioids in inducing consumption are typically much stronger for highly palatable foods, especially when they are sweet or/fatty.90 91 Studies in rodents have shown that µ- and κ-opioid receptor agonists increase intake and reinforce the consumption of energy-dense foods, while they have little effect on intake of less palatable foods.92-94 Similarly, µ-opioid receptor antagonists significantly attenuated the palatable food induced activation of reward-processing regions of the brain in human.95 Furthermore, naltrexone (opioid antagonist) has been shown to decrease intake of preferred foods (carbohydrates in carbohydrate-preferring animals and fats in fat-preferring animals), suggesting that the baseline preference for specific foods has modulatory effect on sensitivity to opioid effects.96 In addition, several studies predominately showed the fat preference effect of the opioids.97-99 Rats administrated opioid-agonist morphine, preferentially consumed more fat, when fed with self-choice of the fat, carbohydrate and protein diets.98 Similarly, an subcutaneously injection of the κ-opioid agonist ketocyclazocine induced a greater increase in intake of a high-fat
diet than of a high-carbohydrate diet when rats were fed either the high-fat or the high-carbohydrate diet and intake of food was measured during the 6 hour trial\textsuperscript{97}.

Although the homeostatic and reward mechanisms have separate mechanisms\textsuperscript{100}, they are highly intertwined. The projections of the nucleus accumbens to the hypothalamus have been suggested to modulate reward-driven food intake, as the accumbens nuclei projections influence the hypothalamic feeding circuits, which are known to be significantly involved in homeostatic regulation of energy balance. The activation of opioid receptors in the nucleus accumbens stimulates the lateral hypothalamic area by removing their tonic inhibition\textsuperscript{101}. Therefore, the accumbens-hypothalamic pathways are an illustration of the interaction between the homeostatic and reward mechanisms that control food intake. Reciprocally, the metabolic signals can influence the sensitivity of certain reward-processing brain areas, such as the amygdala, orbitofrontal cortex, nucleus accumbens, anterior insula and striatum, and modulate reward, processing of food stimuli and food intake. Anorexigenic hormones, such as leptin, reduces the sensitivity of the reward-processing regions of the brain to food, whereas orexigenic signals, such as ghrelin, increases their sensitivity\textsuperscript{102}. Krugel et al showed that leptin depresses the basal and feeding-evoked release of dopamine in rats, suggesting its role in modulating the reward-based system\textsuperscript{103}. Also, a functional imaging study, observing two congenitally leptin-deficient humans, confirmed the role of leptin in the activation of reward-processing pathways. Researchers found increased activity in the striatum in the participants after they were shown images of food, whereas after
receiving leptin treatment (for 7 days), the activation was abrogated\textsuperscript{104}. This suggests that leptin may decrease perception of food reward. Furthermore, animal studies have shown that ghrelin enhances dopamine activity and turnover in the nucleus accumbens\textsuperscript{105}, a reward-processing brain structure. This is consistent with a recent human functional imaging study, suggesting that ghrelin increases the hedonic responses to food cues. In this study, Malik et al, administered ghrelin intravenously to healthy subjects and observed that the neuronal responses to pictures of food were increased in reward-processing regions of the brain, including the amygdala, orbitofrontal cortex, anterior insula, and striatum\textsuperscript{60}.

2.3 Physiology of the regulation of fat intake

Fat intake is also regulated by both the homeostatic and reward systems. These systems are dependent on the brain’s ability to sense dietary fats.

**Sensing of dietary fats:** Regulation of energy intake from fat occurs via the interaction between dietary fat and particular receptors found in the oral cavity and GI tract. It is the enzyme lipase found in the oral cavity that catalyses the release of fatty acids (FA) from the undigested triacylglyceride component of fat\textsuperscript{106, 107}. The receptors that specifically detect the released FA\textsuperscript{106, 107} are located on the apical surface of taste cells that populate the oral cavity and on enteroendocrine cells found in the GI tract\textsuperscript{108}. Their activation induces a regulatory cascade, which stimulates gustatory nerves that convey sensory information to the nucleus of the solitary tract (NTS) of the brainstem. This then
activates important centres involved in the regulation of food intake, such as the subcortical (e.g. hypothalamus, ventral striatum, amygdala) and cortical (e.g. insula) regions\textsuperscript{109, 110}.

Sensitivity of individuals to oral fat varies depending on their differences in the responsiveness to FA induced stimuli. For example, CD36 is a membrane-bound protein that is involved in tasting of fat. CD36 knockout mice show an abrogated brain response to fatty acid stimulation\textsuperscript{109}. Moreover, wild-type mice normally display a strong preference for fatty acid–enriched drinking vs. control solutions, while CD36-null mice show no such preference\textsuperscript{111, 112}. It has also been shown that a lower ability to detect FA (hypo-sensitivity) is associated with higher overall energy intake, fat intake (both absolute and relative) and BMI\textsuperscript{110}.

Species differences in the cortical representation of taste exist, with the most complex processing being seen in humans in whom both the perceptual and affective aspects of feeding behaviour are involved\textsuperscript{113}. Using functional imaging, de Araujo et al investigated how factors of food palatability, texture and fat content, are represented in the human brain. They showed that both anterior and mid-insular regions became activated in response to viscosity of oral stimuli and fatty vegetable oil. The response was proportional to the log of the viscosity\textsuperscript{114}.

Human research has also demonstrated that oral FA induces physiological responses, such as the stimulation of gastric lipase secretion, elevation of serum triglycerides, suppression of ghrelin and reduction of appetite and energy
intake. Therefore, dietary fat sensation is important in the regulation of fat intake.

2.3.1 Homeostatic mechanisms

Fat intake is regulated by the homeostatic system that maintains energy and body fat balance. For example, the presence of fat in the small intestine has been shown to specifically stimulate the secretion of a number of gastrointestinal hormones such as CCK, GLP-1 and PYY, and suppress the secretion of ghrelin (as stated above). Also, fat has a higher energy density and hungry people tend to eat more energy-dense, fat-containing, foods. Studies have demonstrated the important role of AgRP as a hunger signal that stimulates fat intake in particular. Fasted rodents have increased expression of AgRP in their hypothalamus and agouti mice that have increased expression of AgRP and consequent suppression of the melanocortin hormone, consume more fat, when fed a three-choice diet (fat, protein and carbohydrates).

2.3.2 Reward mechanisms

Strong reward mechanisms make people experience a heightened sense of "wanting" and "liking" fatty foods and because of their high palatability many tend to consume large quantities of these energy-dense foods.

In terms of the "wanting" aspect of reward, research suggests that dopamine is also released within nucleus accumbens when fats are sensed. Using microdialysis probes implanted in the brain of rats with gastric fistulae for sham feeding, Liang et al. reported that oral stimulation by corn oil released
dopamine in the nucleus accumbens\textsuperscript{124}. Another rat study showed that pharmacological inhibition of D1 and D2 dopamine receptors decreases preference for a corn oil emulsion in a dose-dependent manner\textsuperscript{125} \textsuperscript{126}. In humans, it has been shown that neural activity in response to actual consumption of high-caloric foods (such as fatty foods) might be reduced in subcortical regions rich in dopamine\textsuperscript{127}, especially in individuals with TaqIA A1 variant, which is associated with diminished dopamine D2 receptor density\textsuperscript{128}.

“Liking” is the appetite for palatable food items\textsuperscript{129}, which is, as mentioned above, predominantly regulated by the opioid system\textsuperscript{102} \textsuperscript{130}. Opioid-agonists stimulate fatty-food intake\textsuperscript{131} \textsuperscript{132}. For example, Zhang et al. showed that the µ-opioid agonist D-Ala\textsubscript{2}-N-Me-Phe\textsubscript{4}-gly\textsubscript{5}-ol-enkephalin (DAMGO) selectively increases appetite for high-fat foods when administered into the nucleus accumbens\textsuperscript{132} \textsuperscript{133}. In addition, opioid antagonists, like Naloxone, can decrease intake of high-fat foods in humans\textsuperscript{134}, and significantly attenuate the activation of reward-processing regions of the brain that are normally triggered by palatable food stimulus, such as the amygdala\textsuperscript{95}.

\textbf{2.4 Amygdala and fat intake and preference}

The amygdala is a brain structure within the temporal lobe that is involved in processing food reward, in regulating feeding behaviour\textsuperscript{135} \textsuperscript{136} and in controlling appetite\textsuperscript{137}. This amygdala contains several receptors for neurotransmitters and hormones involved in the regulation of food intake, such as dopamine, opioids, NPY and insulin\textsuperscript{18} \textsuperscript{138-141}. In addition, animals with a lesion in their amygdala show
hyperphagia and excessive weight gain\textsuperscript{142-145}. Similarly, humans that underwent amygdala surgery as a treatment for epileptic seizures report hyperphagia\textsuperscript{143}. Furthermore, activation of the amygdala by food-related visual stimuli, tastes and odors have been shown in several neuroimaging studies\textsuperscript{146-149}.

The amygdala has also been studied in the context of drug addiction and the regulation of fat intake and fat preference\textsuperscript{150 151}. Thus, human functional imaging studies have shown that high-fat stimuli induce higher activation of the amygdala than low-fat stimuli\textsuperscript{152}. The net outcome of this activation, however, may be a decrease of fat intake, as amygdala volume correlates inversely with fat intake in humans\textsuperscript{153}. Animal studies have also demonstrated that activation of the amygdala by intra-amygdalar administrations of NPY\textsuperscript{18} and enterostatin\textsuperscript{154} decreases fat intake.

With respect to drug addiction, lower amygdala volume has been observed in individuals with alcohol addiction\textsuperscript{19}, they had greater alcohol craving and greater likelihood of relapsing into alcohol consumption\textsuperscript{155}. Lower amygdala volume has also been observed in adolescents and young-adults who are offsprings of individuals with alcohol addiction which suggests that having a smaller amygdala may be a risk factor of alcohol addiction rather than a consequence\textsuperscript{156}.

Furthermore, the results of several studies suggest that activity of the amygdala may be altered in obesity. Studies on gastric distension (as a satiation mechanism) showed that, in lean vs. obese individuals, gastric distension results
in higher activation of the amygdala\textsuperscript{157 158}. Another imaging study investigating functional connectivity between key reward-related brain regions in response to food images found that obese versus lean individuals show a deficiency in the amygdala projections and connectivity to the orbitofrontal cortex and nucleus accumbens\textsuperscript{159}.

2.5 Factors influencing fat intake and preference

People differ in regard to the amount of fatty foods they consume. Various environmental factors play a role in determining this inter-individual variability.

2.5.1 Environmental factors influencing fat preference

Fat intake varies with (1) socio-economic status (SES), determined by education and income, (2) ethnicity, (3) lifestyle choices, such as exercise, smoking, and alcohol intake, and (4) various psychological or physiological states, such as stress, pregnancy and sleep deprivation.

(1) SES: Individuals with lower SES tend to eat more fatty and less nutrient-rich diet compared to those with higher SES\textsuperscript{160 161}. The educational level inversely correlates with intake of fat\textsuperscript{162 163}. For example, a cross-sectional study in Dutch population of 6,008 men and 6,957 women (≥19 years of age) showed that a higher education level was associated with a lower fat intake among men and women (\textit{p}<0.01\textsuperscript{163}). This association was also reported in a study of urban, low-income African Americans\textsuperscript{164}. An important determinant of children’s fat intake is parental educational level where the mean of parental education was
inversely correlated with the percentage of energy intake from fat ($r = -0.39; p = 0.0001$). Household income is also considered as an important factor influencing fat intake. Population-based surveys of individual intake show that those with lower income consume a greater proportion of energy from fat compared to higher income individuals. Similarly, in studies of adolescents, it has been shown that lower intake of fat is associated with both parental higher education and income.

(2) Ethnicity: Different ethnic groups have different dietary fat intake. For example, significant ethnic differences in dietary intake of fat were observed in an urban South African study of 198 women (61 black, 76 of mixed ancestry, 61 white), in which black women consumed less dietary fats than the other groups of women independent of SES.

(3) Lifestyle and individual behavioural choices are other important factors in fat intake. The relationship between dietary fat and physical activity has been demonstrated, with more physically active people consuming less calories from fat. In addition, the use and abuse of various substances strongly is strongly associated with fat intake and fat preference. For example, greater fat consumption was seen in alcohol drinkers compared with abstainers in a cross-sectional study investigating 985 women and 863 men in Finland. Similarly, cigarette smoking was positively associated with the consumption of high-fat foods in adolescents ($p = 0.04$). Similar relationships were observed for the use of marijuana ($p = 0.03$) or alcohol ($p = 0.005$).
(4) **Physiological factors** can also have an important effect on fat preference and intake. Torres and Nowson\textsuperscript{178} studied the relationship between stress and eating behavior and determined that stressful conditions induced higher consumption of food. Furthermore, changes in taste perception, food cravings, and food aversions are widely reported during pregnancy\textsuperscript{179-181}. Hormonal fluctuations and the needs of a healthy pregnancy might drive these taste alterations, but the underlying mechanisms are not fully understood. Recent studies have also shown the link between sleep deprivations and increasing the hedonic value of food as well as the development of obesity\textsuperscript{182-184}. For example, a study in China showed that people who slept for less than 7 h a day ate significantly more fat (as a percentage of total energy intake) than those who slept for 7-9 h per day (\(p = 0.005\))\textsuperscript{182}. It is believed that sleep loss can cause several physiologic changes that together increase appetite; for example, lack of sleep both increases ghrelin and decreases leptin\textsuperscript{185,186}.

Additional factors were also suggested to play a role in fat intake such as exposure to advertisements, time and convenience, and delayed eating that creates hunger. Food availability is of particular importance\textsuperscript{187}, as individuals consume more readily foods that are accessible, such as high-fat snacks and fast foods. In addition, the familiarity and the repeated consumption of certain foods influence individual’s choices, as well as the level of hedonic enjoyment experienced\textsuperscript{188}. A study showed that higher liking for high-fat foods was associated with higher fat intake\textsuperscript{189}. The composition of diet is also an important
factor, since diets restricting intake of sugar may result in increased intake of fat and *vice versa*\(^{165,190,191}\).

Finally, age and sex influence the eating behaviour. Energy intake and fat preference change with age and it seems that food intake (including fat intake) is increased from childhood to young adulthood\(^{192}\), as the body needs more energy in this period, but in elderly people, because of the impairments in several mechanisms involved in food regulation, food intake is decreased\(^{193}\). For example, elderly people often suffer from olfactory\(^{194}\) and/or taste deficits\(^{195}\). A reduced perception of hunger and increased satiation in old age has been also reported in several studies\(^{196-198}\). Differences in fat preference between men and women exist; some studies show that males and females differ in hedonic ratings of fatty foods\(^{199,200}\) and, compared with men, women eat less fatty foods and they were more likely to avoid consuming fats\(^{199,201,202}\).

**2.5.2 Genetic factors influencing fat preference**

Inter-individual differences exist in the relative intake of fat (percent of energy intake). As will be summarized below, previous research suggests that at least some of these differences may be due to inter-individual variations in genetic makeup.

**2.5.2.1 Heritability of fat intake (family and twin studies)**

Heritability is the proportion of total phenotypic variance in the population attributed to genetic factors\(^{203}\). Both genetic and environmental variances should
be taken into account in the calculation of heritability. For the measurement of heritability, two methods of twin and family studies are usually used.

Twin studies are based on the difference between monozygotic (MZ) and dizygotic (DZ) twins; MZ twins share all their genes as well as their upbringing and early environment, whereas DZ twins also share their upbringing and early environment but, on average, only half of their genes. Therefore, if similarity in a phenotype is greater in MZ than DZ twins, a genetic component contributes to the expression of that phenotype; the higher this similarity, the bigger the genetic component. Therefore, to calculate heritability, the difference of correlations between MZ and DZ twins should be measured. The main widely accepted way to calculate the crude estimate of heritability \( h^2 \) is Falconer's formula: \( h^2 = 2 \times (r_{mz} - r_{dz}) \). This calculation is based on the correlations of identical/monozygotic twins \( r_{mz} \) and dizygotic twins \( r_{dz} \).

Family studies are an alternative to twin studies. In family studies, degree of genetic sharing between family members is compared with their degree of phenotype similarity. In sibling analysis, the similarity between the siblings born to the same biological parents is compared. In a simple method, an estimate of heritability can be calculated by double the interclass correlation coefficients for the phenotype of interest between the siblings pairs; \( h^2 = 2 \times r \), where the \( h^2 \) indicates heritability and “r” represents the sibling-pairs correlation.

Family and twin studies have demonstrated familial aggregation of relative fat intake, with heritability estimates ranging from 20% to 48%. In these
studies, food diary, food-frequency questionnaires (FFQ) and 24-hour food recall were used to assess fat intake. In comparison, heritability estimates derived from FFQs (mean = 16%) were generally lower than those from food diaries (mean = 33%) or 24-hour food recalls (mean = 25%). In addition, age was an important factor to consider; individuals from the same generations (e.g., sibling-sibling) showed higher family correlations (mean = 40%) compared individuals from different generations (e.g., parent–offspring; mean = 24%)\textsuperscript{206}.

2.5.2.2 Genetic approaches used to study complex traits

Two main approaches have been used to identify genes of complex traits: (1) linkage studies and (2) association studies. Both qualitative and quantitative traits can be analysed using these approaches. Qualitative traits have discrete categories of phenotypes such as diseased/non-diseased or affected/non-affected status, whereas quantitative traits are continuous in distribution – these include blood pressure, BMI or fat intake.

(1) Genetic linkage analyses: In linkage studies, families with individuals possessing the trait of interest are genotyped for an extended set of markers to identify chromosomal regions/loci that co-segregate with the trait more often than predicted by chance\textsuperscript{209}. There are two types of linkage analysis; parametric (suitable for single-gene traits that have specified parameters, such as the inheritance mode and trait penetrance) and non-parametric (suitable for complex traits that have unclear mode of inheritance, incomplete penetrance and underlying genetic and environmental heterogeneity). When studying quantitative
traits, linkage analysis identifies quantitative trait loci (QTL) which contain genes that possibly affect the continuously distributed phenotypes\textsuperscript{210}.

(2) Genetic association analyses: Genetic association studies correlate phenotypes with specific gene variants (i.e., alleles) in related or unrelated individuals\textsuperscript{211}. Case-control association studies compare the allelic frequencies of affected groups (cases) with that of controls and, if significantly different, genetic associations are indicated to exist between the allele and the studied phenotype. Family-based association studies investigate markers that are more often transmitted along with the trait of interest from parents to offspring and, if specific alleles show an enriched pattern of transmission these are considered to be associated with the trait\textsuperscript{212}. The absence of correlation between the single nucleotide polymorphisms (SNPs) and the trait of interest (null hypothesis), is usually rejected once the threshold p-value of less than <0.05 is attained, thus, suggesting the existence of a genetic association. When several association tests are performed, the derived p-values should be adjusted for multiple testing to avoid the occurrence of false positives (Type I error)\textsuperscript{213}.

Each approach has their advantages and disadvantages. Linkage analysis is successful in localizing variants that are less common but with larger effects (e.g. parametric linkage of a causative gene of a monogenic condition). Whereas, association studies are suitable for common variants with modest effect size, as in the case of complex traits\textsuperscript{214}. Loci singled out by linkage studies usually span over 20–30 million base pairs (bp). Therefore, genetic linkage analyses are not used for fine mapping of complex traits\textsuperscript{215} whereas association analyses can be
employed for this purpose. Linkage analyses use only family-based samples whereas both family-based and case-control samples can be used in association studies. Compared to case-control samples, family based samples: 1) are more robust against population stratification, which can depreciate true association, 2) do not necessitate the recruitment of a controls set\textsuperscript{216}, 3) allow for simultaneous linkage and association analyses\textsuperscript{217} and 4) tend to more homogeneous (within families) regarding environmental exposures\textsuperscript{218}. The main disadvantage of this method is the difficulty of collecting a large number of well-characterized families and this problem is aggravated if trying to study complex traits.

Both linkage and association studies can be used either to investigate: (1) the entire genome (genome wide) or (2) candidate genes.

**1) Genome-wide approach:** circumvents the need for prior knowledge of the biological basis underlying the phenotype and, therefore, has the potential to uncover unpredicted relationships and new pathways.

**Genome-wide association studies (GWAS)** are powerful hypothesis-generating tools that search the whole genome for genes/loci that could be mechanistically involved in specific traits. The approach was designed based on the ‘common disease, common variant’ hypothesis; where complex traits are partially attributed to population variants with frequencies higher than 1-5\textsuperscript{219}. However, causal variant may not be specifically identified in association studies, but a well-designed study is likely to detect one or more common markers in strong linkage disequilibrium (LD) with the causal allele\textsuperscript{211}. GWAS examines

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27
thousands of SNPs, usually > 300,000 spanning the entire genome, for association with a particular phenotype under study\textsuperscript{220}. Because of the large number of association tests performed, the accepted threshold for statistical significance in GWAS is $5 \times 10^{-8}$ \textsuperscript{221}. This approach has been enabled by the availability of high-throughput genotyping platforms and the completion of both the Human Genome and International HapMap (haplotype map) Projects, which list human genetic variants\textsuperscript{222}. The major advantages of GWAS are that they: (1) generate hypothesis, (2) have a simple design, (3) can be used for case-control data sets as well (as opposed to linkage studies that require family-based samples) and (4) have the ability to detect genetic factors with small effects\textsuperscript{223}. However, most common variants have a small effect and, therefore, usually explain only a minor fraction of the heritability of a trait (attributed relative risks usually range around 1.1–1.5 fold)\textsuperscript{224}. Also, GWAS are costly, are known to have high false discovery rates and could omit rare variants important in the pathogenesis of the studied trait\textsuperscript{225,226}.

\textbf{(2) Candidate-gene approach:} The candidate-gene approach is hypothesis driven, and focuses on either or both: (1) genes considered to be physiologically involved in the development of the phenotype (biological candidates), or (2) genes located in regions previously correlated with the trait by genome-wide, linkage or association studies (positional candidates)\textsuperscript{227}. Since most candidate-gene analyses test several SNPs (multiple testing), the statistical threshold (p-values) to attain significance should be adjusted. The main disadvantage of the
candidate-gene approach is its inability to identify novel, previously unsuspected pathways.

2.5.2.3 Genetic study of fat intake

Several of the above-described genetic approaches have been used to study fat intake; these include a few genome-wide linkage studies and more numerous candidate-gene association studies.

Genome-wide linkage studies of fat intake

To date, only a few linkage studies of relative fat intake have been performed\textsuperscript{24, 228}. One such study, working with 347 sibling pairs of Caucasians and 99 sibling pairs of African-Americans, did reveal a significant linkage between fat intake (in grams) and chromosome 12q14.1 in whites\textsuperscript{228}. Another investigation\textsuperscript{24}, involving 816 participants (of extended Mexican American families) from the San Antonio Family Heart Study, reported linkage between fat intake (in grams) and chromosome 2p22; and this region was also linked with body adiposity.

Candidate-gene association studies of fat intake

Fat intake has more often been studied using the candidate-gene approach. Researchers have selected several genes of interest based on either their attributed physiological role in fat intake (e.g. AgRP, TUB, HTR2A, CD36,
and TAS2R38) or previous evidence of associations with obesity (e.g. FTO, MC4R, SH2B1).

The agouti-related peptide gene (AgRP) encodes a potent stimulant of feeding expressed predominantly in the hypothalamic arcuate nucleus\textsuperscript{229}. A specific variant of AgRP was associated with lower energy intake derived from fat and higher energy intake derived from carbohydrates in 478 Caucasian individuals participating in the HERITAGE family study. This variant, Ala67Thr, had been previously shown to be associated with leanness\textsuperscript{230}.

The tubby gene (TUB) encodes an evolutionary conserved protein, which is highly expressed in the hypothalamus and may act there as a transcription factor\textsuperscript{231, 232}. In a study on 1,680 middle-aged Dutch women, a variation in TUB was associated with lower consumption of energy from fat, higher consumption of energy from carbohydrates, and higher body weight and BMI\textsuperscript{233}.

The serotonin type 2A receptor gene (HTR2A) encodes the 5-hydroxytryptamine (serotonin) receptor 2A, which is expressed in several regions of the brain, including those that process rewards, such as the nucleus accumbens, ventral pallidum and olfactory cortex\textsuperscript{234}. A specific variant of HTR2A was associated with lower energy and relative fat intake in the young adult population, but not with weight and BMI\textsuperscript{235}.

The cluster of differentiation 36 gene (CD36) encodes a fatty acid translocase, which is suggested to have a role in oral sensing of fat (described in
section 2.3). Animal and human studies have demonstrated its expression in taste bud cells and it was shown that CD36 deletion decreases fat consumption. In humans, CD36 is not only associated with obesity but also with fat perception and intake. Keller et al. investigated the association of CD36 with oral fat perception, reported liking of high-fat food, and obesity in African Americans. In this study, two SNPs, rs1761667 and rs1527483, were associated with oral fat perception and one of them, rs1761667, was also associated with the liking of added fats and oils. Moreover, Madden et al. showed that genetic variations in CD36 are associated with differences in human metabolism of fat. CD36 has also been linked with BMI and waist circumference.

TAS2R38 (taste receptor, type 2, member 38) encodes a G protein-coupled receptor found in the tongue and was found to be involved with PROP (6-n-propylthiouracil) bitterness tasting. Individuals who could not taste PROP bitterness are unable to differentiate high (40%) vs. low (10%) fat salad, whereas medium and super-tasters possess this ability. It has been demonstrated that lower ability of PROP tasting is associated with higher fat intake and higher BMI. Lacking the ability to detect PROP seems to modulate the perceptions normally induced by oral fat during eating; this could lead to alterations in feedback loops or hedonic responses that compel to over-consume fats.
Other candidate gene loci were identified through GWAS of obesity and were examined for their association with fat intake. These include the fat mass and obesity-associated (FTO), melanocortin 4 receptor (MC4R), and SH2B adaptor protein 1 (SH2B1) genes.

FTO is highly expressed in the hypothalamus, a brain region that regulates energy intake and expenditure. It has been shown that an FTO variant is linked to the predisposition to obesity along with higher intake of fat in grams and of energy as well as increased appetite in children. Another study also demonstrated that FTO has a strong association with obesity in individuals with higher (40.6–58.4%) relative fat intake, compared with those with medium- (35.5–40.6%) or low- (13.4–35.5%) relative fat intake.

MC4R encodes a seven trans-membrane G-protein-coupled receptor that is widely expressed in the central nervous system where it regulates reward, food intake, energy intake and expenditure. Blocking MC4R signalling in animals increases the reward value, preference and consumption of high-fat versus high-carbohydrate foods. Qi et al. studied 3,459 women and showed that a variant of MC4R was associated with an increase in total energy and linked to a greater percentage of intake from fat, but not from carbohydrate.

SH2B1 positively regulates the leptin receptor in the hypothalamus which increases food intake. A Dutch study on 1,700 women described an association between a SH2B1 variant and total fat intake.
**Genome-wide association studies of fat intake**

To the best of my knowledge, GWAS conducted in the aim to identify genetic variations associated with relative fat intake have not yet been reported.
CHAPTER THREE

Rationale, Hypothesis & Objective
3.1 Rationale

Higher relative intake of fat may increase risk for obesity. Relative fat intake is a complex behaviour modulated by genetic factors.

3.2 Hypotheses

Genes play a significant role in determining fat intake and thus possibly the development of obesity. Identifying genes that modulate fat intake may enhance our understanding of the pathways resulting in obesity.

3.3 Objectives

(1) To search genome-wide for genetic variants associated with relative intake of fat.

(2) To test whether the variants identified above are also associated with adiposity.

(3) To replicate our findings in an independent sample of young adults.
CHAPTER FOUR

Material and Methods
4.1 Participants

We studied a total of 598 adolescents (12- to 18-year old) who were recruited, as part of the Saguenay Youth Study, through regional high schools from the genetic founder population living in the Saguenay Lac St. Jean (SLSJ) region of the Canadian province of Quebec.

The SYS is a population-based cross-sectional study of cardiovascular, metabolic and mental health in adolescence; it also investigates the long-term consequences of prenatal exposure to maternal cigarette smoking\textsuperscript{258}. It is a family-based study focused on collecting sib-pairs. A total of 1,024 adolescents (half of whom were exposed prenatally to maternal cigarette smoking) were recruited through regional high schools and included in the SYS. The selection criteria were: (1) Age ranging from 12 to 18 years, (2) One or more siblings in the same age group, (3) Maternal and paternal grandparents of French–Canadian ancestry, and (4) Positive history of maternal cigarette smoking (being exposed was defined as having a mother who smoked >1 cigarette/day during the 2\textsuperscript{nd} trimester and being non-exposed was defined as having a mother who did not smoke one year before the pregnancy). The main exclusion criteria were: (1) premature birth (<35 weeks) or detached placenta, (2) maternal alcohol abuse during pregnancy, (3) positive subject’s medical history of type 1 diabetes and heart disease requiring surgery or sustained medication, and (4) magnetic resonance imaging (MRI) contraindications. Written consent of the parents and assent of the adolescents were obtained. The Research Ethics Committee of the Chicoutimi Hospital approved the study. Exposed and non-exposed sib-pairs
were matched at recruitment by maternal education and participant’s school attended to minimize the potentially confounding influence of socio-economic status. At the time of our GWAS of fat intake, data information for 602 participants were available and after excluding four subjects (explained in section 4.6), 598 adolescents (278 exposed and 314 non-exposed) with quality controlled genome-wide genotypes were included in our study. Among them, 573 adolescents had food recall data.

**Saguenay Lac-Saint-Jean Founder Population**

The SLSJ region is located in the north-eastern part of the Canadian Province of Quebec. The first inhabitants of this region (who were of French origin) settled there between 1838 and 1911. A majority of the settlers (75% of the total 28,656 people) were from a neighbouring region, Charlevoix. The SLSJ, with almost 300,000 inhabitants, is now one of the largest founder populations in North America. This is mainly due to high birth rates and low immigration. Similar to other founder populations, SLSJ has a higher frequency of certain recessive diseases and lower allelic diversity of their genetic determinants, compared with ethnically less homogenous populations. The recessive diseases, occurring almost exclusively in the SLSJ population, include: (1) ataxia of the Charlevoix-Saguenay, (2) agenesis of corpus callosum and peripheral neuropathy and (3) French-Canadian-type Leigh syndrome. Furthermore, the recessive disease, occurring at a higher prevalence in the SLSJ population, include: (1) oculopharyngeal muscular dystrophy, (2) tyrosinemia,
type 1, (3) cystic fibrosis, (4) Leber hereditary optic neuropathy and (5) familial hypercholesterolaemia\textsuperscript{260}. The incidence and carrier frequency of these diseases have been estimated to be 1/207 live birth and 1/7 inhabitants, respectively\textsuperscript{260}.

### 4.2 Founder population

Genetic mapping of complex traits in population isolates (founder populations) may be more effective. Founder populations are defined with two main characteristics: (1) small number of ancestors and (2) isolation (geographic, cultural or religious). When immigration is limited in these populations, mating occurs within the community resulting in limited genetic diversity\textsuperscript{263}. The allelic and locus heterogeneity is always one of the main obstacles in genetic studies of complex diseases, as it usually diminishes association signals; this heterogeneity expected to be reduced in founder populations. Use of founder populations for nutritional and dietary investigations has frequently been suggested and used. For examples, genetic isolates (such as Pima Indians, Finland and Sardinia) have been used in obesity and type 2 diabetes researches as well\textsuperscript{264-266}.

It is important to consider that one of the potential disadvantages of using genetic isolates is the identified genes might not be relevant for other populations\textsuperscript{264}.

### 4.3 Dietary assessment

In our study, we used a 24-hour food recall to obtain information on the foods and drinks consumed during the past 24 hours in a structured interview.
conducted in person by a trained nutritionist on a Saturday during a hospital session of our protocol. Volume models were used to estimate the food portion size. The obtained information on the foods and drinks consumed during the past 24 hours was then analyzed with the recipe file to obtain energy and macronutrients (ie, fat, carbohydrates, and protein) intake. This instrument has been validated for Quebec youth\textsuperscript{267}. The proportions of energy consumed in the form of fat, carbohydrates, and protein, as well as total energy intake were calculated.

Different methods are used to measure food intake. The main dietary assessment methods are: (1) food-frequency questionnaire (FFQ), (2) dietary record, (3) diet history and (4) 24-hour dietary recall. They all have their own advantages, disadvantages and validity depending on the aim of the study.

(1) FFQ: The FFQ\textsuperscript{268} asks individuals to report their usual frequency of consumption of each food and beverages from a list of foods in a specific time duration (a usual week, one or several months). To estimate relative or absolute nutrient intake, many FFQs incorporate questions about portion size. Many FFQs are adapted for different populations and different purposes (e.g., cancer vs. cardiovascular research). For example, the Health Habits and History Questionnaire or Block Questionnaire are commonly used for U.S. adults\textsuperscript{269,270}, whereas the European Prospective Investigation into Cancer and Nutrition (EPIC) is used in Europe\textsuperscript{271}. The strengths of FFQs are that they are inexpensive, have a low respondent burden and aim to estimate the respondent’s food intake over an extended period of time. Because of these factors, FFQs are
commonly used in large epidemiological studies. The major limitation of FFQs is that they show substantial measurement errors\textsuperscript{272, 273}. For example, well-conducted validation study with objective measurements of biomarkers of diet, showed that FFQ methods could pose a high degree of error, thus masking existing relationships between diet and disease risk\textsuperscript{274}. It often does not account for some important details, such as preparation methods, amounts, added condiments, and the quantification of intake is not as accurate as with recalls or records\textsuperscript{272, 273}.

\textbf{(2) Dietary records:} In this method, respondents write down the amounts of food and beverage consumed upon eating over a given period of time. The strength of this method is that respondents do not rely on memory to record intake. However, it has several disadvantages, such as it is biased in the respondent’s selection of what is reported and it is costly. Several studies indicate that energy and protein intakes reported in diet records are underestimated in the range of 4\% to 37\%\textsuperscript{275-277}. Furthermore, individuals with high BMIs, particularly women, are those most likely to under report consumption in food records\textsuperscript{278, 279}. Furthermore, knowing that food recording is required alters the dietary behaviors, such as the types of food chosen and the quantities consumed\textsuperscript{280}. Also, the respondent must be trained to describe adequately the foods and amounts. Usually no more than 3 or 4 days are considered reliable, as the quality of recording decreases due to fatigue and low compliance\textsuperscript{281}. Food records require literate, motivated participants and place a high burden on
subjects, making this method particularly difficult to use in children and adolescent.

(3) Diet history: This method usually consists of a detailed interview (which sometimes includes a 24-hour recall) about patterns of eating and meal preparation, a food list asking for usual frequency and amount of food consumed, and a 3-day diet record\textsuperscript{282}. Depending on the aims of the assessment, usual intake was measured in the past 3, 6, or 12 months. In comparison to records or recall methods, this detailed collection of information is the major strength of the diet history method. A weakness of this approach is that respondents must judge both the usual type and amount of food they have eaten, which is a task that may be difficult for many children and adolescents.

(4) 24-hour food recall: The respondent is asked to remember and report the foods and drinks consumed in the preceding 24 hours. As the food assessments occur retrospectively after consumption, the potential for interfering with dietary behavior is reduced in comparison with dietary record methods. The recall typically is conducted as an interview in person or by telephone\textsuperscript{283, 284}, ideally, by a trained dietitian. The information is obtained with probing questions designed to encourage accurate reporting. Interviewers are knowledgeable about the local food availability and prevalent regional or ethnic food preparation. The 24-hour food recall method is recognized as having high efficacy in comparing groups, high compliance rate, low bias, and minimal burden on subjects. Since the recall period is immediate, respondents are generally able to report most of
their dietary intake, and because the interviewer records the responses, literacy of the respondent is less crucial. Furthermore, the recall can be done quickly, typically in 20 minutes. As such, this method is useful across a wide range of populations, including children and adolescents. Under and over reporting of food and drink intake occurs with a 24-hour food recall, but an energy adjustment (e.g., analyzing relative rather than absolute macronutrient intake) can be made, which reduces this error. For example, relative protein intake (i.e., percent energy intake from protein) determined with a 24-hour food recall was in close agreement to a biomarkers-based measure in the Observing Protein and Energy Nutrition (OPEN) study. Twenty-four-hour food recall was also used in the U.S. National Health and Nutrition Examination Surveys, which are the only nationally representative dietary surveys in the United States. As day-to-day variation occurs in food intake, it is recommended that a minimum of 3 days (including 1 weekend day) are assessed to obtain data for habitual food intake of an individual. A single-day 24-hour food recall is suitable to describe group differences, as the group means are robust and unaffected by within-person variation.

Assessing the diets of children is considered to be particularly challenging, as children’s estimates of portion size have large errors; they are less able than adults to estimate portion sizes. However, our participants were adolescents and reporting discrepancies were minimize as the trained nutritionists used volume models to help the participants estimate food portion size.
4.4 Magnetic resonance imaging of the amygdala

Magnetic resonance (MR) T1-weighted images (1-mm isotropic) of the brain were acquired on a Phillips 1.0-T superconducting magnet (Gyroscan NT, Philips Healthcare, Best, The Netherlands). It lasted between 45 and 60 minutes. Volumes of the whole brain and of the right and left amygdala were obtained with FreeSurfer (FreeSurfer Version 5.0.0)\textsuperscript{291}. Briefly, FreeSurfer is a collection of image-analysis algorithms that assigns automatically anatomical labels to each voxel of the MR volume; this is achieved by combining probabilistic information about the global location of a structure in the atlas space with local spatial relationships between adjacent structures\textsuperscript{292}. In this manner, the volumes of the left and right amygdala were obtained. In the analyses reported here, the total (left + right) volume of the amygdala was used.

4.5 Adiposity assessment

Weight (0.1-kg precision) and height (1-mm precision) were measured and BMI was derived as weight in kilograms divided by height in meters squared. Total body-fat mass was assessed in kilograms using multi-frequency bioimpedance analysis (Xitron Technologies, Inc., San Diego, CA, USA). For the purpose of this analysis, adolescents were asked to refrain from caffeine, alcohol and vigorous activity 24 hours before the measurement, which was made after a 20-min stabilization period during which the participants were resting in a supine position.
4.6 Genotyping

All adolescents (n=602) were genotyped with the Illumina Human610-Quad BeadChip (Illumina, San Diego, CA) containing over 580,000 SNPs distributed across 22 autosomal chromosomes. The genotyping was conducted at the Centre National de Génotypage (Paris, France) where genotype calling was made for 567,726 SNPs. Single nucleotide polymorphisms (SNPs) with call rate <95% and minor allele frequency <0.01, and SNPs that were not in Hardy-Weinberg equilibrium (p<1x10^{-4}) were excluded. A total of 543,159 SNPs passed these quality-control criteria and were included in statistical analyses. In addition, 4 subjects were excluded from our study: 3 subjects based on principal component analysis of population structure performed with HapMap II (release 22) and 1 subject due to >3% of missing genotypes; a total of 598 adolescents had quality-controlled and complete genotype datasets.

4.7 Statistical analyses

The GWAS of relative fat intake was conducted with Merlin-1.1.2. A simple regression model was fitted to each trait and a variance component approach was used to account for correlation between different observed phenotypes within sib-ships. In the SYS sample, fat preference was normally distributed and no statistical outliers (mean ± 3 standard deviations) were present. Age and sex were included as potential confounders.
Secondary analyses were aimed at examining whether the GWAS-identified locus of fat intake (rs2281617) was also associated with energy, carbohydrate and protein intakes, and with adiposity as well as amygdala volume; these analyses were performed with multivariate linear model, while adjusting for age and sex (and height when relevant). Body-fat mass and BMI had positively skewed distributions and were log transformed, using logarithm with base 10, which improved the fit. In addition, statistical outliers (i.e. values outside mean ± 3 standard deviations) were excluded in the case of fat, energy, carbohydrates and protein intakes, body-fat mass, BMI, and amygdala volume (n<8). The number of minor allele T homozygotes at rs2281617 was <10 and, thus, TT-homozygotes were pooled together with TC-heterozygotes.

4.8 Replication study

Participants: Toronto Nutrigenomics and Health Study (TNHS), is a cross-sectional study of diet, genotype and biomarkers of chronic disease in a population of young adults from the University of Toronto. Caucasian, Asian and South Asian were the major ethnocultural groups in this study. This project is aimed to elucidate the genetic basis for variability in response to nutrients and food bioactives, and identify genetic determinants of food preferences and intake. A total of 1,850 subjects (594 men and 1256 women) between 20-29 years of age were recruited. In this study, pregnant or nursing women were excluded and people with temporary inflammatory conditions were included in the study after a two-week recovery period. Our replication study was performed in a subset of 490 Caucasian adults that had no special dietary regime.
**Dietary assessment:** Energy and macronutrient intake were assessed with a one-month 196-item semi-quantitative food-frequency questionnaire (FFQ).

**Adiposity assessment:** Anthropometry was used to measure weight and height and these measures were used to calculate BMI (kg/m²).

**Genotyping:** All participants (n=490) were genotyped with the Affymetrix Genome-Wide Human SNP Array 6.0 (Santa Clara, California, U.S.A) that included > 900,000 SNPs.
CHAPTER FIVE

Opioid Receptor mu 1 Gene, Fat Intake and Obesity in Adolescence

► The following chapter is a reproduction of a manuscript that has been submitted to Molecular Psychiatry.
Opioid receptor mu 1 gene, fat intake and obesity in adolescence

Amirreza Haghighi¹, Melkaye M. Melka¹, Manon Bernard¹, Michal Abrahamowicz², Gabriel T. Leonard³, Louis Richer⁴, Michel Perron⁵, Suzanne Veillette⁵, Chang Jiang Xu², Celia M. T. Greenwood², Andre Dias⁶, Ahmed El-Sohemy⁶, Daniel Gaudet⁷, Tomas Paus⁸, and Zdenka Pausova¹*

¹Hospital for Sick Children, University of Toronto, Toronto, Canada;
²Department of Epidemiology, Biostatistics and Occupational Health, McGill University, Montreal, Canada;
³Montreal Neurological Institute, McGill University, Montreal, Canada;
⁴Department of Psychology, Université du Québec à Chicoutimi, Chicoutimi, Canada;
⁵ÉCOBES, Recherche et transfert, Cégep de Jonquières, Jonquières, Canada;
⁶Department of Nutritional Sciences, University of Toronto, Toronto, Canada;
⁷Community Genomic Centre, Université de Montréal, Chicoutimi Hospital, Canada;
⁸Rotman Research Institute, University of Toronto, Toronto, Canada.

* The author for correspondence:

Zdenka Pausova, MD
Scientist, The Hospital for Sick Children
Associate Professor of Physiology and Nutritional Sciences
University of Toronto, Toronto, Canada
Phone: (416) 813-7654/4340; Fax: (416) 813-5771
E-mail: zdenka.pausova@sickkids.ca
ABSTRACT

Dietary preference for fat may increase risk for obesity. It is a complex behavior regulated in part by the amygdala, a brain structure involved in reward processing and food behavior, and modulated by genetic factors. Here, we conducted a genome-wide association study (GWAS) to search for gene loci associated with dietary intake of fat, and we tested whether these loci are also associated with adiposity and amygdala volume. We studied 598 adolescents (12-18 years) recruited from the French-Canadian founder population and genotyped with 530,011 single nucleotide polymorphisms. Fat intake was assessed with a 24-hour food recall. Adiposity was examined with anthropometry and bioimpedance. Amygdala volume was measured by magnetic resonance imaging. GWAS identified a locus of fat intake in the µ-opioid receptor gene (OPRM1, rs2281617, p=5.2x10^{-6}), which encodes a receptor expressed in the brain-reward system and shown previously to modulate fat preference in animals. The minor OPRM1 allele was associated with lower fat intake (by 4 %) and lower body-fat mass (by ~2 kg, p=0.02). Consistent with the possible amygdala-mediated inhibition of fat preference, this allele was additionally associated with higher amygdala volume (by 69 mm³, p=0.02) and, in the carriers of this allele, amygdala volume correlated inversely with fat intake (p=0.02). Finally, OPRM1 was associated with fat intake in an independent sample of 490 young adults. In summary, OPRM1 may modulate dietary intake of fat and hence risk for obesity, and this effect may be modulated by subtle structural changes of the amygdala.
INTRODUCTION

Research in humans and experimental animals suggests that excessive intake of fat contributes to obesity\textsuperscript{6-8, 295-300}. Fats compared with other macronutrients, namely carbohydrates and proteins, are of higher energy density and higher energy efficiency. Each gram of fats contains double the energy of each gram of carbohydrates or proteins\textsuperscript{301} and, even when the same energy is consumed, almost all calories eaten as fats are stored, whereas 5-10\% and 20-30\% of calories eaten as carbohydrates and proteins, respectively, are lost during their absorption, processing and storage\textsuperscript{9}.

Preference for certain foods, including fats, is a complex behavior regulated by: (a) homeostatic mechanisms, which serve to maintain energy balance, and (b) reward-related mechanisms, which process the hedonic properties of food independently of energy status\textsuperscript{16}. The latter mechanisms may overlap with those processing the hedonic properties of drugs of abuse\textsuperscript{17} and involve brain structures processing reward\textsuperscript{18-19 154-156}.

Amygdala is a brain-reward structure involved potentially in both the development of drug addiction and the regulation of fat preference. Regarding the former, lower amygdala volume is seen in individuals with alcohol addiction\textsuperscript{19 155} and in their adolescent and young-adult offspring, suggesting that lower amygdala volume may be a risk factor for addiction rather than its consequence\textsuperscript{156}. Regarding the role of amygdala in the regulation of fat preference, it has been shown that the amygdala is activated by high-fat versus
low-fat stimuli in human functional magnetic resonance (MRI) studies\textsuperscript{152}. The net outcome of this activation may be a decrease of fat intake, as amygdala volume correlates inversely with fat intake in humans\textsuperscript{153} and its activation by intra-amygdalar administration of neuropeptide Y\textsuperscript{18} or enterostatin\textsuperscript{154} decreases preference for fat in experimental animals. Taken together, the above research indicates that reduced size and/or lesser activation of the amygdala may increase risk for addiction and dietary preference for fat.

Genes play a significant role in determining inter-individual differences in fat preference/intake, as suggested by animal and human studies\textsuperscript{24, 228, 246-304}. Very little is known, however, about individual genes (and hence mechanistic pathways) involved. Therefore, we performed a genome-wide association study (GWAS) to search for such genes. In addition, we tested whether gene loci identified in this GWAS are also associated with differences in adiposity and the size of the amygdala. We performed these studies in a population-based sample of 598 adolescents who were recruited from the French-Canadian population with known genetic founder effects\textsuperscript{260, 261, 305}. The power of genetic analyses is expected to be higher in founder than regular outbred populations due to more homogenous genetic and environmental backgrounds and, in turn, fewer genes contributing to complex traits, such as fat intake studied here\textsuperscript{264}. In addition, we tested our primary findings in an independent sample of 490 young adults of European ancestry.
PARTICIPANTS AND METHODS

The Saguenay Youth Study (SYS)

The SYS included 598 Caucasian adolescents, aged 12–18 years, who were recruited from a genetic founder population of French-Canadians living in the Saguenay-Lac St. Jean region of Quebec, Canada, as part of the SYS. It is expected that, in founder populations, fewer gene variants contribute to the determination of a complex trait, such as fat preference and adiposity studied here. The prevalence of several recessive disorders is higher in the Saguenay-Lac St. Jean region than in other populations, and limited allelic diversity exists among patients with these disorders. The SYS is an ongoing, population-based cross-sectional study of cardiovascular, metabolic and mental health in adolescence. It is a family-based study focused on collecting sib-pairs. Recruitment and selection criteria have been described previously. Written consent of the parents and assent of the adolescents were obtained. The Research Ethics Committee of the Chicoutimi Hospital approved the study. Descriptive characteristics of the studied participants are provided in Supplementary Table 1.

Dietary intake of fat was assessed with a 24-hour food recall, which is a well-established method of assessing diet used, for example, in the U.S. National Health and Nutrition Examination Surveys, the only nationally representative dietary survey in the United States. Using this method, information on the foods and drinks consumed during the past 24 hours is collected in a structured
interview conducted by a trained nutritionist and then analyzed with the Recipe File (USDA) to derive energy and macronutrient (i.e. carbohydrate, fat, and protein) intake. This instrument has been validated for Quebec youth\textsuperscript{267}.

**Magnetic resonance imaging of the amygdala:** Magnetic resonance T1-weighted images (1-mm isotropic) of the brain were acquired on a Phillips 1.0-T superconducting magnet (Gyroscan NT, Philips Healthcare, Best, The Netherlands). The details of magnetic resonance imaging data collection were reported previously\textsuperscript{258}. Volumes of the whole brain and of the right and left amygdala were obtained with FreeSurfer (FreeSurfer Version 5.0.0)\textsuperscript{291}. Briefly, FreeSurfer is a collection of image-analysis algorithms that assigns automatically anatomical labels to each voxel of the MR volume; this is achieved by combining probabilistic information about the global location of a structure in the atlas space with local spatial relationships between adjacent structures\textsuperscript{292}. In this manner, we obtained the volumes of the left and right amygdala. In the analyses reported here, we used the total (left + right) volume of the amygdala.

**Adiposity:** Weight (0.1-kg precision) and height (1-mm precision) were measured and body mass index (BMI) was calculated as body weight in kg divided by height in cm squared. Total body fat was assessed by multi-frequency bioimpedance analysis (Xitron Technologies, Inc., San Diego, CA, USA). Adolescents were asked to refrain from caffeine, alcohol and vigorous activity 24 hours before the measurement. The measurement was made after a 20-min stabilization period during which the participants were resting in a supine position.
**Genotypes:** All adolescents (n=602) were genotyped with the Illumina Human610-Quad BeadChip (Illumina, San Diego, CA) that includes 582,539 single nucleotide polymorphisms (SNPs) distributed across 22 autosomal chromosomes. The genotyping was conducted at the Centre National de Génotypage (Paris, France) where genotype calling was made for 567,726 SNPs. We excluded SNPs with call rate <95% and minor allele frequency <0.01, and SNPs that were not in Hardy-Weinberg equilibrium (p<1x10^{-4}); a total of 530,011 SNPs passed these quality-control criteria and were included in GWAS. On average, the call rate of these SNPs was 99.2%. In addition, 3 participants were removed based on principal component analysis of population structure performed with HapMap II (release 22) and SYS genotypes, and 1 participant was excluded due to >3% of missing genotypes; the final number of adolescents studied here was 598.

**Statistical methods:** GWAS of fat preference was conducted using Merlin-1.1.2, under an additive model. With Merlin, a simple regression model is fitted to the trait under study and a variance component approach is used to account for correlation between observed phenotypes within each family (i.e. to adjust for family relatedness of siblings in the SYS). For individuals with missing genotypes, the Lander-Green algorithm is employed to estimate an expected genotype score. In the SYS sample, fat preference was normally distributed and no statistical outliers (mean ± 3 standard deviations) were present. Age and sex were included as potential confounders.
Secondary analyses were aimed at examining whether the most significant GWAS-identified locus of fat intake (rs2281617) is also associated with energy, carbohydrate and protein intakes and with adiposity and amygdala volume; these analyses were performed with multivariate linear model, while adjusting for age and sex (and height when relevant). For each outcome, a preliminary analysis involved assessing the normality assumption, on which the statistical inference about the linear-model estimates relies. Body-fat mass and BMI had positively skewed distributions and were log transformed, using logarithm with base 10, which improved the fit. In addition, statistical outliers (i.e. values outside mean ± 3 standard deviations) were excluded in the case of fat, energy, carbohydrates and protein intakes, body-fat mass, BMI, and amygdala volume (n<8). The number of minor allele T homozygotes at rs2281617 was <10 and, thus, TT-homozygotes were pooled together with TC-heterozygotes.

The Toronto Nutrigenomics and Health Study (TNHS)

The TNHS is a cross-sectional study of diet, genotype and biomarkers of chronic disease in a population of young adults recruited from the University of Toronto, Toronto, Canada. Recruitment criteria are described elsewhere. A subset of the TNHS including 490 Caucasian adults (20–29 years old) whose food intake was assessed by a one-month, 196-item, semi-quantitative food-frequency questionnaire and who were genotyped with the Affymetrix SNP Array 6.0 (Affymetrix, Santa Clara, CA) were studied here. In these individuals, body weight and height were measured in a laboratory setting. Descriptive
characteristics of the studied participants are provided in Supplementary Table 1. All SNPs that passed quality control and were located within the µ-opioid receptor gene (OPRM1) were tested for association.

RESULTS

GWAS of fat intake in the SYS

The most significant gene-associated locus of fat intake was found in the µ-opioid receptor gene (OPRM1, rs2281617, p=5.2x10^{-6}, Table 1 and Figure 1). At this locus, T-carriers compared with CC-homozygotes showed lower intake of fat (by 4 %, Figure 2) and higher intake of carbohydrates (by 3%, p=0.002) and no differences in intake of protein (p=0.55) and energy (p=0.69, Figure 2).

OPRM1 locus and adiposity in the SYS

At the OPRM1 locus, T-carriers who showed lower fat intake when compared with CC-homozygotes also demonstrated lower body weight (by ~3 kg, p=0.02), BMI (by ~1 kg/m^2, p=0.01) and body-fat mass (by ~2 kg, p=0.02; Figure 2).

OPRM1 locus and amygdala volume in the SYS

Previous human and animal research suggests an inverse relationship between the size/activation of the amygdala and fat preference/intake. Consistent with this research, the OPRM1 T-carriers who showed lower fat intake...
when compared with CC-homozygotes demonstrated higher amygdala volume (by 69 mm$^3$, p=0.02, Figure 2). In addition, only in these T-carriers, amygdala volume correlated inversely with fat intake (T-carriers: $r=-0.13$, p=0.02; CC-homozygotes: $r=-0.01$, p=0.66), and this relationship remained virtually unchanged after additional adjusting for the overall brain volume (T-carriers: $r=-0.13$, p=0.04; CC-homozygotes: $r=-0.01$, p=0.34).

The above results remained virtually unchanged after the additional adjustment for prenatal exposure to maternal cigarette smoking, a known risk factor for adolescent fat intake$^{153}$.

**OPRM1 locus, fat intake and adiposity in the TNHS**

Next, we tested whether OPRM1 is also associated with fat intake in an independent sample of young adults (n=490). We found that a SNP most significantly associated with fat intake (rs518596, p=0.009) was located in the same intron as the SNP most significantly associated with fat intake in the SYS (rs2281617, Supplementary Figure 1). Similar to the SYS, the allele associated with lower fat intake was also associated with lower BMI (p=0.005).

Although the SNPs associated with fat intake in the SYS (rs2281617) and TNHS (rs518596) were located in the same last intron of OPRM1, they did not replicate each other (Table 2). They were not in linkage disequilibrium (LD, $D' < 0.48$). In fact, they were located on opposite sites of a recombination hot spot at which recombination rate was about 60 times higher than that across the genome$^{309}$ (Supplementary Figure 1). This high recombination rate is likely to
preventing direct replication\textsuperscript{310}, but it does not exclude the possibility that, in each sample, the identified SNP is in LD with and detects a signal of the same causal variant. Further studies are required to confirm this possibility.

**DISCUSSION**

The results of the present study suggest that *OPRM1* may modulate dietary intake of fat and hence risk for obesity, and that this effect may be associated with subtle structural changes in the amygdala.

*OPRM1* encodes the mu-opioid receptor, which shows high affinity for endogenous (β-endorphin and enkephalin) and exogenous (morphine, heroin and methadone) opioids. The gene is highly expressed in the brain areas processing reward associated not only with drugs of abuse but also with palatable foods and, in particular, dietary fat\textsuperscript{311,312}. The results of the present study demonstrating that *OPRM1* is associated with fat intake are consistent with previous research in experimental animals showing that administration of μ-opioid receptor agonists increases intake of fat\textsuperscript{312,313-315}. In the present study, the minor T allele was associated with lower fat intake, suggesting that it may be detecting a signal of a loss-of-function variant of the gene. This allele was additionally associated with higher carbohydrate intake and no differences in energy intake (Figure 2). These results are also consistent with previous research in experimental animals showing that while the administration of μ-opioid receptor agonists increases intake of fat, it either decreases or shows no impact on the intake of
carbohydrates; these effects are seen independently of energy status (i.e., in both satiated and hungry animals)\textsuperscript{132}.

In the present study, the \textit{OPRM1} variant most significantly associated with fat intake was rs2281617. The same variant has previously been associated with self-reported euphoria in response to amphetamine in a community-based sample\textsuperscript{316}. In that study, T-carriers (versus CC-homozygotes) who showed lower fat intake in our study demonstrated lower amphetamine-induced euphoria\textsuperscript{316}. Taken together with the results of the present study, these data support the possibility of a mechanistic overlap between processing reward from drugs of abuse and fatty foods.

In the current study, T-carriers versus CC-homozygotes demonstrated not only lower fat intake but also higher amygdala volume, and, in T-carriers, amygdala volume correlated inversely with fat intake. The amygdala is a structure of the brain-reward system studied extensively in the context of both drug addiction and the regulation of fat preference\textsuperscript{19} \textsuperscript{155} \textsuperscript{156}. Inverse relationships between amygdala volume and both fat intake\textsuperscript{153} and alcohol craving\textsuperscript{155} have been reported previously. It has also been shown that activation of the amygdala by intra-amygdalar administration of neuropeptide Y\textsuperscript{18} or enterostatin\textsuperscript{154} decreases preference for fat in experimental animals.

The mechanisms through which the \(\mu\)-opioid receptor may influence amygdala volume are not clear at present. It has been reported that the \(\mu\)-opioid receptor is functionally present on pre-synaptic glutamatergic terminals in the
amygdala and that its activation decreases the release of glutamate\textsuperscript{317}, a major excitatory neurotransmitter. Considering these relationships and the fact that enhanced excitatory neurotransmission and associated metabolic demands may induce cellular adaptations, such as increased vascularization and augmented number of glia, and that these adaptations may translate into an increase in the volume of a given brain structure, a loss-of-function mutation of \textit{OPRM1} would be expected to increase this excitatory neurotransmission and hence volume of the amygdala (Figure 3, left pathway). Alternatively, \textit{OPRM1} may influence embryonic development of the amygdala and, subsequently, its function. It has been shown that \textit{OPRM1} is expressed very early on in the embryonic rat brain, including the amygdaloid complex\textsuperscript{318} and that opioids may inhibit neurogenesis\textsuperscript{319} (Figure 3, right pathway).

In summary, the results of the present study suggest that \textit{OPRM1} may be involved in the regulation of dietary preference for fat and, perhaps through this pathway, in the development of obesity. The results also suggest that this effect of \textit{OPRM1} may be mediated, at least in part, through its influences on amygdala volume.
<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Chromosome</th>
<th>Position*</th>
<th>Nearest known gene</th>
<th>Effect size(\Psi) (standard error)</th>
<th>p-value</th>
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<td>-2.36 (0.50)</td>
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<tr>
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<td>OPRM1</td>
<td>-3.69 (0.81)</td>
<td>5.2x10^{-6}</td>
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<tr>
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<td>2.33 (0.52)</td>
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<td>1.5x10^{-5}</td>
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</table>

Associations were tested with Merlin-1.1.2.

* HapMap II+III, release 27

\(\Psi\) Effect size for minor alleles are reported
Table 2. Top *OPMR1* SNPs associated with fat intake in the Saguenay Youth Study (SYS) and Toronto Nutrigenomics Health Study (TNHS), respectively.

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Position</th>
<th>SYS</th>
<th>TNHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2281617</td>
<td>154,529,113</td>
<td>-3.69</td>
<td>5x10^-6</td>
</tr>
<tr>
<td>rs518596</td>
<td>154,504,070</td>
<td>0.51</td>
<td>0.30</td>
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</table>

* Effect size for minor alleles are reported.
### Supplementary Table 1: Characteristics of the studied participants in the Saguenay Youth Study and Toronto Nutrigenomics Health Study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SYS</th>
<th>TNHS</th>
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<tbody>
<tr>
<td>Sex (male/female)</td>
<td>285/313</td>
<td>156/334</td>
</tr>
<tr>
<td>Age (years)</td>
<td>15.1 ± 1.9</td>
<td>23.2 ± 2.5</td>
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<tr>
<td>Height (cm)</td>
<td>163.3 ± 9.6</td>
<td>169.9 ± 8.8</td>
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<tr>
<td>Weight (kg)</td>
<td>57.3 ± 13.0</td>
<td>67.3 ± 12.5</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>21.3 ± 3.7</td>
<td>23.2 ± 3.4</td>
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<tr>
<td>Body-fat mass (kg)</td>
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<td>-</td>
</tr>
<tr>
<td>Energy intake (kcal)</td>
<td>2,408 ± 899</td>
<td>2,037 ± 637</td>
</tr>
<tr>
<td>Fat intake (%)</td>
<td>31.9 ± 8.1</td>
<td>30.3 ± 6.3</td>
</tr>
<tr>
<td>Carbohydrate intake (%)</td>
<td>53.1 ± 9.7</td>
<td>52.7 ± 7.8</td>
</tr>
<tr>
<td>Protein intake (%)</td>
<td>14.6 ± 4.0</td>
<td>16.7 ± 3.0</td>
</tr>
</tbody>
</table>

Data are presented as means and standard deviations. SYS: Saguenay Youth Study, TNHS: Toronto Nutrigenomics Health Study
Supplementary Table 2: Chromosomal position and minor allele frequency of the top *OPMR1* SNPs associated with fat intake in the SYS and TNHS, respectively

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Chromosome 6 position*</th>
<th>Major/Minor allele</th>
<th>Minor allele frequency</th>
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</thead>
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<tr>
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<td></td>
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<td>HapMap</td>
</tr>
<tr>
<td>rs2281617</td>
<td>154,529,113</td>
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<td>0.17</td>
</tr>
<tr>
<td>rs518596</td>
<td>154,504,070</td>
<td>A/G</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* HapMap II+III, release 27
Figure 1: *OPRM1* locus of fat intake in the Saguenay Youth Study. The *OPRM1* section of the Manhattan plot for a genome-wide association study of fat intake in the Saguenay Youth Study and the structure of *OPRM1* are shown. The rs2281617 SNP was associated with fat intake at $p=5 \times 10^{-6}$. 
Figure 2: Association of the *OPRM1* locus (rs2281617) with fat, carbohydrate, protein and energy intakes and adiposity measures in the Saguenay Youth Study. Means ± standard errors, adjusted for age, sex and, when appropriate, height, are shown.
Figure 3: Two possible scenarios for the role of *OPRM1* in shaping the amygdala volume: “activity-induced neural plasticity” (left) and “embryonic development” (right).
Supplementary Figure 1: SNPs most significantly associated with fat intake in the Saguenay Youth Study and Toronto Nutrigenomics Health Study: significance of association, linkage disequilibrium and recombination rate. P-values of the association between top SNP and relative fat intake in each tested sample (left axis) and recombination rate (right axis) are presented with LocusZoom\textsuperscript{320}. Note that the average recombination rate across the human genome is about 1.3 cM/Mb\textsuperscript{309}, but in the area of the two SNPs, it is about 60 times higher (close to 80 cM/Mb). Linkage disequilibrium between the two SNPs is also shown (Haploview, 4.2.). HapMap II, release 22 data were used for the analyses of linkage disequilibrium and recombination rate.
CHAPTER SIX

Discussion
In the SYS, we identified a variant of *OPRM1* that was associated with lower fat intake and higher carbohydrate intake but no differences in protein or energy intake. In addition, the variant was also associated with lower adiposity and higher amygdala volume. Given the possible role of the amygdala in regulating fat preference, these results suggest that *OPRM1* may modulate dietary intake of fat and hence the risk of obesity, and this effect may be modulated by subtle variations in the volume of the amygdala.

Consistent with our findings, several studies done in rodents have demonstrated that activating the µ-opioid receptors with agonists increases intake of palatable foods (both sweet and fatty), but this effect is greater in animals fed high-fat diets than in those fed high-carbohydrate diets\(^{132} \, 133 \, 321 \, 322\). Furthermore, when both diets are available simultaneously, *OPRM1* stimulation increases preferentially intake of fat over carbohydrates\(^{132}\). Interestingly, some animals naturally prefer fats to carbohydrates, whereas others favor carbohydrates over fats, and this baseline preference influences their response to µ-opioid activation. Animals initially partial to fats showed greater increases in fat consumption with µ-opioid activation when compared to those that originally preferring carbohydrates\(^{132} \, 323\).

In our GWAS, the association between fat intake and the *OPRM1* variant (rs2281617) attained a p-value of \(<5.2 \times 10^{-6}\) and, as such, did not reach the established \(<5 \times 10^{-8}\) cut off for genome-wide significance\(^{324}\). Also, this exact variant was not associated with fat intake in our replication sample; despite this, another variant located in close proximity (rs518596) was associated with fat
intake and BMI (adiposity). These two variants were not in LD. They were located on opposite sites of a recombination hot spot, which showed recombination rates approximately 60 times greater than the average rate across the genome. Recombination affects LD patterns of a population and its occurrence may diminish the LD between different loci, which degrades the statistical power of the association analyses that are based on LD patterns\(^{325}\). Therefore, the high recombination rate of this region is likely to have prevented direct replication. Various explanations could be offered for the results of our replication study, one being that both \(\text{OPRM1} \) SNPs from the SYS (rs2281617) and the TNHS (rs518596) might actually be detecting the same causal variant. Alternatively, there could be different causal variants in the SYS and TNHS, with the rs2281617 detecting one and the rs518596 detecting the other in their respective populations. Finally, false positive results cannot be excluded.

The SNPs associated with fat intake and adiposity in the SYS and TNHS are both located in the last intron of \(\text{OPRM1} \), which is a region of the gene that has not yet been extensively studied. Although most transcription regulatory sites are located in the 5’ end of genes, they are also found on the 3’ end. Our intronic region of interest might have regulatory sequences that can alter gene expression. For example, transcription factors, key proteins that act as co-activators or co-repressors of gene expression, bind to specific sets of short DNA sequences, namely transcription-factors binding sites. Interestingly, the TRANSFAC software indicated that the last \(\text{OPRM1} \) intron contains several predicted transcription-factor binding sites that are altered by the SYS SNP.
(rs2281617); these are sites for the V$Hand (Twist subfamily of class B bHLH) and V$ZBPF (Zinc) transcription factors. Although their specific function is not very well understood, they belong to the matrix family of transcription factors and are expressed in the central nervous system

The OPRM1 variants associated with fat intake and adiposity in the SYS and TNHS are likely not causal. Therefore, further analysis of the identified region in this gene is essential to detect the causal variant(s) and to determine their precise roles in the regulation of fat intake. For this, powerful sequencing techniques are required. ‘Deep-sequencing’ allows to investigate specific regions of the genome. Once candidate sequence-variants are identified, they are tested for their function (predicted by bioinformatics) with experimental in vitro and in vivo models.

Different LD patterns can confound the replication and result in false negative results. We detected differences in the LD patterns of discovery (SYS) and replication (TNHS) samples (Figure 4). This might be because the SYS samples were collected from a Caucasian founder population and therefore genetically homogenous whereas, the TNHS samples, although also Caucasian, were of mixed European origin and thus likely genetically more heterogeneous. Studying the relationship between OPRM1, the intake of fat and obesity in multiple large independent samples of different ethnic background would help to support the association strength.
**Figure 4: LD plot for OPRM1 locus.** LD plots (Haploview 4.2) of OPRM1 in the Saguenay Youth Study and Toronto Nutrigenomics and Health Study are shown. Arrows denote the identified SNPs in each sample sets.
Genes, such as \textit{OPRM1}, most likely influences fat intake through one or more pathways that involve several other genes known to regulate eating behavior. Multiple polymorphisms in these genes can cumulate and together affect a functional pathway that leads to a measurable phenotypic outcome like fat intake. Pathway based GWAS analysis, in summary, would examine whether a set of SNPs from genes (using GWAS data) of a particular pathway has been enriched significantly with high ranks using specific statistical methods\textsuperscript{328}.

To assess macronutrient and energy intake, two different methods were used. In the TNHS, a one-month, 196-item, semi-quantitative food-frequency questionnaire was used. In the SYS, a single 24-hour food recall was conducted on the same day of the week for all SYS participants. The average and variance of fat intake were almost similar in both studies (31.9 % ± 8.1% and 30.3 % ± 6.3% in SYS and TNHS; respectively) that suggest the validity of the methods used.

In the present study, we found a relationship between \textit{OPRM1} and amygdala volume. \textit{OPRM1} is expressed in brain regions processing reward, including the amygdala\textsuperscript{329}. Previous studies have shown that µ-opioid receptors in the amygdala can affect intake of palatable foods and alter dietary preference for fat versus carbohydrates\textsuperscript{138 141 330}. Future studies are required to explain the exact mechanisms through which \textit{OPRM1} influences the volume of the amygdala and fat intake.
In the SYS and TNHS, *OPRM1* was also associated with adiposity. Obesity is a widespread problem, and is associated with adverse conditions, such as cardiovascular diseases, diabetes and cancer. Despite numerous efforts, an effective therapy for obesity remains elusive. The $\mu$-opioid receptor could be an interesting pharmacological target in the treatment of obesity. Modulating the activity of the $\mu$-opioid receptor could alter preference for fatty foods, which would lead to changes in body weight. In addition, identifying genetic variants that have a role in regulating food intake (such as *OPRM1* variants) will help identify the individuals predisposed to this type of obesity.
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