GENETIC VARIATION IN CD36 AND DIETARY FAT INTAKE

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Nutritional Sciences
University of Toronto

Your file Votre référence
ISBN: 978-0-494-45176-2
Our file Notre référence
ISBN: 978-0-494-45176-2

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Abstract

Free fatty acid sensor CD36 has been proposed as a putative 'fat taste' sensor. CD36 is expressed on rodent taste buds, and CD36-null mice consume less fat than wildtype littermates. The objective of this study was to determine whether common variations in the CD36 gene are associated with fat intake in humans, using a Caucasian and an Asian population. Habitual diet was measured with a food frequency questionnaire, and CD36 genotypes (rs1984112A>G, rs1761667G>A, rs1527483G>A, rs1049673G>C) were determined using real time-PCR. Differences in fat intake between genotypes were assessed using generalized linear models. Among Caucasians (n= 387), %energy from fat differed between genotypes for rs1984112A>G and rs1761667G>A polymorphisms, primarily at the expense of carbohydrate. Among Asians (n= 259), no differences in %energy from fat were observed, however, %energy from carbohydrate differed for the rs1049673G>C polymorphism. These results demonstrate that variation in CD36 is associated with habitual fat intake in humans.

ACKNOWLEDGEMENTS

Walking through the halls of FitzGerald I am always struck with the sensation that behind closed doors flasks gurgle feverishly and strings of DNA are amplified enough to reach the moon and back. Scientists become giants and ideas whirlwind from proposal to practice. It is a home that truly encourages and emboldens one to *live* one's question.

Ahmed, you are a mentor to emulate. Your ambition, inquisitive nature, and acceptance of no less than excellence were never hindered by your kindness and generosity. You encourage your students through your own example and because of this you will continue to be a driving force in my future goals and learning. Dr. Bazinet, you keep me sharp. You ask me the same question five times but have a way of making me hear it five different ways. Then you ask me 12 different questions and make my head spin a little. Dr. Hanley, your conscientious and supportive nature has helped develop my fundamental approach to a research question, and indeed a question in general. Together the three of you built a sense of safety that allowed me to open my mind and explore.

Leah and Karen, you are peers, teachers, and most exceptional friends. You have been integral to my Masters, and made this past year a true joy! You are certainly researchers to follow, and I am excited to watch as your imprints on science and education grow. Hyeon Joo your work ethic and kind nature make the lab run smoothly, and Davia and Susana your dedication to the study are unparalleled. Thank you to everyone in the El-Sohemy lab and NGH study, past and present, it has been a pleasure.

Louisa, Emelia, Lucile, and Vijay, you make the department run smoothly under Dr. Archer's watchful eye. Dr. Archer, I feel quite lucky to have been in the department while still under your reign and see a glimpse of what built Ahmed, and thereafter, his students who have come before me, to be the teachers and scientists they are.

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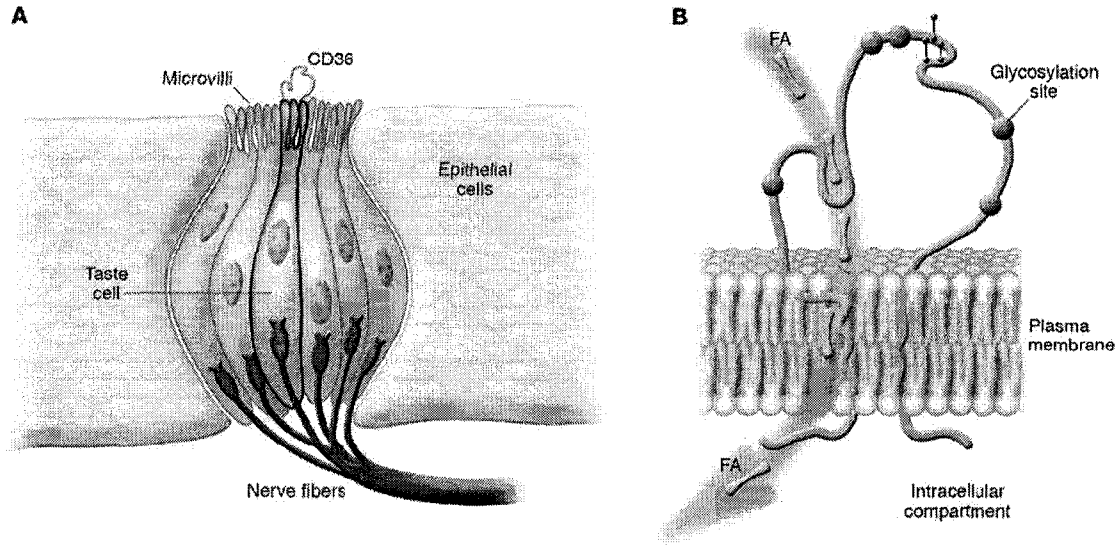
CHAPTER 1

INTRODUCTION

Variation in fat intake exists both within and between populations, with population average intakes usually ranging from less than 15% to 40% of total energy (Hunter et al., 1996; World Cancer Research Fund, 2007). A number of factors influence fat intake, varying from environmental conditions to genetic variation (Calder and Deckelbaum, 2006; van Vliet-Ostaptchouk et al., 2008). In particular, the sensory factors texture and olfaction mediate fat detection and have a strong influence on fat consumption (Drewnowski, 1997). However, blocking the ability to sense these factors does not prevent the recognition of dietary fat (Fukuwatari et al., 2003). This suggests that another oral sensory mechanism, which has tentatively been referred to as 'fat taste', contributes to fat detection and fat intake (Laugerette et al., 2007).

The integral membrane protein CD36 is hypothesized to mediate 'fat taste' by sensing free fatty acids (FFAs) (Figure 1) (Laugerette et al., 2005). Although CD36 has been described as a transporter (Abumrad et al., 1993), a receptor (Abumrad, 2005), and a sensor (Laugerette, et al., 2005), throughout this thesis it will be referred to as a sensor. CD36 is expressed on the taste bud cells of rodents (Figure 1 A) (Fukuwatari et al., 1997; Laugerette, et al., 2005), and in mice a targeted deletion of the CD36 gene results in reduced fat consumption (Laugerette, et al., 2005; Sclafani et al., 2007). This suggests that CD36 may contribute to oral FFA sensing, and that this is associated with fat intake. In humans, several common CD36 polymorphisms have been identified (Ma et al., 2004), however, the effects on habitual fat intake are not known.

Figure 1: Proposed structure and role of CD36 on the taste cells.



CD36 is expressed on the surface of taste cells in rodents (A) and has been proposed to be an oral fatty acid sensor (B) .

This figure has been reproduced with the permission of The Journal of Clinical Investigation from Laugerette et al. *J. Clin. Invest.* **115**: 2965-2967 (2005). doi: 10.1172/JCI26955.

CHAPTER 2

LITERATURE REVIEW

2.1 DIETARY FAT INTAKE

Variation in fat intake exists both within and between populations (Hunter, et al., 1996; World Cancer Research Fund, 2007). Fat intake also varies at the level of the individual, on a day to day basis (Willet, 1998). However, this within person variation may be accounted for by measuring an individual's dietary intake over several time points. If this is done, a population's fat intake distribution more closely reflects the variation between individuals (Willet, 1998). This variation within a population is of interest because it allows potential determinants and consequences of consuming a given amount of fat to be studied. For example, consuming a higher percent energy from fat has been associated with lower fasting triglycerides (TGs) (Holmes et al., 2000; Willett et al., 2001).

The average of percent of energy coming from fat for a population ranges from 30-40 % in highly developed countries to less than 15% to 30 % in less developed parts of the world (World Cancer Research Fund, 2007). The World Health Organization recommends that fat intake should be limited to 15-30% of total energy consumed, based on the prevention of chronic disease (World Health Organiziation, 2003). However, associations between total fat intake and chronic disease are conflicting (Hunter, et al., 1996), and therefore there is not sufficient evidence to make conclusions on the relationship between fat intake and chronic disease. For example, the Panel for the World Cancer Research Fund review of diet and cancer recently stated that only limited evidence suggested that total fat could cause some types of cancer (World Cancer Research Fund, 2007).

A number of determinants influence fat intake, including physiology, psychology, social influences, economics, and sensory factors (Calder and Deckelbaum, 2006; World Cancer Research Fund, 2007; Westerterp-Plantenga, 2004). Industrialization and economics are strong determinants of fat consumption between populations, and account for the large differences in fat intake seen between developed and developing countries (World Cancer Research Fund, 2007). The roles of some determinants, like genetics, which could influence both within and between population variations, are still, in large part, unknown.

2.1.1 Types of fat

Dietary fat is predominantly consumed in the form of triglyceride (TG), which is made up of three fatty acids (FAs) esterified to a glycerol molecule. The characteristics of TGs are influenced by FA chain length, number of carbon-carbon double bonds, type of double bonds (*cis* or *trans*), and the position on the glycerol to which the FA is esterified. Other types of dietary fat include diglycerides, monoglycerides, phospholipids, and sterols (Shils, 1994).

One way to classify FAs found in dietary fat is based on chain length. Although there is some variation in the number of carbons making up each type of FA, typically short chain FA are those with less than 10 carbons, medium chain FA are those with 10-15 carbons, long chain FA (LCFA) are those with 16-20 carbons, and very LCFA are those with more than 20 carbons. Most naturally occurring FA are 16 or 18 carbons in length (Guthrie, 1995).

FAs can also be classified based on their degree of saturation. FAs that do not have any carbon-carbon double bonds are called saturated FA (SFAs). Monounsaturated FAs (MUFAs) have only one double bond, and polyunsaturated FAs (PUFAs) have more than one double bond. The position of the (first) double bond is used in naming MUFAs and PUFAs. From the methyl end, those FA with double bonds in the n-3 and n-6 positions are called essential FA. These cannot be synthesized by humans and therefore must be consumed in the diet or from supplements. Sources of α-linolenic acid (18:3 n-3) include flax oil and marine fish oil, whereas linoleic acid (18:2 n-6) can be consumed from a number of vegetable oils like corn oil and safflower oil. These essential FA are biologically important, and function as precursors for very LCFA like eicosapentaenoic acid and docosahexaenoic acid. These are important for the production of bioactive lipids such as prostaglandins and leukotrienes (Guthrie, 1995).

The digestion of dietary fat involves the mechanical break down of food through chewing and muscular contractions of the gastrointestinal tract, and the hydrolysis of TG via lipase enzymes. Micelles aid in the absorption of the otherwise water insoluble lipids across the intestinal mucosal cells, at which point FAs are re-esterified into TG and packaged into chylomicrons for subsequent transport into the lymphatic system (Guthrie, 1995).

2.1.2 Methods of dietary assessment

Food intake is often measured to study the relationship between dietary exposure and health consequence, though the complex nature of food selection can

make this challenging (World Cancer Research Fund, 2007). Several approaches are used, including diet histories, 24-hour recalls, food records (or food diaries), and food frequency questionnaires (FFQs). Diet histories involve obtaining information about an individual's diet, without following a set template. Twenty-four hour recalls require that an individual reports all food and beverages consumed over the last 24-hours. If used to measure dietary intake of individuals, these are often administered several times over a study period, to capture seasonal variation and account for day to day fluctuation in the diet. Food records or food diaries involve an individual reporting everything consumed over a given period of time. They may require that individuals weigh their food, estimate portion sizes, or collect duplicate portions. FFQs require that an individual records the frequency with which food and beverages are consumed from a set list, over a given duration of time (e.g. 1 month or 1 year). FFQs may also gather information on portion size. Compared to other dietary assessment methods, FFQs are inexpensive and are therefore frequently used in studies with a large number of subjects. Importantly, the FFQ measures habitual diet, which is key in understanding relationships between long term diets and health outcomes.

Although a number of FFQs exist, there are two commonly used formats; the Block FFQ and the Willett FFQ. Recently the National Cancer Institute developed the Dietary History Questionnaire (DHQ). In an effort to validate it and compare the 3 dietary assessment tools, Subar et al. provided some important insight into the use and interpretation of these questionnaires (Subar et al., 2001). Overall, the analyses found that the 3 tools yielded similar results, following energy adjustment. This

implies that the questionnaires should all provide similar energy adjusted fat intake values. Therefore, using any of the questionnaires, relative rankings of subjects based on energy adjusted fat intake should be similar. However, because they are similar types of tools there may be errors intrinsic to the method of measurement. Indeed, although similar attenuation coefficients suggest that there is a high degree of correlation between the tools, it also suggests there may be a maximum effectiveness with which FFQs can measure nutrient intake (Willett, 2001).

FFQs are commonly validated using multiple 24-hour recalls or food records (World Cancer Research Fund, 2007). Specifically for fat, energy adjusted fat intake measured by a FFQ usually has a correlation of about 0.6 with 24-hour recalls and food records (Munger et al., 1992; Subar, et al., 2001; Willett, et al., 2001; Willett et al., 1985). Among a group of women, energy adjusted fat intake measured from a FFQ and from five 24-hour recalls had a correlation of 0.62 (Munger, et al., 1992). During the comparison of the Willett, Block, and DHQ FFQs, four 24-hour recalls administered approximately 3 months apart were used as the reference instrument (Subar, et al., 2001). The correlation of energy adjusted fat intake between 24-h recalls and FFQs ranged from 0.60 for the Block in men to 0.67 for the DHQ in women (Subar, et al., 2001).

Food records, like 24-hour recalls, have also been used to validate FFQs (Willett, et al., 1985). In the original validation of the Willett FFQ, four sets of 7-day food records were collected over one year and used as the dietary reference tool (Willett, et al., 1985). The semi-quantitative FFQ was administered twice; initially prior to any food records, and then again after either the third or fourth set of food

records. Based on the percent of subjects who were in the lowest quintiles and highest quintiles of fat intake, using measurements from both food records and FFQ, the authors concluded that the FFQ and 7-day food records provide similar relative rankings of subjects. Following energy adjustment, total fat intake measured with the FFQ had a correlation of 0.53 with the food records (Willett, et al., 1985). Overall, this suggests that measurements of energy adjusted fat intake from FFQs, 24-hour recalls, and food records are comparable.

Although there is agreement in energy adjusted fat intake measurements from FFQs, 24-hour recalls, and food records, there is also some overlap in error between these methods. For example, all of these techniques will have some reporting error. Therefore, correlations between dietary fat intake and biological markers have also been used to validate measurements of fat intake from FFQs. This type of validation is particularly important because, although imperfect, the source of error is independent of that from dietary measurement. FA isolated from subcutaneous adipose tissue can be used to approximate some dietary FA intakes (Arab, 2003; Bingham, 2002). The percent energy from dietary PUFA, measured with the Willett FFQ, had significant correlations of about 0.3 with total n-3 and n-6 FAs found in adipose tissue samples of African-American men (Holmes et al., 2007). In a group of Boston health professionals, percent energy from PUFA, measured both with the Willett FFQ and food records, also significantly correlated with PUFA found in adipose tissue samples (Hunter et al., 1992). This suggests that PUFA isolated from subcutaneous adipose tissue correlates well with dietary measures of PUFA intake, and that food records and the Willett FFQ are comparable to

measuring 'true' PUFA intake. In the same study, correlations between dietary MUFA measured by the FFQ and MUFA in the adipose tissue were positively correlated, although the relationship was not statistically significant. Unlike dietary PUFA, there is no widely accepted biological marker to validate total fat intake. Fasting TG levels are, however, inversely related to energy adjusted fat intake (Holmes, et al., 2000; Knopp et al., 1997; Willett, et al., 2001), suggesting TG may serve as a type of biomarker for total fat intake. In a clinical trial, hypercholesterolemic men were randomized to one of four diets with different percents of energy coming from fat (Knopp, et al., 1997). After one year, participants who had been assigned to diets with less energy coming from fat had significant increases in TG levels (Knopp, et al., 1997). In an observational study, the percent energy from total fat, measured using the Willett FFQ, was inversely associated with fasting TG (Willett, et al., 2001). This demonstrates that significant variation in total fat intake can be captured with the FFQ. It also provides non-dietary support that the Willett FFQ is accurately measuring total fat intake.

Energy adjusted values, as opposed to absolute values, are commonly used when validating dietary methods of fat intake and biomarkers of fat intake. Although there is some criticism with using energy adjusted values for micronutrients and nutrients not strongly associated with energy intake (Block, 2001), this is not the case for dietary fat. In nutritional epidemiology, energy adjusted nutrient intakes are often more valuable than absolute intakes because they take diet composition into account (Willett, 2001). Therefore, the effect of replacing a given nutrient in the diet with another can be estimated. This is of particular value for dietary fat, because

there have been conflicting reports that substituting complex carbohydrate for fat in the diet may be beneficial in preventing chronic disease (Willett, 2008). Additionally, adjusting for energy reduces potential confounding from caloric intake, thereby avoiding associations with nutrient intake from being made if they are only artifacts of energy intake (Willet, 1998). In addition to reducing potential confounding, energy adjustment can also reduce some of the error associated with dietary measurement. Because over and under reporting of macronutrients and total energy are highly correlated, adjusting for energy can partially correct these errors in reporting (World Cancer Research Fund, 2007). Overall, adjusting fat intake for energy provides a better value with which to study nutrient associations than unadjusted values.

Each tool for measuring food intake has strengths and weaknesses. These are important to understand because they can lead to differences in measurement error, such as dietary misclassification, and may lead to attenuation of an effect estimate. Minimizing such error can, in part, be done by using the most appropriate method of dietary intake.FFQs are a cost-effective way of measuring energy adjusted fat intake in nutritional epidemiological studies. These questionnaires are designed to measure habitual diet, which is useful when studying nutrient associations with health outcomes. As well, they may be useful for studying how determinants of food intake relate to the long term diet.

2.2 'FAT TASTE'

A number of factors influence food intake, including food preferences, which are strongly determined by sensory factors, like taste (Drewnowski, 1997). In

addition to influencing food choice, taste may enhance the digestion of foods consumed (Abumrad, 2005). Taste receptor cells are responsible for receiving taste stimuli from food (Chandrashekar et al., 2006). These cells come together to form taste buds, which in turn group to form papillae. Circumvallate, foliate, and fungiform papillae concentrate in parts of the sensory epithelium, like the oral cavity (Chandrashekar, et al., 2006). Traditionally, five taste modalities have been described: bitter, sweet, sour, salty, and umami (Chandrashekar, et al., 2006). However, there is increasing evidence that 'fat taste' may be a 6^{th} taste modality (Laugerette, et al., 2007; Laugerette, et al., 2005).

Rodents select a high fat over a low fat diet when given the choice, suggesting they have a natural preference for dietary fat (Hamilton, 1964). Humans may have a comparable inclination, as indicated by fatty food preference tests (Mela and Sacchetti, 1991). The mechanisms mediating fat detection have traditionally been attributed to texture and olfaction (Laugerette, et al., 2007). These factors influence the palatability of food and, in addition to post-ingestive cues that can influence gut-brain signaling, contribute to fat preference and fat intake (Abumrad, 2005; Drewnowski, 1997). However, blocking the ability to sense these factors fails to abolish the recognition of dietary fat suggesting another mechanism also contributes (Fukuwatari, et al., 2003).

Mice prefer a range of vegetable oil solutions emulsified in xanthan gum over a non-oil 0.3% xanthan gum solution that masks textural cues (Takeda et al., 2000), demonstrating that fat can be detected in solutions with comparable textures. In conditioned aversion tests, rats were trained to avoid a sucrose-corn oil mixture

(Smith et al., 2000). However, this behaviour was dependent on the corn oil and could not be reproduced when substituted with a non-nutritive mineral oil (Smith, et al., 2000). Presumably, the corn oil and mineral oil provided similar textural cues, and therefore there must have been another type of cue that allowed the rats to identify the corn oil. The trigeminal nerve is responsible for transmitting textural cues to the central nervous system. Interestingly, cutting the gustatory nerves, but leaving the trigeminal nerve intact, was sufficient to abolish the preference for long chain fatty acid (LCFA) (Gaillard et al., 2008), further suggesting that textural cues are not sufficient to detect fat. Fat can also be detected without olfactory cues (Takeda et al., 2001), as anosmic mice that have their sense of smell blocked by zinc sulfate treatment could be conditioned to recognize oil as a reward in conditioned place preference tests (Takeda, et al., 2001). Short term intake tests using low concentration fat solutions limit the amount of fat reaching the gut, and therefore minimize the influence of post-ingestive cues (Tsuruta et al., 1999). In these 5 minute tests, rats preferred LCFA to a texturally comparable 0.3% xanthan gum vehicle, suggesting that post-ingestive cues are also not sufficient to detect fat (Tsuruta, et al., 1999). Finally, attempting to minimize textural, olfactory, and post-ingestive cues in the same rat model still fails to abolish the preference for dietary fat, further supporting the existence of 'fat taste' (Fukuwatari, et al., 2003).

2.2.1 Detection cues

Although dietary fat is predominantly consumed and stored as TG, it appears that FFA, the metabolically active form of fat, is more important for fat detection in

'fat taste'. In a series of two-bottle preference tests, rats were presented simultaneously with two bottles containing different solutions, and were given the choice from which to drink (Fukuwatari, et al., 2003). Rats preferred LCFA oleic acid, over a 0.3% xanthan gum control (Fukuwatari, et al., 2003). However, they did not show the same preference for triolein, the TG form of oleic acid. When given the choice of oleate and triolein, rats preferred the oleate solution, suggesting that FFAs are an important cue (Fukuwatari, et al., 2003). In fact, recognition may be specific for LCFA. Brief two-bottle tests assessed preferences for a number of 18-carbon LCFAs, and found that LCFAs were selected over a xanthan gum control and over their LCFA derivatives. Conversely, rats chose a xanthan gum control over a solution containing an 8-carbon medium chain fatty acid, caprylic acid (Tsuruta, et al., 1999).

In addition to chain length, recognition of and preference for LCFA may depend on the degree of saturation. In whole cell studies with taste receptor cells, a series of FFAs were applied while patch-clamp recording was used to measure the inhibition a group of potassium channels important in intracellular taste receptor cell signaling, called delayed-rectifying potassium channels (Gilbertson et al., 1997). Inhibition of these potassium channels prolonged taste cell depolarization, and was hypothesized to be a mechanism of fat detection. Only cis-polyunsaturated FA caused significant inhibition, while monounsaturated, saturated, and trans-polyunsaturated FA failed to produce a significant effect (Gilbertson, et al., 1997). In addition, two-bottle preference tests, rats preferred α-linolenic acid (18:3 n-3) to

linoleic acid (18:2 n-6), and linoleic acid to oleic acid (18:1 n-9), suggesting degree of saturation may influence choice (Tsuruta, et al., 1999).

Lingual lipase activity is sufficient to yield FFA from dietary TG, and produce an oral FFA cue (Kawai and Fushiki, 2003). In addition, rats have very low FFA detection thresholds, approaching 44 uM for linoleic and oleic acid (McCormack et al., 2006). This suggests that a FFA cue could easily be recognized in the oral cavity.

2.2.2 Physiological relevance

Our ability to detect trace amounts of FFAs, including oleic, stearic, and linoleic acid, on the tongue suggests that they may be oral chemosensory cues in humans (Chale-Rush et al., 2007). Physiologically, such a cue could aid in lipid metabolism by providing an early warning signal of incoming fat. Indeed, oral exposure to fat affects post-prandial fat metabolism (Mattes, 1996; Mattes, 2001; Mattes, 2001). To prevent oral detection, subjects were given encapsulated oil, followed by sham feeding with full-fat or non-fat cream cheese treatment (Mattes, 1996). Plasma TG was measured at set time points, to determine whether the presence of fat in the oral cavity was associated with post-prandial metabolism. Full-fat treatment resulted in greater area under the curve for plasma TG. A similar study compared sham feeding between butter and fat replacer (Mattes, 2001). Again, the fat treatment resulted in greater area under the curve for TG than non-fat treatment. These studies suggest that textural cues are not sufficient to cause these changes in plasma TG. Plasma TGs are thought to reflect acute changes in the diet (Mattes,

2001), and although the mechanism leading to their rise following oral fat exposure cannot be determined from these studies, the observations nonetheless demonstrates that there is likely a 'fat taste' component that causes changes in post-prandial lipid metabolism, as measured by plasma TG. Finally, a study ruled out the possibility that increases in TG were primarily in response to olfactory cues, as opposed to 'taste' chemosensory cues of fat (Mattes, 2001). Following ingestion of oil capsules, subjects either smelt or chewed and spat out the cream cheese stimulus. Olfactory stimulus alone was associated with an increase in plasma TG only at 4 hours after receiving the encapsulated oil, whereas sham feeding was associated with increases in TG at each time point (baseline, 2, 4, and 6 hours) after fat loading with the encapsulated oil (Mattes, 2001). Overall, these experiments demonstrate that humans have a mechanism to orally detect dietary fat that is associated with changes in post-prandial lipid metabolism. Since none of the fat stimuli were swallowed, this mechanism(s) is independent of post-ingestive cues as well as texture and olfaction. Instead, an oral chemosensory fat 'taste' detection system is likely influencing lipid metabolism.

There is evidence, in humans, that this oral detection of fat may depend on its degree of saturation (Mattes, 2005; Tittelbach and Mattes, 2001). Following an encapsulated fat load, subjects were given one of a number of test stimuli, including margarine containing unsaturated fat, jelly alone, jelly and margarine, or butter containing saturated fat. Each of these was given on a small amount of cracker, and tests with cracker alone and no oral stimulus were also completed. In each case, subjects chewed and spat out the treatment. Although at one time point both

margarine alone and margarine with jelly were associated with increases in TG, the margarine alone treatment had a maximum change in TG that was significantly greater than jelly alone, jelly and margarine, and butter alone (Tittelbach and Mattes, 2001). Because oral stimulation with unsaturated fat, but not saturated fat, was associated with changes in post-prandial lipid metabolism, it suggests that fat detection may be influenced by the degree of fat saturation. This is in agreement with taste receptor cell studies that found degree of saturation influenced taste receptor cell response to FFA (Gilbertson, et al., 1997).

In addition to influencing in lipid metabolism, oral fat sensing may function in the detection of traditional taste modalities (Gilbertson, et al., 1997). LCFA can inhibit delayed rectifying potassium channels on taste receptor cells, prolonging cell depolarization and potentially sensitizing the cells to tastants (Gilbertson, et al., 1997). This may explain the increased licking response in rats given a sucrose solution containing LCFA, compared to sucrose solution alone (Pittman et al., 2006). Moreover, the addition of LCFA to sour, salty, and bitter solutions decreased the licking response (Pittman, et al., 2006). The authors suggest that LCFA may intensify the perceived taste, resulting in increased preference for palatable solutions and aversion to less pleasing ones. In humans, however, the addition of LCFA to solutions may reduce the sensitivity for sour, salty, and bitter tastes as subjects' thresholds for tasting these stimuli increased (Mattes, 2007). The intensity of sweet, sour, salty, and bitter tastes was either unaffected or reduced by the addition of LCFA to solutions. This suggests that although LCFA may affect the detection of traditional taste modalities, their primary role in taste is likely to aid in

the detection of dietary fat (Mattes, 2007). It has been hypothesized that this detection is mediated by CD36 (Cluster of Differentiation 36), which is an integral membrane protein (Laugerette, et al., 2005).

2.3 CD36

Although the precise mechanism mediating oral chemosensory detection of fat is not clear, the observation that FAs can inhibit delayed rectifying potassium channels on taste receptor cells provides a good molecular clue (Gilbertson, et al., 1997). Inhibition was specific for LCFA and selective for unsaturated LCFA, and was generally limited to stimuli applied extracellularly, consistent with an apical 'fat taste' sensor. In the same year, CD36 was detected on the apical surface of taste bud cells (Fukuwatari, et al., 1997). CD36 is a multifunctional integral membrane glycoprotein that has a high affinity for unesterified LCFA (Baillie et al., 1996). Although it is commonly known for its role in facilitating FFA transport across the cell membrane (Harmon and Abumrad, 1993), Laugerette et al. have recently proposed that CD36 is an oral fat sensor (Laugerette, et al., 2005).

CD36 has two transmembrane domains creating a large extracellular hydrophobic loop and two short cytoplasmic tails (Figure 1A) (Vega et al., 1991). This molecular structure should facilitate extracelluar FFA sensing, consistent with previous observations (Figure 1B) (Gilbertson, et al., 1997). The extracellular domain is well glycosylated, which may support the protein's multifunctional nature (Armesilla and Vega, 1994; Vega, et al., 1991). Overall, this suggests that CD36 may be involved in the oral chemosensory detection of fat, by sensing FFA.

Though CD36 was first identified on platelets (Kieffer et al., 1988; Tandon et al., 1989), it is now recognized as being widely expressed on a number of cells and tissues, including taste cells (Fukuwatari, et al., 1997; Laugerette, et al., 2005), adipocytes (Abumrad, et al., 1993), skeletal muscle (Van Nieuwenhoven et al., 1995), smooth muscle (Kwok et al., 2007), intestine (Chen et al., 2001), and monocytes and macrophages (Huh et al., 1996).

The factors regulating CD36 expression are cell and tissue dependent. On monocytes/macrophages, differentiation can influence expression (Huh, et al., 1996). In other cases, CD36 expression can be regulated by its ligands. For example, binding with oxidized low-density lipoprotein (LDL) can activate PPARγ and upregulate CD36 expression (Feng et al., 2000).

Throughout the body, CD36 has a number of different ligands. It is a receptor for oxidized (Endemann et al., 1993) and acetylated (Acton et al., 1994) LDL, as well for native high-density lipoprotein (HDL), LDL, and very low-density lipoprotein (VLDL) (Calvo et al., 1998). On platelets, it is a receptor for thrombospondin-1 (Kieffer, et al., 1988). CD36 can also scavenge erythrocytes infected with *Plasmodium falciparum* malaria (Oquendo et al., 1989). Its interaction with some of these ligands has led to CD36 being classified as a member of the class B scavenger receptor family (Febbraio et al., 2001). The association of CD36 with almost all of its ligands, except LCFA, has been described as that of an endocytic receptor (Febbraio and Silverstein, 2007). Although CD36 has traditionally been described as binding or transporting LCFA (Abumrad, et al., 1993), some controversy exists regarding whether or not it directly acts as a transporter

(Hamilton, 2007). Febbraio and Silverstein (2007) propose that while direct CD36-LCFA interaction may facilitate membrane transport, the process may also be assisted by fatty acid binding proteins. As well, they suggest that a CD36-directed change in membrane permeability may facilitate transport or alternatively, that CD36 protein oligomerization shapes a LCFA-friendly opening (Febbraio and Silverstein, 2007). Regardless of the mechanism, the ability of CD36 to sense LCFA is important for normal fatty acid transport in cells and tissues throughout the body.

2.3.1 Signaling

Following ligand-specific interaction, CD36 signaling is cell and tissue specific. For example, thrombospondin-1 binding to CD36 results in *src* kinase *fyn* phosphorylation, which leads to further activation of caspases and mitogen activated protein (MAP) kinase p38 in endothelial cells (Jimenez et al., 2000). Whereas, modified LDL binding to CD36 on macrophages can activate *src* kinase *lyn*, followed by MAP kinase kinase and *jnk* kinase (Rahaman et al., 2006). Recent work suggests how CD36 signaling may occur in taste cells (El-Yassimi et al., 2008). This involved understanding the molecular mechanisms that allow a LCFA stimulus to increase intracellular calcium in taste cells expressing CD36 (CD36-positive) (Gaillard, et al., 2008). El-Yassimi et al. demonstrated that the rise in calcium was from an intracellular source, because the increase was still observed when tested in a medium that lacked calcium (El-Yassimi, et al., 2008). This gives important clues on the molecular steps following a LCFA stimulus. A Linoleic acid stimulus led to the production of inositol 1,4,5-triphosphate (IP_3) signaling molecule, and this response

was blocked with the CD36 inhibitor sulfo-N-succinimidyl oleic acid ester (SSO). This suggests that in taste cells, linoleic acid interacts with CD36 to produce IP_3, which results in an increase in intracellular calcium. The production of IP_3 implies that phospholipase C (PLC) is also involved. In resting platelets, CD36, *src* kinase, and PLCγ2 co-localize, and following cold-induced activation, *src* kinases are phosphorylated and PLCγ2 is activated (Gousset et al., 2004). Previous to this, the *src* family of protein tyrosine kinases were also associated with CD36 signaling (Huang et al., 1991). This suggests that taste cells may be activated by LCFA-CD36 interaction that results in *src* kinase phosphorylation and PLC activation, which causes increased production of IP_3 leading to a rise in intracellular calcium (El-Yassimi, et al., 2008).

Increases in intracellular calcium are also associated with taste signaling via afferent nerve fibers (El-Yassimi, et al., 2008). CD36 is required for an oral LCFA stimulus to activate taste neurons in the nucleus of the solitary tract, which is the brainstem connection between taste in the oral cavity and the central nervous system (Gaillard, et al., 2008). However, the mechanism of signaling is not clear. The mRNA of enzymes involved in monoamine neurotransmitter production is present in CD36-positive taste cells (El-Yassimi, et al., 2008). Moreover, stimulating these cells with linoleic acid led to 5-hydroxytryptamine (serotonin) and noradrenalin release (El-Yassimi, et al., 2008). This was both CD36-specific and calcium dependent, as treatment with SSO abolished this response, and blocking the channels that allow intracellular calcium stores to be replenished also abolished this response. Additionally, *src* kinase inhibitors also prevented neurotransmitter release.

Overall, this suggests that following LCFA stimulus in CD36-positive cells, phosphorylation of *src* kinases leads to a rise in intracellular calcium via PLC and IP_3, and this rise in calcium is associated with the release of neurotransmitters, which may be involved in afferent nerve signaling (El-Yassimi, et al., 2008).

2.3.2 Genetic variations

The human CD36 gene is located on chromosome 7q11.2 (Fernandez-Ruiz et al., 1993). This region has been associated with symptoms of the metabolic syndrome in genome wide association studies (An et al., 2005; Arya et al., 2002). Polymorphisms in the CD36 gene have been associated with HDL and TG levels, in a cohort of related and unrelated African American subjects (Love-Gregory et al., 2008). However, the significance of these results do not necessarily extrapolate to other ethnoracial groups, as the frequency of some genetic variants in CD36 appear to be much more common in populations of African and Asian descent (Curtis and Aster, 1996). Because CD36 is involved in cytoadherence of erythrocytes infected with *Plasmodium falciparum* malaria, it has been suggested that this high frequency of variation may, in part, be due to evolutionary pressures to protect against malaria (Oquendo, et al., 1989; Urwijitaroon et al., 1995). However, it has also been suggested that the variation is due to another adaptive pressure, such as another infection (Aitman et al., 2000).

Ma et al. found that polymorphisms located in the 5' flanking region of exon 1A (rs1984112 A>G and rs1761667 G>A), intron 11 (rs1527483 G>A), and the 3'-UTR of exon 15 (rs1049673 G>C) were 'tag' single nucleotide polymorphisms

(SNPs) that can be used to identify 2 major linkage disequilibrium blocks in the CD36 gene (Ma et al., 2004). In Caucasian males, a haplotype combination of these polymorphisms was associated with higher FFA and TG levels (Ma et al., 2004). The same haplotype was more common in type 2 diabetic males and females that also had coronary artery disease than in diabetic subjects who were free of coronary artery disease (Ma et al., 2004). These same polymorphisms were linked to greater changes in fasting plasma TG following 6 weeks of treatment with fish oil supplements while maintaining a habitual diet (Madden et al., 2008). Both studies reported frequencies for Caucasian populations (Ma et al., 2004; Madden, et al., 2008) and, to our knowledge, are the only studies that have investigated these specific polymorphisms. The NCBI database reports frequencies for Asian populations for rs1049673 and rs1527483, and these frequencies are similar to those reported in our Asian population. However, there are no reference frequencies for non-Caucasian ethnoracial groups for rs1984112 and rs1761667. It has been hypothesized that these common polymorphisms may result in reduced functionality of CD36 (Ma, et al., 2004), a phenotype that has already been documented for rare polymorphisms in the CD36 gene (Kashiwagi et al., 1994; Kashiwagi et al., 1995).

Genetic variation in CD36 may influence protein levels, as evidenced by patients who have rare genetic variants (Kashiwagi et al., 1994; Kashiwagi et al., 1995) . These variants result in changes in CD36 functionality, and are diagnosed by characterization of platelets that lack CD36 (Kashiwagi et al., 1995) or by platelets and macrophages that lack CD36 (Kashiwagi et al., 1994). These conditions are low among Caucasian populations (0.3%), but can reach frequencies

of 3-10% in some populations of Asian and African descent (Curtis and Aster, 1996; Rac et al., 2007). This state of reduced CD36 functionality can have symptoms similar to the metabolic syndrome (Hirano et al., 2003). These patients frequently have elevated plasma glucose, insulin resistance, elevated blood pressure, raised TG, and low HDL (Hirano, et al., 2003). The ability of the heart to uptake and therefore utilize LCFA is impaired, which may result in the development of cardiomyopathy and other pathologies (Tanaka et al., 2001). It appears that the binding of oxidized LDL to CD36 is also impaired (Nozaki et al., 1995), and that the normal oxidized LDL-induced expression of inflammatory cytokines is reduced (Janabi et al., 2000). It has been suggested that this profile is partially attributable to the dysregulation of LCFA metabolism (Miyaoka et al., 2001).

Experiments with CD36-null mice suggest that the reduced cardiac uptake of FAs is compensated for by increased glucose uptake (Febbraio and Silverstein, 2007). Though there are conflicting results on how a targeted deletion of the CD36 gene affects insulin resistance (Febbraio et al., 1999; Hajri et al., 2002), it appears that this may be tissue specific (Goudriaan et al., 2003). Unlike in humans, where a lack of CD36 is associated with elevated blood glucose, CD36 knockout mice tend to have lowered blood glucose (Febbraio, et al., 1999). These mice have greater insulin-mediated glucose uptake in whole body and muscle-specific tissue experiments, compared to wildtype littermates (Goudriaan, et al., 2003). However, CD36-null mice failed to suppress endogenous glucose production when exposed to hyperinsulinemic conditions, compared to wildtype mice (Goudriaan, et al., 2003). This suggests that despite increased insulin sensitivity in the rest of the body, CD36-

null mice are insulin resistant in the liver. These mice also tend to have increased fasting FA and poor lipid profiles, in comparison to their wildtype littermates (Febbraio, et al., 1999).

2.3.3 Function as an oral lipid sensor

Genetic variation in CD36 can result in functional changes, as evident from individuals with rare genetic variants (Kashiwagi, et al., 1994; Kashiwagi, et al., 1995). In the context of a LCFA sensor, such a functional modification could result in variation in the ability to sense dietary fat in the oral cavity. Although the precise mechanism by which CD36 senses FAs is not clear, its topology supports extracellular sensing, consistent with a 'fat taste' sensor. The sensing ability appears to be specific to longer chain FA, but both saturated and unsaturated LCFA can caused depolarizing increases in intracellular calcium in CD36-positive taste bud cells (Gaillard, et al., 2008). This suggests that both saturated and unsaturated dietary fat may be sensed on the tongue. Finally, CD36-specific inhibitor sulfo-N-succinimidyl oleic acid ester attenuated LCFA-induced depolarizations, strengthening the theory that oral CD36 is involved in FA sensing (Gaillard, et al., 2008). Functionally, this may be important in influencing dietary fat intake and preparing the digestive system for incoming fat (Laugerette, et al., 2005).

2.3.3.1 Regulation of fat intake

Direct evidence that CD36 may act as a putative 'fat taste' sensor comes from animal studies using knockout mice (Laugerette, et al., 2005; Sclafani, et al.,

2007). In a two-bottle preference test, mice were provided with two bottles over 48 hours (Laugerette, et al., 2005). The first bottle contained 2% linoleic acid emulsified with 0.3% xanthan gum and the control bottle contained a water (0.3%) xanthan emulsion to mimic the texture of fat. Wildtype mice preferred the linoleic acid choice, whereas mice with a targeted deletion of CD36 did not distinguish between the two (Laugerette, et al., 2005). It is not surprising that wildtype mice preferred the fatty emulsion, as mice normally prefer dietary fat over a texturally comparable alternative (Takeda, et al., 2000). Similar preferences were also observed in emulsions with higher fat concentration. These observations appear to be specific for fat, as opposed to a general loss of taste among CD36-null mice, as indicated by the maintenance of a preference for sweet and aversion to bitter (Laugerette, et al., 2005). To reduce input from post-ingestive cues, short term (0.5 h) intake tests were conducted with the same test solutions. Again, wildtype, but not CD36-null mice, consumed significantly more of the linoleic acid containing emulsion. Finally, preferences were tested with fat-enriched solid diets. Fasted mice were presented with a 5% linoleic acid solid diet and 5% paraffin control solid diet for 1 hour. CD36-null mice did not distinguish between the two, but wildtype mice showed a significant preference for the linoleic acid enriched diet. These experiments demonstrate that CD36 is required to recognize FFA and mediate fat intake.

Although dietary fat is predominantly in the form of TG, lingual lipase may yield sufficient FFA to act as a chemosensory cue to CD36. Lipase secretion is constant and yields FFAs quickly enough to be detected by an oral fat sensor (Kawai and Fushiki, 2003). Inhibition of lingual lipase reduced the intake of TG, but

failed to reduce the intake of FFAs (Kawai and Fushiki, 2003). This strengthens the evidence that FFAs are the primary chemosensory cue by which dietary fat is recognized, and further suggests that FFA recognition is involved in fat intake.

Lipid naïve wildtype mice that have not previously been exposed to dietary fat, were more likely to choose a FA emulsion over xanthan gum than lipid naïve CD36-null mice (Sclafani, et al., 2007). Wildtype mice were also more likely to choose TG over 0.15% Emplex vehicle (sodium stearoyl lactylate, American Products, Kansas City, MO) than CD36-null mice (Sclafani, et al., 2007). This indicates that TG can be hydrolyzed to FA rapidly enough to be sensed orally and influence fat intake. However, at increasing concentrations of FA and TG CD36-null mice exhibited increased fat preferences, though they continued to consume less total fat than their wildtype littermates (Sclafani, et al., 2007). The authors suggest that post-oral conditioning effects may, in part, be responsible for this 'rescued' phenotype (Sclafani, et al., 2007).

2.3.3.2 Early phase fat digestion

Although the more obvious role of sensory cues is to guide food intake, they also contribute to digestion (Abumrad, 2005). A possible physiological advantage of 'fat taste' is to prepare the digestive system for incoming fat while still in the oral cavity, thereby promoting efficient utilization of nutrients. Rats that have been esophagectomized, to prevent nutrient ingestion, can still increase the protein content of pancreatobiliary secretions following oral exposure to LCFA (Hiraoka et al., 2003). This means that rats can detect LCFA in the oral cavity, and this detection

is associated with changes in digestive secretions. Another study found that esophagectomized rats had increased protein content and flux of pancreatobiliary secretions in response to oral LCFA stimulus, compared to water (Laugerette, et al., 2005). This observation was dependent on FA chain length and degree of saturation, as only unsaturated LCFA induced a response and saturated LCFA and medium (10-carbon) chain FA did not (Laugerette, et al., 2005).

The ability of unsaturated LCFA to induce changes in digestive secretions appears to be dependent on CD36 (Laugerette, et al., 2005). When linoleic acid was applied to the soft palate, where CD36 is not expressed, no changes in secretions were observed. Moreover, although wildtype mice showed changes in pancreatobiliary secretions similar to those of rats, CD36-null mice failed to produce a rise in protein content or flux in response to oral linoleic acid stimulation. This demonstrates that orally induced changes in pancreatobiliary secretions are CD36 dependent. Overall, this suggests that CD36 is sensing unsaturated LCFA in the oral cavity, and that this detection prepares the digestive system for incoming fat.

2.3.3.3 Involvement of the gustatory pathway

Experiments studying the role of the gustatory nerves in oral LCFA sensing have clarified that the CD36 lipid sensor is part of the gustatory pathway, as opposed to a tangential pathway of the textural or olfactory systems. Fungiform and circumvallate papillae are clusters of taste buds that express CD36, and are innervated by the chorda tympani and glossopharyngeal gustatory nerves, respectively (Fukuwatari, et al., 1997; Gaillard, et al., 2008; Laugerette, et al., 2005).

Bilateral transection of these nerves completely abolished the ability of the mice to distinguish between linoleic acid solution and the xanthan gum vehicle (Gaillard, et al., 2008). Whereas sham operated animals continued to have strong preferences for the FA solution over vehicle, demonstrating that the gustatory nerves are required to detect FA (Gaillard, et al., 2008). As expected, denervation also abolished the ability to distinguish sweet sucrose solutions and bitter denatonium solutions from water controls, while sham operated animals continued to show significant preferences for sweet solutions and avoidance of bitter solutions (Gaillard, et al., 2008). Cutting the gustatory nerves also significantly attenuated pancreatobiliary flux in esophagectomized mice following oral LCFA stimulation, compared to sham operated mice (Gaillard, et al., 2008). This demonstrates that the oral phase of lipid digestion is dependent on intact gustatory nerves.

Conditioned taste aversion tests showed that the gustatory nerves are required to recognize LCFA (Gaillard, et al., 2008). Previously, rats were conditioned to have a taste aversion to unsaturated LCFA in the micromolar range (McCormack, et al., 2006). More recently, mice were conditioned to avoid linoleic acid (Gaillard, et al., 2008). These mice continued to prefer sweet tastes, suggesting that conditioned recognition was specific to FA. Transection of gustatory nerves abolished the conditioned avoidance behaviour, implying that LCFAs are indeed gustatory cues (Gaillard, et al., 2008). Finally, depositing linoleic acid on the tongue of wildtype mice can elicit a response in the nucleus of the solitary tract, where taste signals are first received in the brain stem (Gaillard, et al., 2008). The same response, however, cannot be replicated in mice with a targeted deletion of CD36 (Gaillard, et al., 2008).

These observations provide support that oral CD36 requires gustatory nerves to sense LCFA and influence its actions.

2.3.4 Summary

CD36 is involved in the gustatory pathway (Gaillard, et al., 2008). It influences the oral phase of fat detection and directly affects fat intake in mice (Laugerette, et al., 2005; Sclafani, et al., 2007). Although there are differences in fat intake in humans, CD36 has not been explored as a potential determinant of this diversity. Genetic variation in proteins that receive taste stimuli may be responsible for some of the variability in food preferences and food intake (Garcia-Bailo et al., 2008). While common polymorphisms have been identified in CD36 (Ma, et al., 2004), it is not yet known whether these contribute to variation in the ability to detect fat or and subsequent variation in fat intake.

CHAPTER 3

RATIONALE, HYPOTHESIS,

AND OBJECTIVE

Rationale: The FFA sensor CD36 has recently been proposed as a putative 'fat taste' sensor (Laugerette, et al., 2005). CD36 is expressed on the taste bud cells of rodents and CD36-null mice consume less fat than their wildtype littermates (Laugerette, et al., 2005; Sclafani, et al., 2007). This suggests that CD36 FFA sensing is associated with fat intake. Although several common polymorphisms in the CD36 gene have been identified in humans (Ma, et al., 2004), the effects on habitual fat intake are not known.

Hypothesis: Common polymorphisms in the CD36 gene are associated with variation in habitual fat intake among humans.

Objective: To determine whether common variations (rs1984112 A>G, rs1761667 G>A, rs1527483 G>A, rs1049673 G>C) in the CD36 gene are associated with habitual fat intake in humans using a Caucasian and an Asian population.

CHAPTER 4

MATERIALS AND METHODS

4.1 Participants. Subjects are from the Toronto Nutrigenomics and Health Study. This is an ongoing cross-sectional study aimed at investigating gene-diet interactions on biomarkers of chronic disease in a young, healthy population. Females (n= 519) and males (n= 222) ranging in age from 20-29 years were recruited using University of Toronto advertising media, including postings, newspapers, internet and classroom announcements. Studying food preference and food intake in a young population is advantageous as orosensation can diminish with age (Duffy, 2007). Subjects were excluded if they were pregnant or breastfeeding because these life stages are associated with changes in metabolism and food intake. Subjects were also excluded if they could not communicate in English, as all questionnaires were only available in English.

For the purposes of these analyses participants were further excluded if their reported caloric intake was below 800kcal/day (n= 15 in females and n= 1 in males) or exceeding 3500kcal/day in females (n= 22) and 4000kcal/day in males (n=18) to avoid under and over-reporting, respectively. Females (n=33) and males (n=5) were also excluded if they self-reported being on a diet restricting carbohydrate, fat, or calories, or a diet that required fasting. One participant with Type I diabetes was also excluded. Participants were grouped by self-declared ethnoracial group as Caucasian, Asian, South Asian, or Other (all subjects that do not fit into one of the aforementioned groups). In these analyses only Caucasian (n= 387) and Asian (n= 259) groups were studied, because South Asian (n= 86) and Other (n= 61) had very small sample sizes. Consequently, 449 females and 197 males were included in these analyses. However in a few cases individuals could not be genotyped for a

polymorphism and those instances are noted in the *Results* section. Informed consent was obtained from all participants and the University of Toronto Research Ethics Board approved this study.

4.2 Study protocol. Subjects were screened for age during an initial phone call or e-mail. They were subsequently recruited to the study office to meet with a trained study coordinator whereupon informed written consent was obtained. During this visit subjects' height, weight, waist circumference and blood pressure were recorded. Subjects were instructed on how to complete 4 self-administered questionnaires: a 1-month food frequency questionnaire (FFQ), a food preference checklist (FPC), a general health and lifestyle questionnaire (GHLQ) and a physical activity questionnaire. Subjects were given a requisition for venous blood samples to be drawn at a MDS laboratory between 8:30 and 10:30am following a 12 hour overnight fast. Once questionnaires and blood samples were received by the study office subjects received $20 compensation for their participation.

4.3 Dietary assessment. The Toronto Nutrigenomics and Health Study uses a self administered semi-quantitative196-item food frequency questionnaire (FFQ) to measure habitual dietary intake over the past 1 month. The FFQ was modified, from the Willett FFQ, to improve dietary assessment of whole grains, fruits and vegetables, glycemic index, and caffeine. These include the addition of breads and cereals (6), fruits (6), vegetables (7), tree nuts (3), and drinks (4) (See Appendix).

Subjects were instructed on how to complete the FFQ and shown cups of standard portion sizes to help estimate their intake of each item over the last month. For most food items 9 possible responses were provided as options, ranging from never to 4 or more times per day. Open-ended questions allowed subjects to provide further information about brand names and other commonly consumed items that were not part of the questionnaire. Questionnaires were scanned and analyzed at Harvard University and incorporated into our database upon completion. Subjects' responses are converted into average grams of daily fat intakes, and can be sub-divided from total fat intake into type of fat and source of fat consumed. Energy from fat was computed from these data and total energy intake.

4.4 Anthropometrics. Height (to the nearest 0.1cm), weight (to the nearest 0.1kg), and waist circumference (to the nearest 0.1cm) were measured at time of recruitment and BMI (kg/m^2) was calculated. Resting blood pressure was measured twice and then averaged.

4.5 Physical activity questionnaire. MET (metabolic equivalent) level was used to measure usual physical activity over the past month. One MET is the energy required at rest, and is approximately equal to 1kcal/kg of body mass/hour at rest. This accounts for the intensity of activity, and thus includes contribution from light activity such as occupational activity, but excludes sleeping. A questionnaire was used to assess the number of hours per weekday and weekend day spent on light (2.3 MET), moderate (3.6 MET), and intense (7.5 MET) activity. MET-hours per

week is determined by tallying the total hours/activity x MET/activity x # of days/week (5 for weekdays and 2 for weekends).

4.6 Laboratory analyses. Venous blood samples were drawn after an overnight fast. Plasma FFA, triglycerides, total cholesterol, and HDL cholesterol were measured by MDS laboratories (Toronto, Canada). The Friedewald equation was used to calculate LDL cholesterol.

4.7 Genotyping. DNA was isolated from whole blood samples using a GenomicPrep Blood DNA Isolation kit (Amersham Pharmacia Biotech, Piscataway, NJ) or MasterPure Genomic DNA Purification kit (EPICENTRE, Cat No. MG71100). Real time - PCR was used to genotype subjects for 4 single nucleotide polymorphisms (SNPs) in CD36 including rs1984112 A>G, rs1761667 G>A, rs1527483 G>A, and rs1049673 G>C using the TaqMan allelic discrimination assays C___12093946_10, C___8314999_10, C___8315330_10, and C___8315317_20 respectively from Applied Biosystems (Foster, City, CA).

Approximately 20-200ng of DNA, 0.125ul of TaqMan 40x concentration assay, and 2.5ul of TaqMan Mastermix were used to amplify DNA sequences of interest on an ABI 7000 Sequence Detection System. PCR conditions were 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. The polymorphisms were detected using the probe sequences GTTA[A/G]GAAG (rs1984112 A>G), CAGC[A/G]CCAT (rs1761667 G>A), GGTC[A/G]ATTT (rs1527483 G>A), and TCCT[C/G]TTG (rs1049673 G>C), respectively.

4.8 Statistical analyses. Statistical analyses were performed using SAS Statistical Analysis Software version 9.1 (SAS Institute, Cary, NC). General linear models were used to examine differences across genotypes for continuous variables. The χ^2 test was used for categorical variables.

Nutrient (MUFA, PUFA, and protein) and other (weight, BMI, waist circumference, HDL, TG, and insulin) variables that were non-normally distributed were log transformed. Potential confounders in models examining nutrient intake included sex as a categorical variable, and BMI and physical activity as continuous variables. Nutrient density methods were used to create energy adjusted variables for dietary nutrient intake (Willet, 1998). Caucasians and Asians were examined separately because allele frequencies varied between the two populations. Previous work has also found that frequencies of CD36 genetic variants are different between ethnoracial populations (Curtis and Aster, 1996; Rac, et al., 2007).

Based on our findings for individual polymorphisms, SNP combinations were created with the two SNPs that were associated with percent energy from fat, rs1984112 A>G and rs1761667 G>A. The SNPs studied here have previously been used to identify 2 major linkage disequilibrium blocks in the CD36 gene (Ma, et al., 2004). Therefore, THESIAS software version 3.1 (Tregouet, Paris, France)(Tregouet and Garelle, 2007) was used to estimate haplotypes and conduct haplotype analyses. All the SNPs were examined for deviations from Hardy-Weinberg equilibrium based on a χ^2 test with 1 degree of freedom. Significant p-values are two-sided and ≤ 0.05.

CHAPTER 5

RESULTS

Table 1 shows the genotype frequencies for each of the SNPs for Caucasians and Asians. Similar frequencies as those for our Caucasian population have previously been reported for other Caucasian populations for these SNPs (Ma, et al., 2004; Madden, et al., 2008). Due to the small sample size of subjects homozygous AA for rs1527483 G>A (Caucasians n=2 and Asians n=15) this SNP was excluded from further analyses. Allele frequencies did not deviate from Hardy-Weinberg equilibrium for the population as a whole or for each ethnoracial group for any of the SNPs. Because one of our main variables of interest, total fat intake, was different in males and females we adjusted for sex for all nutrient analyses. Among Caucasians, total cholesterol differed for rs1049673 G>C. Among Asians, glucose differed for rs1049673 G>C. Remaining subject characteristics did not vary by genotype for the 3 SNPs (Table 2).

Among Caucasians, dietary intake expressed as grams/day did not differ significantly between the genotypes for any of the SNPs (Table 3). Energy intake and energy expenditure also did not differ. However, dietary intake expressed as percent (%) energy was significantly different. For rs1984112 A>G, % energy from total fat, MUFA, and PUFA differed between the genotypes, with the A allele being associated with lower energy coming from fat. Although energy from saturated fat followed a similar trend, it was not significant (p= 0.48). For rs1761667 G>A, % energy from total fat, MUFA, and PUFA also differed between the genotypes, with the A allele being associated with less energy coming from fat.

Table 1 Genotype frequencies as numbers and percents for each SNP by ethnoracial group.

	n (%)			
rs1984112 A>G	AA	AG	GG	Total
Caucasian	156 (40)	169 (44)	61 (16)	386 (100)
Asian	108 (42)	121 (47)	29 (11)	258 (100)
rs1761667 G>A	GG	GA	AA	
Caucasian	94 (24)	189 (49)	103 (27)	386 (100)
Asian	119 (46)	119 (46)	21 (8)	259 (100)
rs1049673 G>C	GG	GC	CC	
Caucasian	78 (20)	189 (49)	120 (31)	387 (100)
Asian	72 (28)	141 (54)	46 (18)	259 (100)
rs1527483 G>A	GG	GA	AA	
Caucasian	328 (85)	56 (14)	2 (1)	386 (100)
Asian	140 (55)	100 (39)	15 (6)	255 (100)

Subjects were classified based on self-identified ethnoracial group. For rs1984112, 1 Caucasian and 1 Asian subject could not be genotyped. For rs1761667, 1 Caucasian subject could not be genotyped. For rs1527483, 1 Caucasian and 4 Asian subjects could not be genotyped.

Table 2 General characteristics for Caucasians and Asians.

	rs1984112 A>G				rs1761667 G>A				rs1049673 G>C			
Caucasian	AA	AG	GG	p	GG	GA	AA	p	GG	GC	CC	p
n	156	169	61		94	189	103		78	189	120	
Age (y)	23.2 ± 2.4	23.1 ± 2.6	22.7 ± 2.2	0.47	23.0 ± 2.4	23.1 ± 2.5	23.2 ± 2.4	0.85	22.9 ± 2.5	23.2 ± 2.5	22.9 ± 2.3	0.54
Female (%)	100 (64)	120 (71)	45 (74)	0.25	69 (73)	132 (70)	64 (62)	0.20	60 (77)	128 (68)	78 (65)	0.19
BMI (kg/m²)	23.1 ± 3.3	23.0 ± 3.3	23.2 ± 3.7	0.89	23.1 ± 3.5	23.1 ± 3.3	23.1 ± 3.3	1.00	22.9 ± 3.4	23.1 ± 3.4	23.2 ± 3.2	0.76
WC (cm)	75.4 ± 8.5	74.0 ± 8.1	75.5 ± 8.9	0.85	75.2 ± 8.4	75.3 ± 8.3	75.2 ± 8.6	0.99	74.6 ± 8.0	75.5 ± 8.7	75.2 ± 8.1	0.73
TC (mmol/L)	4.1 ± 0.8	4.1 ± 0.8	4.1 ± 0.9	0.89	4.1 ± 0.8	4.1 ± 0.8	4.1 ± 0.8	0.82	4.1 ± 0.9 [a b]	4.0 ± 0.7 [a]	4.2 ± 0.8 [b]	**0.03**
LDL (mmol/L)	2.1 ± 0.6	2.1 ± 0.6	2.1 ± 0.7	0.96	2.1 ± 0.7	2.2 ± 0.6	2.1 ± 0.6	0.49	2.1 ± 0.7	2.1 ± 0.6	2.2 ± 0.7	0.20
HDL (mmol/L)	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.3	0.87	1.6 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	0.21	1.5 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	0.62
TG (mmol/L)	1.0 ± 0.5	1.0 ± 0.6	1.0 ± 0.4	0.75	0.9 ± 0.4	1.0 ± 0.6	1.0 ± 0.5	0.53	1.0 ± 0.4	0.9 ± 0.4	1.1 ± 0.7	0.10
FFA (µmol/L)	501 ± 372	483 ± 255	454 ± 230	0.64	477 ± 243	483 ± 260	497 ± 413	0.97	481 ± 233	495 ± 360	474 ± 243	0.93
Glucose (mmol/L)	4.7 ± 0.4	4.7 ± 0.3	4.7 ± 0.4	0.47	4.7 ± 0.4	4.7 ± 0.3	4.7 ± 0.3	0.34	4.7 ± 0.3	4.7 ± 0.3	4.7 ± 0.3	0.58
Insulin (pmol/L)	48.3 ± 30.5	45.9 ± 24.4	48.5 ± 38.0	0.73	47.7 ± 35.5	48.7 ± 29.8	44.4 ± 21.4	0.50	47.4 ± 26.2	48.5 ± 34.3	45.1 ± 22.1	0.87
Asian												
n	108	121	29		119	119	21		72	141	46	
Age (y)	22.3 ± 2.3	22.3 ± 2.2	22.1 ± 1.9	0.93	22.4 ± 2.3	22.1 ± 2.1	22.5 ± 2.5	0.64	22.3 ± 2.3	22.2 ± 2.2	22.4 ± 2.0	0.91
Female (%)	73 (68)	85 (70)	25 (86)	0.14	94 (79)	76 (64)	13 (62)	**0.02**	52 (72)	96 (68)	35 (76)	0.55
BMI (kg/m²)	21.4 ± 2.8	21.5 ± 2.6	21.4 ± 2.8	0.96	21.1 ± 2.5	21.8 ± 2.8	21.4 ± 2.4	0.11	21.5 ± 2.5	21.4 ± 2.8	21.4 ± 2.5	0.96
WC (cm)	70.4 ± 7.4	70.7 ± 6.7	69.4 ± 6.7	0.67	69.4 ± 5.9	71.5 ± 7.7	70.6 ± 7.8	0.08	70.9 ± 6.5	70.2 ± 7.4	70.5 ± 6.4	0.73
TC (mmol/L)	4.2 ± 0.8	4.2 ± 0.7	4.3 ± 0.6	0.52	4.2 ± 0.6	4.3 ± 0.8	4.1 ± 0.7	0.36	4.2 ± 0.7	4.2 ± 0.8	4.1 ± 0.7	0.67
LDL (mmol/L)	2.2 ± 0.6	2.1 ± 0.6	2.3 ± 0.6	0.46	2.1 ± 0.6	2.2 ± 0.7	2.1 ± 0.4	0.42	2.1 ± 0.6	2.2 ± 0.7	2.2 ± 0.6	0.92
HDL (mmol/L)	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.3	0.97	1.6 ± 0.4	1.6 ± 0.4	1.5 ± 0.2	0.57	1.6 ± 0.4	1.6 ± 0.4	1.5 ± 0.3	0.39
TG (mmol/L)	1.0 ± 0.7	1.0 ± 0.8	1.0 ± 0.4	0.79	0.9 ± 0.4	1.0 ± 0.8	1.1 ± 1.4	0.19	1.0 ± 0.8	1.0 ± 0.4	1.1 ± 1.2	0.89
FFA (µmol/L)	451 ± 208	501 ± 255	490 ± 173	0.19	478 ± 214	477 ± 244	498 ± 221	0.81	460 ± 175	482 ± 238	499 ± 270	0.98
Glucose (mmol/L)	4.8 ± 0.3	4.8 ± 0.4	4.8 ± 0.4	0.66	4.8 ± 0.4	4.8 ± 0.3	4.8 ± 0.2	0.24	4.7 ± 0.3 [a]	4.8 ± 0.3 [a]	4.9 ± 0.4 [b]	**0.005**
Insulin (pmol/L)	49.2 ± 38.2	54.2 ± 65.6	49.3 ± 23.6	0.75	51.7 ± 41.2	52.1 ± 63.8	46.7 ± 22.8	0.86	46.6 ± 28.5	49.6 ± 38.0	64.9 ± 96.6	0.08

Abbreviations: BMI= body mass index, WC= waist circumference, TC= total cholesterol, LDL= low density lipoprotein, HDL= high density lipoprotein, TG= triglyceride, FFA= free fatty acid. Values are means ± standard deviation. BMI, WC, HDL, TG, FFA, and insulin were log-transformed. P-values shown are for log-transformed data, whereas variable values are for non-transformed data. P-values are from χ^2 test for categorical variables and from generalized linear models for continuous variables.

Table 3 Dietary intakes for Caucasians and Asians.

	rs1984112 A>G				rs1761667 G>A				rs1049673 G>C			
Caucasian	AA	AG	GG	p	GG	GA	AA	p	GG	GC	CC	p
Fat (g/day)	66 ± 2	70 ± 2	73 ± 3	0.22	72 ± 3	69 ± 2	65 ± 3	0.20	69 ± 3	70 ± 2	66 ± 2	0.41
MUFA (g/day)	25.9 ± 1.0	28.0 ± 0.9	29.2 ± 1.5	0.16	28.5 ± 1.2	28.0 ± 0.9	25.3 ± 1.2	0.11	27.1 ± 1.4	28.1 ± 0.9	26.3 ± 1.1	0.30
PUFA (g/day)	12.1 ± 0.5	12.8 ± 0.5	13.6 ± 0.7	0.24	13.6 ± 0.6	12.6 ± 0.4	12.1 ± 0.6	0.23	12. ± 0.7	12.9 ± 0.4	12.2 ± 0.5	0.39
SFA (g/day)	22.2 ± 0.7	22.8 ± 0.7	23.1 ± 1.2	0.73	23.3 ± 0.9	22.7 ± 0.7	21.9 ± 0.9	0.54	22.5 ± 1.0	23.1 ± 0.7	21.9 ± 0.8	0.50
CHO (g/day)	278 ± 7	270 ± 7	262 ± 11	0.48	267 ± 9	271 ± 7	281 ± 9	0.52	261 ± 10	275 ± 7	275 ± 8	0.46
Protein (g/day)	86 ± 2	86 ± 2	86 ± 4	0.88	86 ± 3	85 ± 2	86 ± 3	0.94	86 ± 4	88 ± 2	83 ± 3	0.27
Fat (% energy)	29 ± 1 [a]	30 ± 0 [b]	31 ± 1 [b]	**0.01**	31 ± 1 [a]	30 ± 0 [a]	28 ± 1 [b]	**0.007**	30 ± 1	30 ± 0	29 ± 1	0.38
MUFA (% energy)	11.2 ± 0.3 [a]	12.1 ± 0.3 [b]	12.6 ± 0.4 [b]	**0.008**	12.2 ± 0.4 [a]	12.1 ± 0.3 [a]	10.9 ± 0.3 [b]	**0.005**	12.0 ± 0.4	11.9 ± 0.3	11.5 ± 0.3	0.45
PUFA % energy)	5.3 ± 0.1 [a]	5.5 ± 0.1 [ab]	5.9 ± 0.2 [b]	**0.02**	5.8 ± 0.2 [a]	5.4 ± 0.1 [ab]	5.2 ± 0.2 [b]	**0.05**	5.7 ± 0.2	5.5 ± 0.1	5.3 ± 0.2	0.24
SFA (% energy)	9.6 ± 0.2	9.9 ± 0.2	10.0 ± 0.3	0.48	10.1 ± 0.3	9.9 ± 0.2	9.5 ± 0.2	0.20	10.0 ± 0.3	9.8 ± 0.2	9.7 ± 0.2	0.68
CHO (% energy)	54 ± 1	52 ± 1	52 ± 1	0.15	52 ± 1	53 ± 1	54 ± 1	0.12	53 ± 1	53 ± 1	54 ± 1	0.25
Protein (% energy)	17 ± 0	17 ± 0	17 ± 0	0.96	17 ± 0	17 ± 0	17 ± 0	0.87	17 ± 0	17 ± 0	16 ± 0	0.65
Energy (kcal/day)	2066 ± 50	2069 ± 49	2056 ± 80	0.99	2070 ± 65	2065 ± 46	2074 ± 61	0.99	2014 ± 71	2105 ± 46	2042 ± 57	0.47
EE (kcal/day)	2925 ± 22	2925 ± 22	2972 ± 35	0.46	2957 ± 29	2909 ± 21	2933 ± 27	0.36	2970 ± 32	2925 ± 20	2921 ± 25	0.40
Asian												
Fat (g/day)	60 ± 2	62 ± 2	58 ± 5	0.64	62 ± 2	61 ± 2	56 ± 5	0.57	65 ± 3	61 ± 2	54 ± 4	0.07
MUFA (g/day)	23.1 ± 1.0	24.2 ± 1.0	22.0 ± 1.9	0.63	23.7 ± 1.0	23.6 ± 1.0	21.2 ± 2.2	0.59	25.2 ± 1.2	23.4 ± 0.9	20.9 ± 1.5	0.06
PUFA (g/day)	11.1 ± 0.5	11.9 ± 0.5	10.7 ± 1.0	0.80	11.8 ± 0.5	11.3 ± 0.5	10.6 ± 1.2	0.42	12.2 ± 0.7	11.5 ± 0.5	10.2 ± 0.8	0.12
SFA (g/day)	20.0 ± 0.8	19.9 ± 0.8	19.0 ± 1.5	0.83	20.1 ± 0.8	19.9 ± 0.8	18.2 ± 1.7	0.61	21.2 ± 1.0	19.8 ± 0.7	17.8 ± 1.2	0.08
CHO (g/day)	242 ± 9	250 ± 8	248 ± 17	0.81	241 ± 9	252 ± 8	239 ± 19	0.57	250 ± 11	245 ± 8	243 ± 13	0.88
Protein (g/day)	86 ± 3	86 ± 3	83 ± 6	0.96	88 ± 3	85 ± 3	81 ± 7	0.53	92 ± 4	85 ± 3	79 ± 5	0.12
Fat (% energy)	29 ± 1	29 ± 0	28 ± 1	0.45	30 ± 1	29 ± 0	29 ± 1	0.43	30 ± 1	29 ± 0	28 ± 1	0.07
MUFA (% energy)	11.3 ± 0.3	11.3 ± 0.2	10.6 ± 0.5	0.26	11.4 ± 0.3	11.1 ± 0.2	10.9 ± 0.6	0.71	11.6 ± 0.3	11.3 ± 0.2	10.5 ± 0.4	0.10
PUFA % energy)	5.4 ± 0.1	5.5 ± 0.1	5.3 ± 0.3	0.63	5.7 ± 0.1	5.3 ± 0.1	5.4 ± 0.3	0.15	5.6 ± 0.2	5.5 ± 0.1	5.2 ± 0.2	0.26
SFA (% energy)	9.7 ± 0.2	9.4 ± 0.2	9.1 ± 0.4	0.34	9.6 ± 0.2	9.5 ± 0.2	9.3 ± 0.4	0.89	9.6 ± 0.2	9.6 ± 0.2	9.0 ± 0.3	0.21
CHO (% energy)	52 ± 1	53 ± 1	54 ± 1	0.40	52 ± 1	54 ± 1	54 ± 2	0.17	52 ± 1 [a]	53 ± 1 [a]	55 ± 1 [b]	**0.03**
Protein (% energy)	19 ± 0	18 ± 0	18 ± 1	0.66	19 ± 0	18 ± 0	18 ± 1	0.12	19 ± 0	18 ± 0	18 ± 1	0.30
Energy (kcal/day)	1853 ± 62	1890 ± 59	1839 ± 120	0.87	1865 ± 62	1885 ± 59	1773 ± 136	0.75	1952 ± 76	1854 ± 54	1769 ± 94	0.28
EE (kcal/day)	2358 ± 19	2381 ± 19	2390 ± 38	0.60	2381 ± 20	2366 ± 18	2331 ± 43	0.54	2371 ± 24	2359 ± 17	2400 ± 30	0.47

Abbreviations: MUFA= Monounsaturated fat, PUFA= Polyunsaturated fat, SFA= Saturated fat, CHO= Carbohydrate, EE= Energy expenditure. Data are means ± standard error. MUFA, PUFA and protein were log-transformed. P-values shown are for log-transformed data, whereas variable values are for non-transformed data. All variables were adjusted for sex, BMI, and physical activity although this did not materially alter the results. P-values are from generalized linear models for continuous variables.

Although energy from saturated fat followed the same trend, it was not significant (p= 0.20). The decrease in energy coming from fat appeared to be largely at the expense of carbohydrates for both rs1984112 A>G and rs1761667 G>A. No significant differences were observed between genotypes for rs1049673 G>C for any of the nutrients (Table 3). Among Asians, dietary intake expressed as grams/day did not differ between genotypes. Only % energy from carbohydrate for rs1049673 G>C differed significantly. However, for rs1049673 G>C total fat intake expressed as grams/day and as % energy followed trends between genotypes that approached statistical significance (p=0.07 for both). MUFA expressed as grams/day showed a similar trend (p=0.06). Energy intake and energy expenditure also did not differ (Table 3).

Table 4 shows each of the possible genotypic combinations for rs1984112 A>G and rs1761667 G>A, and the combination frequencies for Caucasians and Asians. Among Caucasians, combination 5 (AG/GA) (n=142) was the most common followed by combination 3 (AA/AA) (n=103) and 7 (GG/GG) (n=60). Prior to analyzing genotype data, subjects with combination 3 and 7 were hypothesized to have the lowest and highest percent energy from fat, respectively, and were therefore combinations of special interest. Together these 2 combinations represent just over 40% of our Caucasian population. We predicted that the AG/GA combination would have a phenotype intermediate to these two. Nutrient intake was investigated for these 3 combinations (Table 5). Among Caucasians dietary intake in grams/day did not differ significantly between the combinations. Energy intake and energy expenditure also did not differ.

Table 4 Combinations and frequencies for Caucasians and Asians.

	rs1984112 A>G	rs1761667 G>A	**Caucasian** n (%)	**Asian**
Combination 1	AA	GG	6 (1.6)	28 (10.9)
Combination 2	AA	GA	46 (11.9)	61 (23.6)
Combination 3	AA	AA	103 (26.6)	19 (7.4)
Combination 4	AG	GG	27 (7.0)	62 (24.0)
Combination 5	AG	GA	142 (36.7)	58 (22.5)
Combination 6	AG	AA	-	1 (0.004)
Combination 7	GG	GG	60 (15.5)	29 (11.2)
Combination 8	GG	GA	1 (0.0025)	-
Combination 9	GG	AA	-	-

Two Caucasian subjects and 1 Asian subject did not fit into a combination because they were missing genotype data for one of the SNPs. Combinations that were not possessed by any subjects are indicated by - .

Table 5 Combination dietary characteristics for Caucasians and Asians.

	Caucasian				Asian			
	Combination 3	Combination 5	Combination 7	p	Combination 3	Combination 5	Combination 7	p
n (%)	103 (26.6)	142 (36.7)	60 (15.5)		19 (7)	58 (22.5)	29 (11)	
Fat (g/day)	65 ± 3	69 ± 2	72 ± 3	0.19	57 ± 6	62 ± 4	60 ± 5	0.77
MUFA (g/day)	25.0 ± 1.2	27.8 ± 1.0	29.0 ± 1.6	0.10	22.2 ± 2.7	24.4 ± 1.6	22.9 ± 2.3	0.91
PUFA (g/day)	11.9 ± 0.6	12.4 ± 0.5	13.5 ± 0.7	0.31	10.8 ± 1.4	11.8 ± 0.8	11.1 ± 1.2	0.81
SFA (g/day)	21.7 ± 0.9	22.4 ± 0.8	23.0 ± 1.2	0.65	18.6 ± 1.9	19.7 ± 1.1	19.4 ± 1.7	0.87
CHO (g/day)	278 ± 9	267 ± 8	258 ± 12	0.35	238 ± 23	255 ± 14	246 ± 20	0.79
Protein (g/day)	86 ± 3	85 ± 3	86 ± 4	0.91	82 ± 8	84 ± 5	85 ± 7	0.79
Fat (% energy)	28 ± 1 [a]	30 ± 1 [b]	32 ± 1 [b]	**0.004**	29 ± 1	29 ± 1	28 ± 1	0.94
MUFA (% energy)	10.9 ± 0.3 [a]	12.1 ± 0.3 [b]	12.7 ± 0.4 [b]	**0.002**	11.2 ± 0.6	11.1 ± 0.4	10.9 ± 0.6	0.77
PUFA % energy)	5.2 ± 0.2 [a]	5.4 ± 0.2 [a b]	5.9 ± 0.2 [b]	**0.03**	5.4 ± 0.3	5.4 ± 0.2	5.4 ± 0.3	0.99
SFA (% energy)	9.5 ± 0.2	9.9 ± 0.2	10.0 ± 0.3	0.31	9.4 ± 0.5	9.3 ± 0.3	9.2 ± 0.4	0.94
CHO (% energy)	54 ± 1	53 ± 1	52 ± 1	0.09	53 ± 2	54 ± 1	53 ± 1	0.75
Protein (% energy)	17 ± 0	17 ± 0	17 ± 0	0.08	18 ± 1	18 ± 0	18 ± 1	0.35
Energy (kcal/day)	2037 ± 62	1986 ± 53	1984 ± 82	0.80	1756 ± 159	1870 ± 91	1782 ± 129	0.76
EE (kcal/day)	2923 ± 74	2792 ± 63	2904 ± 97	0.35	2383 ± 152	2455± 87	2325 ± 123	0.68

Combination 3= rs1984112 A>G AA and rs1761667 G>A AA, combination 5= rs1984112 A>G AG and rs1761667 G>A GA, combination 7= rs1984112 A>G GG and rs1761667 G>A GG. Abbreviations: MUFA= Monounsaturated fat, PUFA= Polyunsaturated fat, SFA= Saturated fat, CHO= Carbohydrate, EE= Energy expenditure. Data are means ± standard error. MUFA, PUFA and protein were log-transformed. For these variables, p-values shown are for log-transformed data, whereas the variable values shown are for non-transformed data. N is the number of subjects with the stated combination, and % is the percent of the ethnoracial group that have the combination. All nutrient variables were adjusted for sex, BMI, and physical activity although this did not materially alter the results. Energy and EE were not adjusted. P-values are from generalized linear models for continuous variables.

However, dietary intake expressed as % energy was significantly different. As expected, AA/AA subjects consumed less energy from fat than GG/GG subjects. As with rs1984112 A>G and rs1761667 G>A individual SNP data this was observed for total fat, MUFA, and PUFA. As expected, subjects with the AG/GA combination had a greater % of energy from fat than subjects with the AA/AA combination and less % energy from fat than subjects with the GG/GG combination, however, this was only significantly different between the AG/GA and AA/AA for % energy from total fat and MUFA. Again, differences in fat intake appeared to be at the expense of carbohydrate. Protein intake did not differ between the combinations. Among Asians, no differences in dietary intake were observed between combinations. For both Caucasians and Asians no differences in general subject characteristics were observed between combinations (data not shown).

Based on the results from combination analyses, THESIAS software was used to analyze the relationship between nutrient intake and inferred haplotypes for Caucasians and Asians. Table 6 shows inferred haplotypes that were estimated to occur at a frequency of ≥5% in our population, based on our genotypic data. Haplotypes are shown in order as they were predicted to estimate energy from fat intake. All haplotype effects were analyzed relative to the reference haplotype, or most common haplotype. For Caucasians the reference haplotype was AAC, and for Asians it was AGG. Among Caucasians, dietary intake in grams/day was not significantly different for any of the haplotypes, compared to the reference haplotype. Energy intake and energy expenditure also did not differ.

Table 6 Estimated haplotypes and frequencies for Caucasians and Asians.

	rs1984112 A>G	rs1761667 G>A	rs1049673 G>C	Caucasian %	Asian
AAG	A	A	G	5	8
AAC	A	A	C	47	23
AGG	A	G	G	9	30
GGG	G	G	G	31	17
GGC	G	G	C	7	17

However, dietary intake expressed as % energy was significantly different between the GGC haplotype and the reference haplotype, AAC, for total fat and PUFA (Table 7). This was true after adjusting for multiple comparisons with Bonferroni correction ($p \leq 0.0125$). Energy from total fat ($p= 0.013$), MUFA ($p= 0.020$), and PUFA ($p= 0.017$) was close to significance for differences between the GGG and AAC haplotypes, but after Bonferroni correction was no longer significant. All of these observations were in the directions expected, such that AAG had the lowest energy from fat and GGC had the highest. Haplotype analyses suggested that increases in energy from fat may be at the expense of both carbohydrate and protein (Table 7). Among Asians, dietary intake in grams/day was not significantly different for any of the haplotypes, compared to the reference haplotype. However, % energy from MUFA and from carbohydrate were both significantly different between GGC haplotype and the reference haplotype, AGG.

Table 7 Estimated haplotype dietary characteristics for Caucasians and Asians.

Caucasian	AAG	AAC	AGG	GGG	GGC
Estimated n (%)	19 (5)	182 (47)*	35 (9)	120 (31)	27 (7)
Fat (% energy)	28 ± 1	30 ± 1 [a]	32 ± 1	32 ± 0	34 ± 1 [b]
MUFA (% energy)	10 ± 0.8	12 ± 0.5	13 ± 0.5	13 ± 0.3	14 ± 0.5
PUFA % energy)	6 ± 0.3	6 ± 0.2 [a]	6 ± 0.3	6 ± 0.1	7 ± 0.3 [b]
SFA (% energy)	9 ± 0.5	10 ± 0.3	11 ± 0.3	10 ± 0.2	10 ± 0.3
CHO (% energy)	54 ± 2	55 ± 1	53 ± 1	53 ± 1	53 ± 1
Protein (% energy)	15 ± 1	15 ± 1 [a]	14 ± 0	15 ± 0	12 ± 0 [b]
Asian					
Estimated n (%)	21(8)	60 (23)	78 (30)*	44 (17)	44 (17)
Fat (% energy)	32 ± 1	34 ± 1	36 ± 2	36 ± 1	32 ± 1
MUFA (% energy)	12.8 ± 0.7	14.0 ± 0.3	14.8 ± 0.8 [a]	14.6 ± 0.4	12.8 ± 0.4 [b]
PUFA % energy)	5.4 ± 0.4	5.4 ± 0.2	6.1 ± 0.4	6.0 ± 0.2	5.5 ± 0.2
SFA (% energy)	11.3 ± 0.5	11.7 ± 0.3	12.2 ± 0.6	11.7 ± 0.3	11.1 ± 0.3
CHO (% energy)	52 ± 2	50 ± 1	46 ± 2 [a]	48 ± 1	52 ± 1 [b]
Protein (% energy)	18 ± 1	18 ± 0	20 ± 1	19 ± 0	19 ± 0

Haplotypes are described in Table 6. Abbreviations: MUFA= Monounsaturated fat, PUFA= Polyunsaturated fat, SFA= Saturated fat, CHO= Carbohydrate. Data are means ± standard error. MUFA, PUFA and protein were log-transformed. For these variables, p-values shown are for log-transformed data, whereas the variable values shown are for non-transformed data. N is the number of subjects estimated to have the stated haplotype, and % is the estimated percent of the ethnoracial group that have the haplotype. All variables were adjusted for sex, BMI, and physical activity although this did not materially alter the results. The reference haplotype, is estimated to be the most common haplotype, and is indicated by *. For Caucasians, the reference haplotype is AAC, and for Asians it is AGG. Significant differences of <0.05 from the reference (REF) haplotype after Bonferroni adjustment for multiple comparisons are indicated with letters.

CHAPTER 6

DISCUSSION

These results show that genetic variation in CD36 is associated with differences in habitual dietary fat intake in a young Caucasian population. Carrying the G allele for both rs1984112 A>G and rs1761667 G>A was associated with a higher percent energy, though not total amount (grams/day), consumed from fat as measured using a 1-month food frequency questionnaire. These results indicate that CD36 may be involved in fat sensing and contribute to individual variation in fat intake. The variation in fat intake appears to largely be at the expense of carbohydrate. Similar results were not, however, observed in a young Asian population.

Previous work has shown that mice with a targeted deletion of CD36 failed to differentiate between diets with and without FFA, unlike wildtype littermates (Laugerette, et al., 2005). The inability to distinguish diets appeared to be specific to fat, as a preference for sweet and aversion to bitter was maintained. We found that subjects with the A allele (rs1984112 A>G and rs1761667 G>A) consumed less fat, and this appeared to largely be made up for by carbohydrate intake. Because the aforementioned CD36-null mice study did not test individual macronutrient intake we are unable to conclude whether these mice also adjusted their food intake accordingly (Laugerette, et al., 2005). The A allele polymorphisms (rs1984112 A>G and rs1761667 G>A) may represent a decreased function in CD36 that results in lower fat intake, similar to that seen in CD36-null mice. We hypothesize that this results in reduced acuity in fat sensing which translates to reduced fat intake. Alternatively, a higher sensitivity to sensing FFA may result in greater ability to recognize dietary fat and reduce intake accordingly. From an evolutionary

perspective, the ability to sense fat may have conferred a nutritional advantage, aiding in the selection of high energy foods and foods with essential fatty acids and fat-soluble vitamins. Taken together, these results suggest that CD36 is a fat sensor in both mice and humans.

Subjects with the A allele (rs1984112 A>G and rs1761667 G>A), which we have hypothesized may result in reduced CD36 function, would presumably still be able to detect fat by textural, olfactory, and post-ingestive cues. A recent study showed that CD36-null mice that had not previously been exposed to lipid, showed minimal preferences for emulsions with FFA or TG, compared to wildtype littermates (Sclafani, et al., 2007). However, at increasing concentrations of both FA and triglyceride, CD36-null mice exhibited increased fat preferences, though their total fat intake continued to be less than that of their wildtype littermates. The authors suggest that post-ingestive conditioning effects may in part be responsible for this 'rescued' phenotype (Sclafani, et al., 2007). In our study, subjects with genotypes that likely result in reduced CD36 functionality, may have part of their ability to sense fat restored by the post-ingestive conditioning effects of fat and other sensory cues. This theory could be tested by conducting fat threshold or intensity tests based on CD36 genotype, while minimizing the input from other cues by plugging the nostrils and having the subject compare the stimuli to texturally comparable ones. Such a short term experiment should also eliminate post-ingestive cues.

Previous work suggests that the ability of CD36 to detect fat is limited to FFA and selective for long chain unsaturated FA (Gilbertson, et al., 1997). Although it is likely that CD36 is sensing FA in multiple tissues, CD36 has been isolated on taste

bud cells (Fukuwatari, et al., 1997; Laugerette, et al., 2005). Short term intake tests, eliminating post-ingestive cues, suggest that oral fat detection is involved in dietary fat intake (Tsuruta, et al., 1999). We found that CD36 genotype is closely associated with dietary MUFA and PUFA intake, suggesting that sensing ability may be selective for these types of dietary fats. These observations are consistent with the protein's known affinities and selectiveness (Abumrad, et al., 1993; Baillie, et al., 1996; Gilbertson, et al., 1997). Although previous work has suggested that CD36 may be selective for FA with more double bonds, we found a stronger association between genotype and MUFA intake, compared to PUFA intake. This may be because our population consumed more MUFA than PUFA. It is also possible that foods containing MUFA also contained PUFA, and thus MUFA may be a marker of PUFA. It is possible that our FFQ may be better at capturing MUFA intake than PUFA intake in this population, or dietary PUFA may be misclassified as dietary MUFA. This potential failure to accurately capture PUFA intake may result in a conservative association between PUFA intake and CD36 genotype being reported here. Although not significant, consumption of saturated fat followed a trend suggesting intake may be associated with CD36 genotype. This may be because most of the saturated fat was coming from longer chain saturates, including stearic and palmitic fats. Previous work has shown that palmitic acid stimulated increases in intracellular calcium in CD36 positive taste bud cells on par with unsaturated LCFA (Gaillard, et al., 2008).

Previous work has studied the same polymorphisms in Caucasian populations, and found genotypic frequencies similar to those in our Caucasian

population (Ma, et al., 2004; Madden, et al., 2008). Ma et al. (2004) observed that rs1049673 G>C polymorphism was associated with higher FFA in males and females and that rs1984112 A>G and rs1761667 G>A were associated with higher FFA in males. In our population, we did not observe any significant differences in FFA levels. It is possible that we did not observe differences in FFA due to the younger age of our subjects. Another study examining the effect of CD36 deficiency on aerobic exercise used a study population closer in age to ours (Yanai et al., 2007). They found that although reduced CD36 functionality was associated with failure to reduce FFA levels at peak work rate, unlike subjects without reduced functionality, resting FFA levels were not different between the groups. That defective CD36 phenotype was physiologically significant, yet not apparent by resting FFA levels, suggests that we would not necessarily expect to see differences in fasting FFA levels in our population.

What is not understood is why our estimated haplotypes differed from those of Ma et al. (2004). Our combination analyses, which used actual genotypic data, as opposed to estimated haplotypes, indicate that some of the haplotypes observed by Ma et al. (2004) would only occur in substantial numbers if subjects who were heterozygotes for both rs1984112 A>G and rs1761667 G>A were inherited in certain patterns from their parents. One possible explanation is that our populations had different patterns of inheritance. Although most genotypic frequencies were similar, frequencies differed up to 12 % for the AA rs1984112 A>G genotype. Conversely, our genotypic frequencies are very similar to those found by Madden et al. in an American Caucasian population and did not differ by more than 3% for the GG

rs1527483 genotype (Madden, et al., 2008). This group, however, did not report genotype combinations or haplotypes.

The purpose of studying data by ethnoracial group was to account for possible cultural differences in food intake that may override genetic determinants of food selection. Failure to see the same associations between CD36 genotype and fat intake in Asians as we did in Caucasians could be for several reasons. For rs1984112 A>G, rs1761667 G>A, and rs1049673 G>C the frequency of the homozygote for the minor allele (GG, AA, and CC respectively) is less than that observed among the Caucasian population. In addition, the total sample size is smaller for Asians than Caucasians. This could mean that there are too few subjects in the minor allele homozygote group to observe statistically significant results. Among Asians, rs1049673 G>C was the polymorphism that had the largest number of subjects in the minor allele homozygote category (n=46). For this polymorphism, % energy from carbohydrate was significantly different between the genotypes, and fat intake expressed both in grams/day and % energy neared statistical significance (p=0.07 for both). Moreover, haplotype analyses found that % energy from MUFA and from carbohydrate was different between the GGC haplotype and the reference haplotype, AGG. Therefore, it is possible that genotype frequency and sample size limited our ability to observe statistically significant differences in fat intake between genotypes among Asians.

It is also possible that the polymorphisms studied here may not have the same functional impact in other ethnoracial groups. This could happen if other sensory cues such as smell and texture override gustatory cues. This may have

occurred if culturally specific food is more likely to stimulate texture or olfaction. Alternatively, if these food choices frequently couple fat with another taste modality, like sweet, there may be redundancy in the sensory mechanisms to detect fat. It is also possible that the regularity with which certain types of fat are encountered in the diet could be culturally affected and thus influence familiarity (Lermer and Mattes, 1999). Finally, it is possible that our FFQ did not capture habitual fat intake in Asians as well as it did for Caucasians. Although the Willett FFQ has been validated among Caucasians, it is recognized that its use may depend on ethnoracial factors (Holmes, et al., 2007). Our FFQ has been used to study the relationship between a genetic variant and carbohydrate intake in a multi-ethnic population (Eny et al., 2008). The same relationship was observed in a second population where dietary intake was measured using multiple food records (Eny, et al., 2008). Although this suggests that our FFQ can capture dietary intake among different ethnoracial groups, it is possible that the marked cultural variability in dietary fat intake (Arab, 2003) presents additional challenges in accurately measuring the intake of this nutrient across different ethnoracial groups.

The reliability of our FFQ to capture PUFA intake is evidenced by the concordance with PUFA intake in the Canadian trial of Carbohydrates in Diabetes (CCD) study, which used two 3-day food records administered 2 weeks apart (Fontaine-Bisson et al., 2008 (in press)). Using both methods of dietary assessment, a genetic variant modified the relationship between dietary PUFA and HDL cholesterol. This suggests that our results could be reproducible by measuring fat intake with 3-day food records. Additionally, the Willet, Block, and Diet History

Questionnaire have a high correlation for measuring energy adjusted fat intake, suggesting our results are independent of the type of FFQ used (Subar, et al., 2001).

Overall, these results demonstrate that variation in fat intake exists in this Caucasian population. Determining if there are health implications associated with this may require that the population be followed over the long term to measure changes in blood lipids and chronic disease relative to genotype and dietary composition.

6.1 EXPERIMENTAL CONSIDERATIONS AND FUTURE WORK

This work demonstrates that genetic variation in CD36 is associated with differences in % energy from fat. Previous work suggests that CD36 is an oral fat sensor in mice, and that functional changes in this sensor may be responsible for changes in fat intake (Laugerette, et al., 2005). From our work, however, we did not determine how genetic variation in CD36 influences oral fat sensing and how oral fat sensing, in turn, relates to fat intake. Thus, examining the relationship between inherited variations in CD36 with fat consumption and oral chemosensory response to fat may help identify individuals predisposed to prefer foods higher in dietary fat. Taste tests in the form of threshold detection tests, intensity tests, and preference tests may clarify genotype-phenotype relationships. In this way, we may be able to clarify whether people who are more sensitive to FFA are those who are consuming more or less energy from fat.

Although studies with knockout mice provide functional evidence that CD36 is an oral lipid sensor and expression studies demonstrate its presence in rodent taste buds, evidence of 'fat taste' mediated by CD36 in humans has until now been missing. Previous studies have shown that an oral fat stimulus can result in a greater release of TG into the plasma, indicating that there may be an oral fat sensor involved in fat metabolism (Mattes, 1996; Mattes, 2001; Mattes, 2001). A recent study investigating the effect of CD36 genotype on fish oil supplementation, found that variation in CD36 can is associated with differences in human fat metabolism (Madden, et al., 2008). In the mouse, an oral fat stimulus can trigger increases in protein content and flux of pancreatobiliary secretions, which indicates that an oral

fat sensor may be involved in fat metabolism in mice as well (Laugerette, et al., 2005). This change in secretion did not occur in CD36-null mice, indicating that CD36 is involved in this pathway. Therefore, future studies might consider further investigating the role of CD36 on human fat metabolism and the mechanism by which it is involved.

We detected this gene-diet association despite potential misclassification with the FFQ, suggesting the effect reported here may be more conservative than the actual relationship. Measuring dietary intake by another method, such as multiple day food records or a series of 24-hour recalls would confirm the reliability of the results reported here. However, we have some sense of the reliability of the FFQ used here to capture PUFA intake based on the concordance of gene-diet-biomarker associations, using 3-day food records to measure PUFA intake (Fontaine-Bisson, et al., 2008 (in press)). Studying the relationship between CD36 genotype and fat intake in another population would demonstrate the strength of this association. Finally, CD36 is a large gene with many variants (Rac, et al., 2007). For a more comprehensive analysis of how genetic variation in this gene is associated with dietary fat intake it may be necessary to consider a number of other variants as well.

6.2 IMPLICATIONS

Research investigating CD36 and fat taste is exciting and has potential to modify our traditional thoughts surrounding taste and food intake. However, this

work is still in its infancy and it would be premature to suggest what the impact may be here.

At this stage, our work has implications for genetic epidemiologists. There is growing interest in CD36 and its relationship with the development of the metabolic syndrome. Genome wide association studies have linked the chromosomal region in which the CD36 gene is located to characteristics of the metabolic syndrome (An, et al., 2005; Arya, et al., 2002). More recently, polymorphisms of the gene have been tied to HDL and TG levels (Love-Gregory, et al., 2008). Our work should serve as a caution in linking the CD36 gene directly to a disease outcome, as it is possible that the gene may simply be a marker of dietary preference. In a relevant example, genetic variation in the *TUB* gene that was originally linked to BMI was subsequently also linked to variation in macronutrient intake (van Vliet-Ostaptchouk, et al., 2008). This failure to recognize environmental factors, like diet, in genetic epidemiology is an area that needs to be improved. On the other hand, nutritional epidemiological studies associating a particular nutrient with a disease outcome may likewise be confounded by genetic variation that could directly affect metabolic pathways and influence health outcomes. Clarifying the interaction between diet and genes may improve our understanding of the etiology of complex diseases. In particular, clarifying how genes influence our diet may improve our understanding of food intake and related health outcomes.

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