Genetic variation in Innate Immunity, Diet and Biomarkers of the Metabolic Syndrome

by

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Abstract

Chronic low-grade inflammation is associated with the Metabolic Syndrome (MetS) and may contribute to its development. A diet high in saturated fat (SFA) has been associated with increased inflammation and development of the MetS. SFAs have been shown to elicit pro-inflammatory signaling through proteins of innate immunity, TLR4 and Nods 1 and 2. We determined whether common polymorphisms in the genes of these proteins could modify the association between fat intake and biomarkers of the MetS. Fat intake was measured using a food frequency questionnaire and genotyping was completed using real-time PCR. The TLR4 Asp299Gly (rs4986790) polymorphism was associated with decreased insulin sensitivity while an intronic polymorphism (rs5030728) modified the association between dietary SFA and HDL-cholesterol. The Nod1 Glu266Lys polymorphism modified the association between dietary SFA and HOMA-IR. These results suggest a role for innate immunity in mediating some of the effects of dietary SFAs on factors associated with the MetS.
Acknowledgments

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List of Abbreviations

**ABI** – Applied Biosystems Incorporated

**ANOVA** – Analysis of variance

**BMI** – Body mass index

**CARD** – Caspase recruitment domain

**COX-2** – Cyclooxygenase 2

**CRP** – C-reactive protein

**DHA** – Docosahexaenoic acid

**DNA** – Deoxyribonucleic acid

**E** – Energy

**EPA** – Eicosapentaenoic acid

**FFQ** – Food frequency questionnaire

**HDL** – High density lipoprotein

**HOMA-β** – Homeostasis model assessment of insulin resistance

**HOMA-IR** - Homeostasis model assessment of beta cell function

**hs-CRP** – high sensitivity C-reactive protein

**IL-6** – Interleukin - 6

**IL-8** – Interleukin – 8

**IκB** – Inhibitor kappa B
IMTG – Intramuscular triglycerides

IRS-1 – Insulin receptor substrate - 1

JNK – c-Jun N-amino-terminal kinase

LDL – Low density lipoprotein

LPS - lipopolysaccharide

LRR – Leucine rich repeat

MD-2 – Myeloid differentiation protein - 2

MDP - muramyldipeptide

MET – Metabolic equivalent

MetS – Metabolic syndrome

mRNA – Messenger ribonucleic acid

MUFA – Monounsaturated fatty acid

MyD88 - Myeloid differentiation protein 88

NACHT - NAIP (neuronal apoptosis inhibitor protein), C2TA HET-E TP1

NLR – Nucleotide binding and leucine rich repeat

NF-κB – Nuclear factor kappa B

Nod – Nucleotide binding oligomerization domain

OGTT – Oral glucose tolerance test

PAMP – Pathogen-associated molecular pattern

PBMC – Peripheral blood mononuclear cell
PCR – Polymerase chain reaction

PPAR – Peroxisome proliferators-activated receptor

PUFA – polyunsaturated fatty acid

RIP2 – Receptor interacting protein 2

SAS – Statistical analysis software

SE – Standard error

SEM – Standard error of the mean

SFA – Saturated fatty acid

SNP – Single nucleotide polymorphism

T2D – Type II diabetes

TG – Triglyceride

TIR – Toll-IL-1R domain

TIRAP – TIR domain containing adaptor protein

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor alpha

TRAM – TRIF related adaptor molecule

TRIF – TIR domain containing adaptor inducing interferon-beta
CHAPTER ONE:
Introduction
Global incidence of diabetes was estimated to be 171 million in the year 2000, and there were 2.9 million deaths from diabetes, making it the fifth leading cause of death worldwide (Roglic et al., 2005). In Canada, 1.3 million individuals were type II diabetics in 2000 (CDC, 1997; Ohinmaa, 2004). Similarly, incidence of the metabolic syndrome (MetS) has also seen an increase, paralleling that of diabetes (Ford et al., 2004), and its prevalence may be as high as 33% among Canadian adults (Ley et al., 2009). Both type II diabetes (T2D) and the MetS are polygenic diseases involving complex interactions with the environment (Uusitupa et al., 2000; Malecki, 2005), and in both conditions, obesity is accompanied by an underlying chronic low grade inflammatory state, which impairs insulin signaling (de Luca & Olefsky, 2008).

Modifiable lifestyle factors such as diet and exercise are important in the prevention and management of T2D and the MetS (Uusitupa et al., 2000; Gehling, 2001; Grundy, 2006). In fact, if implemented early, lifestyle modification has been found to be superior to drug intervention in its ability to delay risk-factor development and the need for drug therapies (Grundy, 2006). Multiple dietary strategies are used in both the prevention and management of these diseases (Gehling, 2001), including modifying fat intake. Insulin sensitivity is affected by the type of dietary fat, independent of its effects on body weight (Riccardi et al., 2004). Epidemiological evidence and intervention studies indicate that saturated fatty acids (SFAs) significantly worsen insulin-resistance, while polyunsaturated fatty acids (PUFAs) improve it (Riccardi et al., 2004). While a number of mechanisms are thought to mediate the effect of fatty acids on insulin sensitivity, one hypothesis is through modulation of inflammatory responses.
Recent research has shown that receptors of the innate immune system respond to fatty acids, in addition to bacterial components (Lee, 2003b; Zhao et al., 2007). SFAs can act as agonists of Toll-like receptor 4 (TLR4) signaling cascades, initiating a pro-inflammatory response, while PUFAs are antagonists of this pathway (Lee, 2003b). Similarly, SFAs have also been found to elicit Nucleotide binding Oligomerization Domain (Nod) mediated activation of pro-inflammatory signaling and PUFAs can inhibit this effect (Zhao et al., 2007). Genetic polymorphisms in these components of the innate immune system may affect the extent to which dietary fatty acids can initiate inflammatory responses and subsequent development of the MetS.

Polymorphisms in the TLR4 gene has been studied in relation to diabetes risk with varying results (Illig et al., 2003; Kolek et al., 2004; Buraczynska et al., 2009). The Asp299Gly polymorphism in the extracellular domain of the TLR4 receptor has been identified as less responsive to stimuli (Arbour et al., 2000). This polymorphism has also been associated with decreased risk for clinical diabetes in individuals without acute myocardial infarction undergoing coronary angiography. The minor allele was associated with a lower prevalence of diabetes, as 11% of carriers had diabetes compared to 18% of non-carriers (Kolek et al., 2004); however other studies show no protective benefit of this polymorphism (Illig, 2003; Buraczynska et al., 2009). Single nucleotide polymorphisms (SNPs) in the Nod1 and Nod2 genes also play a significant role in different inflammatory diseases, such as Crohn's disease (Molnar et al., 2007), and atopic diseases (Weidinger et al., 2005), resulting in differing pro-inflammatory activation. However, it is not known whether SNPs in these genes are associated with the MetS or its biomarkers.
While it is evident that both genetics and nutritional sciences have independently made contributions to understanding the causes of the MetS and their role in development of T2D, a more complete understanding can only be achieved by integrating these two experimental approaches to simultaneously assess the role of diet and genetic variation. Currently, it is unknown how TLR4 and Nods 1 and 2 interact with lifestyle interventions, in the risk and management of the MetS.
CHAPTER TWO:
Literature Review
2.1 Type II Diabetes and the Metabolic Syndrome

Projections estimate that the number of Canadians with diabetes will increase from approximately 1.3 million in 2000 to 2.4 million in 2016, with one of the highest increases occurring within Ontario. In addition, the cost of diabetes to the Canadian healthcare system was $4.66 billion in 2000, and is expected to increase to $8.14 billion by 2016 (Ohinmaa, 2004). T2D is also linked with a number of other conditions which together comprise the MetS. This clustering of symptoms includes insulin resistance, dyslipidemia and central obesity (Black, 2003) and diagnosis of MetS is based on the presence of three of the following: high blood glucose, low HDL cholesterol, elevated triglycerides, hypertension and abdominal obesity (Pradhan, 2007). Potential causes of this growing epidemic include changes in dietary patterns, physical inactivity and obesity, but may also include yet unidentified genetic and environmental determinants. Experimental data provide evidence for a direct link between obesity and subclinical inflammation and support the concept that the MetS and T2D are, in part, inflammatory conditions (Pradhan, 2007).

Subclinical inflammation, detectable by measurement of plasma-based inflammatory biomarkers, has been implicated in the development of insulin resistance, T2D and the MetS. One inflammatory cytokine in particular, tumor necrosis factor-alpha (TNF-α), can inhibit the insulin-stimulated glucose uptake and decrease the insulin-stimulated autophosphorylation of the insulin receptor and the phosphorylation of insulin receptor substrate 1 (IRS-1) (Hotamisligil, 1994). Inhibition of TNF-α in obese rats significantly improves insulin sensitivity (Hotamisligil, 1993). In humans, an inverse association has been found between TNF-α and whole body glucose disposal (Paolisso
et al., 1998) and TNF-α infusion inhibits peripheral insulin-dependent glucose uptake (Rask-Madsen, 2003).

Detection of inflammatory markers would be a beneficial clinical tool in the assessment of T2D and MetS risk (Pradhan, 2007) and studying factors which affect these inflammatory markers such as diet and genetics would give insight into disease pathology.

2.2 The role of dietary fat in insulin resistance

Insulin resistance is an important risk factor for both T2D and MetS, as its adverse effects on glucose homeostasis also represents a pathogenic link underlying the abnormality clustering of the MetS. Insulin sensitivity can be affected by different environmental factors, particularly different dietary fatty acids, which appear to influence insulin sensitivity independent of their effects on body weight (Riccardi et al., 2004). Evidence from animal experiments indicate that SFAs impair insulin action, whereas ω-3 PUFAs improve it and monounsaturated fatty acids (MUFA) and ω-6 PUFAs have a less negative effect on insulin sensitivity than SFAs (Storlien et al., 1991). In humans, higher SFA intake is associated with impaired insulin action. Cross sectional studies have found a positive association between SFA intake and hyperinsulinemia, independent of body fat (Maron et al., 1991; Mayer et al., 1993; Feskens et al., 1994; Marshall et al., 1997; Parker, 2002). Others have consistently found that increased unsaturated fatty acid levels, measured in the plasma or muscle, are associated with improved insulin sensitivity (Pelikanova et al., 1989; Borkman et al., 1993; Vessby et al., 1994; Pan et al., 1995).
Clinical studies performed in healthy individuals and obese type 2 diabetics have shown that a moderate substitution of SFAs in the diet with unsaturated fatty acids is able to significantly improve insulin sensitivity (Vessby et al., 2001; Summers et al., 2002). The largest intervention trial on this topic, the KANWU study, involved 162 healthy individuals from five countries (Vessby et al., 2001). Participants were randomly assigned to consume either high SFA or high MUFA diets without any change in other dietary constituents. A subset within each group was also given a fish oil supplement (3.6 g daily) or a placebo. Insulin sensitivity was significantly impaired in the SFA group but remained unchanged in the MUFA group. The fish oil group also experienced no change in insulin sensitivity, however, this effect is not consistent as others have shown a beneficial effect (Delarue et al., 1996). Supplementation with ω-3 PUFAs in premenopausal, non-diabetic females has been reported to decrease insulin response to an oral glucose load in those with a high level of inflammatory markers, while the effect was not significant in those with low inflammatory status (Browning, 2003). These results suggest that inflammation plays an important role in determining the effect of diet on T2D and the MetS and markers of inflammation are gaining significance in the treatment of these disorders (Athyros, 2008). Mechanisms linking the relationship between diet and inflammation still remain unknown, although it appears as though proteins of innate immunity may be playing a role.
2.3 Innate immunity and insulin resistance

The innate immune system is the first line of defense against microbial pathogens in humans (Le Bourhis et al., 2007). It constantly monitors the host’s environment and identifies foreign invading organisms. Recognition of a foreign entity involves interactions between microbial structural motifs and host receptors which will elicit a non-specific response to handle the pathogen (Litman, 2005). Recent evidence indicates that SFAs can be sensed by innate immunity and elicit a pro-inflammatory response. Such recognition occurs through Toll-like Receptor 4 (TLR4) which is found in myocytes, adipocytes and macrophages (Shi, 2006; Tsukumo et al., 2007; Radin et al., 2008), linking chronic low grade inflammation and dietary SFA with the MetS. Nucleotide blinding Oligomerization Domains (Nods) 1 and 2 are also part of the innate immune system shown to be activated in response to SFA in cell culture (Inohara et al., 1999; Zhao et al., 2007). Through these proteins of innate immunity, different fatty acids found in the circulation and intracellularly, could affect inflammation providing a mechanism for diet-induced insulin resistance.

2.3.1 Toll-like receptor 4

Toll-like receptors (TLRs) act to induce and regulate innate immune responses in mammals through recognition of conserved pathogen-associated molecular patterns (PAMPs). These PAMPs are structural motifs specific to microorganisms and include lipids, carbohydrates, nucleic acids and proteins (Medzhitov & Janeway, 1997; Aderem & Ulevitch, 2000; Akira et al., 2001; Akira, 2003). Although a direct interaction between potential ligands and receptors has still not been demonstrated (Lee & Hwang, 2006),
each TLR senses different PAMPs resulting in a variety of immunomodulatory responses accompanied by the expression of pro-inflammatory proteins.

TLR4 is a transmembrane receptor characterized by the presence of extracellular leucine-rich repeat motifs and a cytoplasmic Toll-interleukin-1 receptor (TIR) domain (Figure 2.1), required for the downstream activation of the pro-inflammatory transcription factor nuclear factor kappa-B (NFκB) (Medzhitov et al., 1997). Upon PAMP recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with TIR domains. There are four TIR domain containing adaptor proteins relevant to TLR4: MyD88, TIRAP, TRIF and TRAM. The first two of these molecules signal for pro-inflammatory cytokine production while the latter two signal for Type I interferon production (Lu, 2008) (Figure 2.1).

The ligand for TLR4 is lipopolysaccharide (LPS), found in the cell wall of gram negative bacteria (Medzhitov, 2001). Most of the biological activity of LPS is contained within the ‘Lipid A’ moiety, which is acylated with SFAs. This lipid component of LPS alone is sufficient to trigger TLR4 signaling, and removal of these acylated SFAs results in a complete loss of endotoxic activity (Munford & Hall, 1986; Kitchens et al., 1992). Lipid A containing unsaturated fatty acids does not elicit TLR4 signaling and acts as an antagonist to administered endotoxin (Krauss et al., 1989; Qureshi et al., 1991). This provides an intriguing situation whereby fatty acids are capable of affecting inflammatory response through TLR4.

In vitro studies have shown that TLR4 can respond to nonbacterial ligands such as SFAs and trigger pro-inflammatory responses (Lee, 2001; Lee, 2003a). In fact, incubation of isolated mouse macrophages with a mixture of oleate and palmitate, fatty
acids commonly found in the diet, can induce NFκB dependent TLR4 signaling and subsequent TNF-α and IL-6 up regulation. However, TLR4 deficient macrophages did not respond (Shi, 2006). In addition, the same study found that TLR4 knockdown in diet induced obesity control mice, substantially prevented fatty acid and LPS induced cytokine expression, measured through quantification of IL-6 and TNF-α mRNA (Shi, 2006).

Studies using TLR4 null mice have also shown TLR4 to be responsive to lipids. In vivo lipid infusion into wild type mice has shown that nuclear translocation and DNA binding of NFκB in adipose tissue of wild these mimicked that of LPS binding to TLR4. The lipid infusion also increased NFκB binding to the IL-6 promoter and subsequent stimulation of TNF-α and IL-6 (Shi, 2006). However, in TLR4 null mice these effects of lipid infusion were prevented (Shi, 2006).

TLR4 null mice on a high fat diet do not become insulin resistant and do not have impaired glucose turnover, insulin stimulated glucose uptake into skeletal muscle and adipose or impaired insulin stimulated glycolysis (Shi, 2006; Tsukumo et al., 2007). Administration of a TLR4 antagonist antibody to wild type mice prevents palmitate induced decreases in insulin stimulated glucose uptake and glycogen synthesis and also decreases the effects of LPS and palmitate on insulin signaling (Tsukumo et al., 2007).

Insulin signaling in these TLR4 null mice appears to be affected through prevention of improper phosphorylation of insulin signaling molecules. Control animals on a high fat diet show a significant increase in inhibitory serine phosphorylation of IRS-1 and a decrease in Akt phosphorylation in the liver, muscle and adipose tissue when
compared to TLR4 null mice (Shi, 2006; Tsukumo et al., 2007; Radin et al., 2008). TLR4 deficiency also prevented c-Jun amino-terminal kinase (JNK) phosphorylation and decreased IκB protein expression in livers, skeletal muscles and adipose tissues after consuming a high fat diet (Tsukumo et al., 2007). Administration of a TLR4 antagonist antibody to wild type mice also decreased palmitate induced IκBα degradation and JNK phosphorylation (Tsukumo et al., 2007). IκBα prevents NFκB action, and thus its degradation will increase NFκB activity and subsequent inflammatory effects. JNK is activated by inflammatory cytokines and associates with IRS-1, promoting phosphorylation of serine residues, thereby contributing to insulin resistance (Aguirre et al., 2000).

Conversely, PUFAs appear to antagonize both SFA and LPS-induced NFκB activation and the ω-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) appear to be the most potent inhibitors (Lee, 2003b). Human clinical studies have shown that fish oil supplementation of 18 g daily inhibited the effects of LPS on inflammation, measured by IL-2, IL-1 and TNF-α cytokine production (Endres, 1989; Endres et al., 1993). Monocytes isolated from human subjects receiving 9g of fish oil daily had suppressed expression of the inflammatory marker COX-2, which is also activated by TLR4, when exposed to LPS in vitro (Lee, 2003b). In addition, administration of fish oil also decreases the production of various cytokines, including TNF-α, in LPS stimulated monocytes (Endres, 1989; Endres et al., 1993). The target of PUFAs appear to be the actual TLR4 receptor itself or the events leading to TLR4 activation, as induction of the downstream signaling components could not be inhibited by DHA (Lee, 2003b).
While TLR4 may respond to extracellular fatty acids, the etiology of insulin resistance is also thought to involve elevated levels of metabolites of intramuscular triglycerides (IMTGs) (Corcoran et al., 2007). In a study of TLR4 null mouse skeletal muscle, the reversal of insulin resistance after a palmitate infusion was not complete and hypothesized to be due to a measured accumulation of IMTG metabolites (Radin et al., 2008). Fatty acids are rapidly released intracellularly by the action of various phospholipase A₂s and monoacylglycerol and diacylglycerol lipases in response to extracellular stimuli (Lee, 2001). As muscles also produce their own inflammatory cytokines when exposed to SFAs (Jove et al., 2005; Jove et al., 2006), there may be additional inflammatory mechanisms occurring intracellularly which respond to fatty acids.

High dietary intake of SFAs also encourage IMTG accumulation compared with unsaturated fatty acid–rich diets, however, the overall significance of this is still speculative (Corcoran et al., 2007). In humans, insulin resistance is directly correlated with increased SFAs in skeletal muscle triacylglycerols (Manco et al., 2000), while animal studies have shown that high SFA intake decreases PUFA and increases SFA levels in IMTG (Lee et al., 2006). High PUFA diets also decrease the proportion of all SFAs in IMTG compared with control and SFA feeding. Animals fed a high SFA diet also had increased insulin resistance despite similar weight gain, and PUFA intake appeared to protect animals from insulin resistance (Lee et al., 2006). The mechanism by which intramuscular triglyceride accumulation is associated with insulin resistance has not yet been elucidated and could also involve innate immunity.
Figure 2.1. Basic Structure of a Toll-like receptor.
Figure 2.2. TLR4 mediated signaling pathways. TLR4 signaling can be separated into MyD88-dependent (TIRAP and MyD88) or MyD88-Independent (TRAM and TRIF) pathways which mediate the activation of pro-inflammatory cytokine and Type I interferon genes, respectively.
2.3.2 Nucleotide binding domains (Nods) 1 and 2

Nucleotide-binding oligomerization domain (Nod) 1 and Nod2 are members of the Nod-like receptor (NLR) proteins, which recognize specific intracellular PAMPs. NLRs are a family of proteins characterized by their three part structure: a leucine rich repeat, which likely recognizes ligands, the NACHT domain and the N-terminal effector CARD domain which generates downstream signaling (Figure 3) (Le Bourhis et al., 2007). These proteins detect different muropeptides released from bacterial peptidoglycans and regulate pro-inflammatory responses by inducing the NFκB signaling pathway (Tattoli et al., 2007). Nod1 is ubiquitously expressed, while Nod2 is specific to monocytes, macrophages, dendritic cells and intestinal Paneth cells (Inohara et al., 2005). Upon ligand recognition, Nod1 and Nod2 undergo conformational changes and self-oligomerization, followed by recruitment of Rip2, a serine-threonine kinase (Tattoli et al., 2007). Oligomerization of Rip2 results in the activation of the IκB kinase complex which subsequently releases NFκB. Rip2 is also involved in the activation of the JNK pathway after Nod1 activation, although this pathway is not well characterized (Tattoli et al., 2007; Shaw et al., 2008) (Figure 4).

Recent evidence has shown that both Nod1 and Nod2 pathways are also activated by SFAs, whereas ω-3 PUFAs inhibit their activation (Zhao et al., 2007). In human colonic epithelial cells, stimulation of Nods by lauric acid (C12:0) activated NFκB and induced IL-8 in a dose dependent manner. In addition, ω-3 PUFAs were found to inhibit TNF-α induced NFκB transactivation through Nod proteins and also inhibited Nod oligomerization (Zhao, 2007). DHA and EPA were both found to be the most potent
inhibitors of the Nod2 ligand muramyl dipeptide (MDP) in eliciting IL-8 expression, as compared to other unsaturated fatty acids (Zhao et al., 2007).

As Nod proteins are found intracellularly and are able to initiate inflammation in response to SFAs, there is potential for Nod proteins to act as sensors for intracellular lipids and contribute to insulin resistance. Skeletal muscles exposed to SFAs produce TNF-α and IL-6 (Inohara et al., 1999; Zhao et al., 2007), however, no mechanism has been found to explain this occurrence. Given the role of increased intramuscular triglycerides in the etiology of insulin resistance, intramyocellular SFAs may interact with Nod1, similar to their effects on TLR4 extracellularly, thereby initiating immune pathways from within the muscle cell and could contribute to insulin resistance in that tissue.

Given the different cellular locations of TLR4 and Nod proteins, one would assume that the two function independently of each other; however, there is evidence to suggest both complementary and non-redundant functions between the two. Although TLR4 and Nod are stimulated by different bacterial agonists, both Nods1 and 2 and TLR4 utilize similar signaling pathways. In dendritic cells and monocytes, Nod1 and Nod2 agonists can synergize with TLR agonists to induce proinflammatory mediators. Specifically, Nods 1 and 2 synergize with TLR4 resulting in enhanced production of TNF-α, IL-6 and IL-8 (Fritz et al., 2005; Netea et al., 2005).
There are many probable signaling pathways that could be acting cooperatively to regulate TLR and Nod cross talk. Nod1 and Nod2 mRNA expression is induced by TLR activation and cytokines such as TNF-α (Rosenstiel et al., 2003). Positive feedback is evident after activation of TLRs and NLRs which can be blocked by anti-TNF-α treatment, indicating the involvement of autocrine signaling (Takahashi et al., 2006). Additionally, more evidence to support this cross-talk comes from the Nod signaling molecule RIP2. This protein kinase also binds to members of the TRAF family, such as TRAF6, which is involved in TLR signaling and RIP2 knockout mice show defective TLR4 signaling (Meylan & Tschopp, 2005). It has been suggested that RIP2 is involved in TLR inflammasome activation, making it a likely candidate for interconnection between these two pathways (Kufer & Sansonetti, 2007). However, the relevance of this relationship in SFA sensing is still unknown.

Further understanding of the relationship between diet and innate immunity in insulin resistance can be accomplished through integration of genetic approaches. A more complete understanding of the roles of both diet and genetics on development of the MetS can only be achieved by integrating these two fields simultaneously.
Figure 2.3 schematic representations of mammalian Nod1 and Nod2 proteins.
Figure 2.4 Overview of Nod activation and signaling.
2.4 Nutrigenomics and innate immunity in insulin resistance

Specific genetic polymorphisms have been found to influence the effect of dietary fatty acids on inflammation and subsequent insulin resistance in both healthy and type 2 diabetic populations (Fontaine-Bisson, 2007; Fontaine-Bisson & El-Sohemy, 2008). Single nucleotide polymorphisms (SNPs) in the tumor necrosis factor-alpha (TNF-α) gene affecting the rate of transcription and production of the pro-inflammatory cytokine, have been found to modify both high density lipoprotein (HDL) and apolipoprotein A-I (apo A-I) concentrations in response to dietary PUFA (Fontaine-Bisson, 2007; Fontaine-Bisson & El-Sohemy, 2008). TNF-α has been associated with an altered lipid profile, insulin resistance and increased risk of cardiovascular disease (Jovinge, 1998; Warne, 2003). Its transcription is also up regulated via induction by NFκB, which is activated by the innate immune system. Individual variation in the TLR4, Nod1 or Nod2 genes may affect the response of these proteins to dietary fatty acids in humans.

Epidemiological studies show that polymorphisms of TLRs decrease the susceptibility and tend to protect against the development of many chronic inflammatory diseases, including Alzheimer’s disease, atherosclerosis, diabetes and cancer. Two genetic variants (Asp299Gly, Thr399Ile) of the human TLR4 gene have been characterized as impairing inflammatory signal transduction (Arbour et al., 2000). Both SNPs result in missense mutations in exon 4, altering the extracellular domain of the TLR4 receptor and decreasing its responsiveness to stimuli (Arbour et al., 2000).
The Asp299Gly and Thr399Ile SNPs are common in humans but are also highly linked, cosegregating in 88-100% of study populations (Arbour et al., 2000; Kiechl et al., 2002; Boekholdt et al., 2003). Thr399Ile has also never been found independently of Asp299Gly and not usually studied for this reason (Kolek et al., 2004). Heterozygosity for the Asp299Gly has been associated with hyporesponsiveness to inhaled LPS as humans and cultured cells transfected with the SNP demonstrate reduced NFκB activity (Arbour et al., 2000). The presence of the Glycine allele has been reported to increase the risk of gram negative bacterial infection and increased risk of septic shock (Lorenz et al., 2002), also supporting hypofunctional activity. The SNP has also been found to decrease the risk of acute carotid atherosclerosis (Kiechl et al., 2002), modify the efficacy of statin therapy in patients with coronary artery disease (Boekholdt et al., 2003) and is associated with reduced prevalence of clinical diabetes (Kolek et al., 2004). The inflammatory marker, C-reactive protein (CRP), was also reduced in individuals carrying the Asp299Gly SNP (Kolek et al., 2004), however, levels of other inflammatory markers were not measured. X-ray crystallography has indicated that although the SNP is not directly involved in the binding of the LPS/MD-2 complex required for TLR4 signaling, the polymorphism is close to this binding area (Rallabhandi et al., 2006). The polymorphism may increase the rotational freedom of the peptide bond between TLR4 and the LPS/MD-2 complex and also changes the charge of this area from negative to neutral (Rallabhandi et al., 2006). The rs5030728 intronic SNP in TLR4 replaces a guanine with an adenine. This particular SNP has not been previously investigated for its role in risk for the MetS, however, it has a relatively high frequency and is in close proximity to exon 3, indicating that it could affect mRNA processing.
Variations in the Nod1 and Nod2 genes have been associated with diseases which also have altered inflammatory status. Some Nod2 polymorphisms have been associated with Crohn’s disease (Molnar et al., 2007), asthma or atopic eczema (Weidinger et al., 2005). The Ser268Pro polymorphism in Nod2 (rs2066842) replaces a thymine with a cytosine at position 907 in exon 4 of the Nod2 gene, however its functional effects are currently unknown. The Nod1 Glu266Lys (rs2075820) SNP affects the nucleotide binding domain of the protein. It replaces a guanine with an adenine at position 1319 in exon 6 of the Nod1 gene, and is a significant risk factor for irritable bowel syndrome in some populations and Helicobacter pylori induced duodenal ulcers. This lysine replacement for glutamine results in a slight decrease of the helix-formatting potential and may lead to an altered activation of NFκB (Molnar et al., 2007). It is not clear if any of these genetic polymorphisms also affect inflammation in metabolic tissues or whether they respond differently to dietary fatty acids to impact the development of the MetS.
Table 2.1 Polymorphisms in TLR4 and Nods 1 and 2 investigated in this thesis for their effects on biomarkers of the MetS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>SNP</th>
<th>Location</th>
<th>Base Change</th>
<th>Minor Allele Frequency (~%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLR4</strong></td>
<td>rs4986790 (Asp299Gly)</td>
<td>Exon 3, Chromosome 9</td>
<td>A &gt; G</td>
<td>4.6- 11.1% (Illig, 2003; Kolek et al., 2004; Henckaerts et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>rs5030728</td>
<td>Intron 2, Chromosome 9</td>
<td>G &gt; A</td>
<td>30- 34.7% (Watanabe et al., 2008)</td>
</tr>
<tr>
<td><strong>Nod1</strong></td>
<td>rs2075820 (Glu266Lys)</td>
<td>Exon 6, Chromosome 7</td>
<td>G &gt; A</td>
<td>19- 27.9% (Molnar et al., 2007; Van Limbergen et al., 2007; Watanabe et al., 2008)</td>
</tr>
<tr>
<td><strong>Nod2</strong></td>
<td>rs2066842 (Ser268Pro)</td>
<td>Exon 4, Chromosome 7</td>
<td>C &gt; T</td>
<td>31- 36% (Ince et al., 2008; Watanabe et al., 2008)</td>
</tr>
</tbody>
</table>
CHAPTER THREE:
Rationale, Hypothesis and Study Design
3.1 Rationale

The MetS and T2D are associated with a chronic low-grade inflammatory status which is thought to be mediated, in part, by innate immunity. Recently, TLR4, Nod1 and Nod2 proteins of the innate immune system have been found to detect SFAs resulting in pro-inflammatory signaling. TLR4 null mice fed a high fat diet do not develop insulin resistance as their wild-type littermates do, suggesting that innate immunity could mediate some of the effects of dietary lipids on biomarkers of the MetS. There are several common polymorphisms in each of these three genes, however, it is not known if modify the response to dietary lipids.

3.2 Hypothesis

Common polymorphisms in the TLR4, Nod1 and Nod2 genes modify the association between dietary fat and biomarkers of the MetS.
3.3 Objectives

The study used a candidate gene approach along with dietary assessment tools to examine the relationship between gene-diet interactions affecting innate immunity and biomarkers of the MetS. Specifically, we aimed to determine:

1. The association between Nod1, Nod2 and TLR4 genotypes and biomarkers of the MetS.

2. If Nod1, Nod2 and TLR4 genotypes modify the association between dietary fatty acid intake and biomarkers of the MetS.

3.4 Study Design

Subjects were participants of the Toronto Nutrigenomics and Health Study (n=1274), which is a cross-sectional examination of young men and women between 20-29 years of age recruited from the University of Toronto campus. This age category is optimal for studying gene-diet interactions because it avoids the growth spurt of younger individuals with rapidly changing diets, and it also avoids the confounding effects of older age on biomarkers of the MetS. Furthermore, the onset of the MetS seems to be occurring earlier in life, and this study will add to our understanding of the development of the MetS in early adulthood. Finally, by studying a multi-ethnic group we will be able to determine whether gene-diet interactions differ by ethnicity, and how such interactions affect components of the MetS. Participants for this study were recruited between Sept 2004 and August 2009. Women who were pregnant or breast feeding
were excluded from the study. The three major ethnocultural groups are Caucasian, Asian and South Asian. The study was approved by the Research Ethics Board at the University of Toronto and informed consent was obtained from all subjects.

Dietary intake was assessed using a 196-item semi-quantitative food frequency questionnaire (FFQ) and subjects also completed a general health and lifestyle questionnaire, a physical activity questionnaire and a 63-item food preference checklist. Overnight fasting blood samples were collected and serum has been stored at -80°C for measuring various biomarkers. DNA was isolated from whole blood using the GenomicPrep™ Blood DNA Isolation kit and genotyping will be conducted using real-time PCR.

In total, four common polymorphisms were genotyped. Through the analysis of the FFQ, the intake of total fat and specific types of fatty acids were examined for potential association with fasting serum glucose, insulin, HOMA-IR, HOMA-β, hs-CRP, HDL, LDL, triglycerides and free fatty acids, BMI and waist circumference. Genotype will then be incorporated into these relationships to determine whether the observed effects of diet on specific biomarkers are modified by genetic variation in the innate immune proteins previously described. The results of this study provide support for the role of innate immunity in mediating some effects of diet on biomarkers of the MetS.
CHAPTER FOUR:
Genetic polymorphisms in Toll-like receptor 4
4.1 Abstract

**Background:** Toll-like receptor 4 (TLR4) is a protein of the innate immune system implicated in mediating the effects of a high fat diet on inflammation and insulin resistance, both factors associated with T2D and the MetS. If such a relationship does exist, then genetic variation in this receptor could affect how biomarkers of the MetS respond to dietary fat.

**Objectives:** To determine the effects of two TLR4 polymorphisms (rs4986790 Asp299Gly and rs5030728 G>A) on factors associated with the MetS and to see if genotype modifies the relationship between dietary fat and these factors.

**Design:** The study involved healthy men and women between the ages of 20-29 from the Toronto Nutrigenomics and Health Study (n= 1274). Subjects completed a 1 month semi-quantitative food frequency questionnaire and gave fasting blood samples for determination of genotype and biomarkers of the metabolic syndrome.

**Results:** The Asp299Gly polymorphism in TLR4 was associated with increased insulin, HOMA-IR and HOMA-β (p= 0.03, 0.03, 0.02; one-way ANOVA) and family history of diabetes (p=0.0002, chi-square). The intronic polymorphism (rs5030728) modified the relationship between dietary saturated fatty acids (SFA) and HDL cholesterol (p for interaction=0.003). SFA intake was inversely associated with HDL cholesterol in individuals who were homozygous for the G allele (β = -0.015 ± 0.007 mmol/L, p=0.04) while a positive relationship was seen for heterozygotes (β = 0.025 ± 0.01 mmol/L, p=0.02). There was no association between dietary SFA and HDL cholesterol in
individuals who were homozygous for this polymorphism. No other effects were observed with other fatty acids or other components of the MetS.

**Conclusions:** The TLR4 Asp299Gly polymorphism was associated with certain factors of the MetS. The rs5030728 (G>A) modified the relationship between dietary SFA and HDL, suggesting that both diet and immunogenetics play a role in this condition.

### 4.2 Introduction

The metabolic syndrome (MetS) is an important risk factor for development of type 2 diabetes (T2D) and cardiovascular disease. Although the pathophysiology of this syndrome still remains unknown, accumulating evidence is suggesting that a subclinical inflammatory state may contribute to its development (Festa *et al.*, 2002; Wellen & Hotamisligil, 2005; Pradhan, 2007). Several genetic and lifestyle factors are also important to the development of the MetS (Giugliano *et al.*, 2008; Peeters *et al.*, 2008) and the two may interact to affect the condition.

Inflammation and the MetS are both affected by various environmental factors, including dietary habits (Basu *et al.*, 2006; Mann, 2006b). Consumption of a high saturated fat (SFA) diet is associated with higher concentrations of pro-inflammatory markers (King *et al.*, 2003; Basu *et al.*, 2006) and has also been shown to increase pro-inflammatory gene expression (van Dijk, 2009). Clinical trials have shown dietary SFA to negatively affect inflammation and other factors associated with the MetS (Vessby *et al.*, 2001; Baer *et al.*, 2004; Klein-Platat *et al.*, 2005), including HDL-cholesterol. Current recommendations suggest that no more than 10% of energy be from SFAs, mostly due
to their adverse effects on cholesterol levels (Uauy, 2009). Relative to other dietary fatty acids, high SFA intake has been shown to decrease HDL cholesterol (Adams et al., 2009). Mechanistically, there are many ways by which dietary fats could affect inflammation and the MetS, one of which could be through innate immunity.

Toll-like receptor 4 (TLR4) is a receptor of the innate immune system which responds to lipopolysaccharide (LPS) and results in NF-κB translocation to the nucleus (Miggin & O'Neill, 2006). It is ubiquitously expressed in the body, including macrophages as well as tissues involved in glucose homeostasis: the liver, muscle and adipose (Bes-Houtmann et al., 2007; Tsukumo et al., 2007). Recent evidence is suggesting that TLR4 signaling could be activated by saturated fatty acids (SFAs) (Lee et al., 2003a; Lee et al., 2003b), although this has been debated (Erridge & Samani, 2009). Most of the biological activity of LPS is supported by its lipid A moiety which is acetylated with SFAs. These SFAs are essential to LPS activity and when they are removed or replaced with unsaturated fatty acids, LPS loses its activity or could even act antagonistically (Krauss et al., 1989; Raetz, 1990; Kitchens et al., 1992).

Studies with TLR4 deficient mice indicate that TLR4 could mediate at least some of the effects of dietary fats on inflammation and insulin resistance (Shi et al., 2006; Poggi et al., 2007; Tsukumo et al., 2007; Davis et al., 2008), both important factors of the MetS. A study completed in mice showed a diet high in fat to be capable of TLR4 activation, subsequently leading to inflammatory cytokine production and insulin resistance (Shi et al., 2006). However, in the absence of TLR4, this effect was blunted. TLR4 null mice were also protected from the ability of a lipid infusion to suppress insulin signaling and insulin mediated changes in glucose metabolism (Shi et al., 2006).
Common polymorphisms in the gene encoding for TLR4 could affect susceptibility to factors of the MetS. The Asp299Gly (rs4986790) single nucleotide polymorphism (SNP) is common in most populations and replaces an adenine with a guanine at position 1187 in the TLR4 gene. X-ray crystallography has indicated that although the SNP is not directly involved in the binding of the LPS/MD-2 complex required for TLR4 signaling, the polymorphism is close to this region (Rallabhandi et al., 2006). The polymorphism may increase the rotational freedom of the peptide bond between TLR4 and the LPS/MD-2 complex and also changes the charge of this area from negative to neutral (Rallabhandi et al., 2006). Previous studies have indicated that Asp299Gly may be hypofunctional, resulting in a blunted response to LPS (Arbour et al., 2000), while others have found it to have no functional consequence (van der Graaf et al., 2005). Still others suggest that there may be a TLR4 haplotype that exists because the Asp299Gly SNP often cosegregates with another SNP, Thr399Ile, which could change the effect of Asp299Gly (Ferwerda et al., 2007). The rarity of this haplotype, however, makes it difficult to study. The Asp299Gly SNP has been associated with lower C-reactive protein (CRP) levels and reduced risk for diabetes and coronary artery disease (Kolek et al., 2004), however, the findings have been equivocal (Illig et al., 2003; Buraczynska et al., 2009). It is possible that the polymorphism interacts with other risk factors, such as diet, and this may explain some of the inconsistencies between studies.
In the present study, two common SNPs in TLR4 were examined. The first SNP investigated was the Asp299Gly polymorphism (rs4986790) and the second is an intronic SNP (rs5030728) replacing a guanine with an adenine. This intronic SNP has not been previously investigated for its role in risk for diabetes or the MetS, however, it was chosen based on its relatively high frequency and close proximity to exon 3, indicating that it could affect mRNA processing. The objective of this study was to determine whether these SNPs affected risk factors for the MetS and if they affected the relationship between dietary fat and these risk factors in a young, healthy population.

4.3 Methods

4.3.1 Study Population

Please refer to chapter 3 for general information regarding study population.

For the current analysis, individuals who may have under-reported (≤800 kcal/day) or over-reported (≥ 3500 kcal/day for female, ≥ 4000 kcal/day for male, n=39), those with diabetes, Crohn’s disease, ulcerative colitis or arthritis (n=14) and individuals with inflammation due to ‘external factors’ (n=54) were excluded from the analyses. These factors included elevated inflammatory levels, identified if an individual had CRP ≥ 10 mg/L, as this might represent undeclared acute illness. Individuals who reported infection, fever, dental surgery, or being tattooed or pierced within the week before their blood draw were also included in this group. Individuals who were missing data relevant to the MetS were also excluded (n=51). Finally, all individuals of East Asian descent were excluded (n=440) as the TLR4 polymorphisms studied were not
present in this ethnocultural group (i.e. minor allele frequency ≤ 1%). The final population consisted of 676 individuals. Written informed consent was obtained from all participants and the study was approved by the Ethics Review Committee at the University of Toronto.

4.3.2 Dietary Assessment

*Food Frequency Questionnaire.* A 196-item Toronto-modified Willett food-frequency questionnaire (FFQ) was used to assess habitual dietary intake over the past month. Each subject was instructed on how to complete the FFQ using visual aids of portion sizes to improve the accuracy of self-reported food intake. Subject responses to each food item were converted to daily number of servings for each. Total, saturated, monounsaturated and polyunsaturated fat intake was assigned to a serving of each food item based on the nutrient contents listed for the food in the US Department of Agriculture database.

4.3.3 Anthropometrics and Energy Expenditure

Anthropometric measurements including height, weight, and waist circumference were measured, and BMI (kg/m²) was calculated. Height was measured using a standard stadiometer and weight was measured using a platform scale. Subjects wore light clothing and no shoes for these measurements. Waist measurements were taken using a flexible measuring tape at the midpoint between lower border of the ribs and upper border of the pelvis. Modifiable activity was measured by questionnaire and a metabolic equivalent (MET) hours per day was calculated. This calculation represents both leisure and occupational activity, not including sedentary hours of sleeping or
sitting. One MET is equal to 1 kcal expended per kg body weight per hour sitting at rest (Ainsworth et al., 1993).

4.3.4 Laboratory Measurements

Each subject had venous blood drawn after a 12 hour overnight fast to measure biomarkers of glucose and lipid metabolism (glucose, insulin, total-, and HDL-cholesterol, triglycerides, and free fatty acids) and inflammation (hs-CRP) using standard clinical procedures.

4.3.5 Genotyping

DNA was isolated from whole blood using the GenomicPrep Blood DNA Isolation kit (Amersham Pharmacia Biotech, Piscataway, NJ). The two polymorphisms were detected using TaqMan® allelic discrimination assays (Asp299Gly ABI no. C__11722238_20; intron ABI no. C__26954831_10) from Applied Biosystems (Foster City, CA), with real-time PCR on an ABI 7000 Sequence Detection System. PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.
4.4 Statistical Analysis

All statistical analyses were performed using SAS version 9.1 1 (SAS Institute Inc, Cary, NC). The GLM procedure in SAS was used to perform a one-way analysis of variance to test for differences in the characteristics between genotypes (Asp/Asp vs. Gly carriers and G/G, A/G, A/A for the exonic and intronic SNPs, respectively) and the CONTRAST statement was used for pair-wise group comparisons. The Chi-squared test was used to analyze categorical variables (ethnicity, gender, smoking status, family history of diabetes). Non-normally distributed variables (BMI, waist circumference, free fatty acids, insulin, HOMA-IR, HOMA-β, triglycerides (TG), monounsaturated fat intake and polyunsaturated fat intake) were loge transformed for analysis and their antilogs are reported. Hs-CRP was transformed following a gamma distribution using the GENMOD procedure, and the median and interquartile range values for this variable are given. Any anthropometric, dietary or lifestyle variables that were significantly different across genotypes were adjusted for in analysis of metabolic characteristics and all subject characteristics are presented in Table 4.2.

The two TLR4 polymorphisms were not in linkage disequilibrium ($r^2 = 0.001$) and this was calculated using the HAPLOVIEW software package (Barrett et al., 2005). The frequency for the Gly/Gly genotype in TLR4 was rare (n=5) so it was combined with the Asp/Gly heterozygotes, creating a carrier group, in order to facilitate analysis. Homozygotes of the TLR4 intronic polymorphism were common and the SNP could be analyzed by its three genotypes.
The GLM procedure was also used to test whether the effect of dietary total, SFA, MUFA or PUFA as a continuous or categorical variable on different components of the MetS varied across the three genotypes for each polymorphism. A diet-gene interaction was found for dietary SFA and the TLR4 intronic SNP (rs5030728) on HDL. The model included HDL-cholesterol as the response variable and genotype, SFA and their interaction as the predictor variables. Covariates that were associated with the outcome and reduced the variance of the relationship between dietary SFA and HDL were included in the model. These covariates included TG, PUFA (% Energy), ethnocultural group, alcohol intake, energy intake, age and hs-CRP. Potential covariates that were associated with HDL or differed between genotypes were added individually to the model to test whether the interaction changed (protein intake, insulin, glucose, BMI, LDL-cholesterol, total fat and MUFA intake, fibre intake). These variables were not included in the final model. No differences or interactions were found between the TLR4 genotypes and any of these potential confounders. Dietary SFA was adjusted for total energy intake by using the nutrient density method (% of energy from dietary fatty acids) (Willet, 1998). Slopes of the three lines were estimated using the GLM procedure on a fully adjusted model including the genotype and genotype × SFA interaction terms. Departure of genotype distributions from Hardy–Weinberg equilibrium was assessed using a Chi-square test with 1 df and confirmed using the HAPLOVIEW software. Significant \( p \)-values are two-sided and less than 0.05. Tukey’s post-hoc test was used to correct for multiple comparisons when appropriate.
4.5 Results

Frequencies of the polymorphisms in each ethnocultural group are listed in Table 4.1. The minor allele frequencies in the total population without East Asians were 6.8% for the Asp299Gly polymorphism and 27.9% for the intronic G>A polymorphism (rs5030728). The distribution of both SNPs in the population were in Hardy-Weinberg equilibrium (p= 0.51 and p=0.60 for the Asp299Gly and intronic SNP, respectively). Differences in subject characteristics across the genotypes are listed in Table 4.2. Individuals who were carriers of the Gly allele were older, consumed more carbohydrates and less alcohol than Asp/Asp homozygotes in this population, and these variables, along with ethnicity, were controlled for when analyzing metabolic characteristics. Carriers of the Gly allele had significantly higher insulin, HOMA-IR, HOMA-β (p= 0.03, 0.03, 0.02; one-way ANOVA) and family history of diabetes (p=0.0002; chi-squared) compared to Asp/Asp homozygotes. Because of the high frequency of this polymorphism in South Asians, we also analyzed these variables by ethnocultural group and only family history of diabetes maintained its significance in Caucasians (p=0.004; chi-squared). The insulin and HOMA indices followed the same pattern within each ethnocultural group, however, did not reach significance, probably due to decreased sample size. Across the intronic SNP (rs5030728), differences in protein consumption were noted as the G/G homozygotes consumed significantly less protein than individuals with the A/A or A/G genotypes in this population. This variable as well as ethnicity were controlled for when analyzing metabolic characteristics and no differences were noted across the genotypes.
The TLR4 genotypes were tested to see if they modified the association between dietary fatty acids (total, saturated, monounsaturated and polyunsaturated) and any factors associated with the metabolic syndrome (waist circumference, triglycerides, HDL cholesterol, glucose, blood pressure, HOMA-IR and hs-CRP). As outlined in Table 4.3, a significant interaction was found between TLR4 intronic (rs5030728) genotypes and dietary SFA on serum HDL-concentrations ($p=0.003$, adjusted for TG, PUFA (% Energy), ethnocultural group, alcohol intake, energy intake, age and hs-CRP). Total protein intake, which was different between the genotypes, and other factors that were associated with HDL levels but did not reduce the variance of the relationship, were added individually to the model. These variables did not alter the results and were not included in the final model. Dietary SFA was inversely related to serum HDL concentrations in individuals homozygous for the G allele; however, a positive trend was found among heterozygotes. This positive relationship was significantly different from the G/G homozygotes ($p=0.001$) but not A/A homozygotes ($p=0.15$). The relationship between dietary SFA and HDL-cholesterol was not significant in A/A homozygotes and the slope of this relationship did not differ significantly from G/G homozygotes ($p=0.60$) or G/A heterozygotes ($p=0.15$). There were no further interactions with any of the other covariates in the model.

Dietary SFA intake was also divided according to dietary intake recommendations (≤10% of calories from SFA). The interaction was not significant when SFA intake was divided into a binary variable ($p=0.13$, adjusted for TG, PUFA (% Energy), ethnocultural group, alcohol intake, energy intake, age and hs-CRP). Contrasting those who consumed SFAs above the recommendations to those who met recommendations in each genotype, mean HDL-cholesterol concentrations were
significantly lower in G/G homozygotes consuming diets high in SFA (p=0.05). No differences were noted in the other two genotypes (G/A p= 0.35 and A/A p= 0.59, Figure 4.1).
## TABLE 4.1. TLR4 genotype frequency by ethnicity.

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Asp299Gly</th>
<th>Intronic (rs5030728)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asp/Asp</td>
<td>Asp/Gly + Gly/Gly</td>
</tr>
<tr>
<td>Caucasians</td>
<td>537 (89%)</td>
<td>66 (11%)</td>
</tr>
<tr>
<td>East Asians</td>
<td>435 (99%)</td>
<td>5 (1%)</td>
</tr>
<tr>
<td>South Asians</td>
<td>100 (74%)</td>
<td>35 (26%)</td>
</tr>
<tr>
<td>Other</td>
<td>84 (88%)</td>
<td>12 (12%)</td>
</tr>
</tbody>
</table>
### TABLE 4.2. Clinical and metabolic characteristics and dietary intake by Toll-like receptor 4 genotype

<table>
<thead>
<tr>
<th></th>
<th>Asp299Gly</th>
<th>Intronic (rs5030728)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asp/Asp</td>
<td>Asp/Gly + Gly/Gly</td>
</tr>
<tr>
<td><strong>Age (yrs)</strong></td>
<td>23.0 ± 0.1</td>
<td>22.4 ± 0.3</td>
</tr>
<tr>
<td><strong>Gender (% Female)</strong></td>
<td>67.0%</td>
<td>61.6%</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>23.3 ± 0.2</td>
<td>23.4 ± 0.4</td>
</tr>
<tr>
<td><strong>Smoking Status (%)</strong></td>
<td>Never</td>
<td>80.7</td>
</tr>
<tr>
<td></td>
<td>Past</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>8.7</td>
</tr>
<tr>
<td><strong>Physical Activity</strong></td>
<td></td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td><strong>Waist Circumference (cm)</strong></td>
<td>75.5 ± 0.5</td>
<td>76.1 ± 0.99</td>
</tr>
<tr>
<td><strong>Systolic Blood Pressure (mmHg)</strong></td>
<td>115.3 ± 0.6</td>
<td>116.4 ± 1.2</td>
</tr>
<tr>
<td><strong>Diastolic Blood Pressure (mmHg)</strong></td>
<td>69.5 ± 0.5</td>
<td>70.7 ± 0.9</td>
</tr>
<tr>
<td><strong>Total Cholesterol (mmol/L)</strong></td>
<td>4.2 ± 0.04</td>
<td>4.4 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>Value 1 ± Margin 1</td>
<td>Value 2 ± Margin 2</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/L)</td>
<td>2.3 ± 0.04</td>
<td>2.4 ± 0.07</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/L)</td>
<td>1.5 ± 0.02</td>
<td>1.5 ± 0.04</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.94 ± 0.03</td>
<td>1.03 ± 0.05</td>
</tr>
<tr>
<td>Free Fatty Acids (µmol/L)</td>
<td>470 ± 13.6</td>
<td>455 ± 26.6</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>0.5 ± 1.0</td>
<td>0.5 ± 1.2</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.8 ± 0.02</td>
<td>4.8 ± 0.04</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>52.0 ± 1.6</td>
<td>60.4 ± 3.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.6 ± 0.05</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>HOMA-ß</td>
<td>117.2 ± 4.2</td>
<td>137.5 ± 8.2</td>
</tr>
<tr>
<td>Family History Diabetes (%)</td>
<td>10.3</td>
<td>24.2</td>
</tr>
<tr>
<td>Energy Intake (kcal)</td>
<td>2019 ± 26.6</td>
<td>1926 ± 67.5</td>
</tr>
<tr>
<td>Total Fat (% Energy)</td>
<td>30.2 ± 0.3</td>
<td>29.1 ± 0.7</td>
</tr>
<tr>
<td>SFA (% Energy)</td>
<td>9.8 ± 0.1</td>
<td>9.6 ± 0.3</td>
</tr>
<tr>
<td>MUFA (% Energy)</td>
<td>12.1 ± 0.2</td>
<td>11.5 ± 0.4</td>
</tr>
<tr>
<td>PUFA (% Energy)</td>
<td>5.6 ± 0.07</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
<td>242.3 ± 5.5</td>
<td>233.0 ± 13.9</td>
</tr>
<tr>
<td>Carbohydrates (% Energy)</td>
<td>52.5 ± 0.4</td>
<td>54.5 ± 0.9</td>
</tr>
<tr>
<td>Fibre (g/100 carbohydrates)</td>
<td>9.4 ± 0.1</td>
<td>8.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td>Protein (% Energy)</td>
<td>16.9 ± 0.1</td>
<td>17.0 ± 0.4</td>
</tr>
<tr>
<td>Alcohol (g/d)</td>
<td>7.6 ± 0.4</td>
<td>4.7 ± 1.1</td>
</tr>
</tbody>
</table>

1 hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of beta cell function; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

2 Metabolic characteristics are adjusted for subject characteristics and dietary variables that were different between each genotype (ethnicity, age, carbohydrate intake and alcohol or ethnicity and protein intake). Metabolic characteristics include: waist circumference, systolic and diastolic blood pressures, total, LDL and HDL cholesterol, triglycerides, free fatty acids, hs-CRP, glucose, insulin, HOMA-IR and HOMA-β.

3 P values for differences between genotypes were obtained by using a one-way ANOVA, and the chi-square test was used to test for differences between genotypes in categorical variables.

4 median ± interquartile range given for hs-CRP.
TABLE 4.3
Association of SFA intake (% energy) and HDL-cholesterol (mmol/L) concentrations for TLR4 intronic (rs5030728) genotypes in a young, healthy population (n= 676)

<table>
<thead>
<tr>
<th>Genotypes (n)</th>
<th>HDL-Cholesterol ( \beta \pm SE )</th>
<th>P for Slope</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G (361)</td>
<td>-0.015 ± 0.007</td>
<td>0.04</td>
<td>0.005</td>
</tr>
<tr>
<td>G/A (260)</td>
<td>0.025 ± 0.01</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>A/A (55)</td>
<td>-0.014 ± 0.02</td>
<td>0.53</td>
<td></td>
</tr>
</tbody>
</table>

Values of \( \beta \) regression coefficients ± S.E. and were obtained using a general linear model adjusted for TG, PUFA (% Energy), ethnocultural group, alcohol intake, energy intake, age and hs-CRP. \( p \)-value for the slope between SFA intake and HDL cholesterol for each TLR4 genotype, and the effect of genotype, SFA intake and the interaction between the two in the model.
Figure 4.1

TLR4 intronic polymorphism (rs5030728) modifies the association between dietary SFA intake and fasting HDL-cholesterol concentrations (mmol/L) in a young, healthy population.

Values are means ± S.E.M. adjusted for TG, PUFA (% Energy), ethnocultural group, alcohol intake, energy intake, age and hs-CRP.

![Bar chart showing HDL-cholesterol levels by TLR4 intronic genotypes and SFA intake.](chart.png)
4.6 Discussion

The objective of this study was to determine whether two polymorphisms in TLR4 are associated with components of the MetS and whether they modify the association between diet and these components. The first SNP investigated was the Asp299Gly polymorphism, which has been found to cause hypo-responsiveness to lipopolysaccharide (LPS) in human alveolar macrophages and airway epithelial cells (Arbour et al., 2000). However, this result has not been consistent, as others have found no effect of this polymorphism in monocytes derived from blood (Erridge et al., 2003; von Aulock, 2003; van der Graaf et al., 2005) and even hyperresponsiveness in whole blood from individuals homozygous for the polymorphism (Ferwerda et al., 2007). The impact of this polymorphism on risk of diabetes and cardiovascular disease has also been inconsistent. The polymorphism has been associated with lower CRP levels and reduced odds of having diabetes and coronary artery disease in patients undergoing coronary angiography (Kolek et al., 2004). Others have found no effect on risk for diabetes (Illig et al., 2003; Buraczynska et al., 2009) or cardiovascular disease related pathogenicities (Norata et al., 2005). Only one study has found an increased risk of myocardial infarction, which was seen in male carriers of the Gly allele in a case control study (Edfeldt et al., 2004). Studies on the effects of the Asp299Gly SNP in TLR4 have been in populations that were older and had already developed the disease of interest. An advantage of the present study population is that it allows for the investigation in a young, healthy population to examine how the SNP could affect factors of the metabolic syndrome in individuals who have a low-inflammatory profile and are disease free.
We found that individuals who were carriers of the Gly allele had significantly higher serum insulin levels and higher HOMA-IR and HOMA-β indices, suggesting that they had lower insulin sensitivities compared to the Asp/Asp homozygotes. This is a novel finding since no studies have compared these biomarkers between genotypes for this polymorphism in a healthy population. If the Gly allele is hyporesponsive to LPS, then the ability of these individuals to detect and remove a pathogen would be decreased. Such has been seen, as the Gly allele has been associated with increased septic shock and gram negative infections (Lorenz et al., 2002). As LPS is capable of inducing insulin resistance (Cani et al., 2007), these increased levels of LPS could be continually stimulating the less functional TLR4 receptor for longer, eventually leading to increased insulin levels and decreased insulin sensitivity. We did not see an effect of genotype on inflammation as measured through hs-CRP, however, this is not a complete measurement of inflammatory status and other cytokines such as TNF-α or IL-6 may have yielded additional insight.

The second polymorphism in TLR4 was an intronic SNP (rs5030728) which has not previously been studied in regards to its effects on metabolic syndrome or its associated factors. While the polymorphism was not associated with differences in factors associated with the condition, it did modify the interaction between dietary SFA and serum HDL levels. An inverse association was observed in individuals who were homozygous for the G allele; however, a positive association between dietary SFA and HDL was observed in heterozygous. No significant relationship was observed in individuals homozygous for the A allele.
Stimulation of TLR4 by LPS has been shown to down regulate peroxisome proliferator-activated receptor γ (PPARγ) expression (Necela et al., 2008), an important nuclear factor involved in lipid metabolism (Akiyama et al., 2002; Babaev et al., 2005). PPARγ agonists can increase expression of ATP binding cassette transporter A1 (ABCA1), a major regulator of HDL biosynthesis (Chinetti et al., 2001). Furthermore, macrophages deficient in ABCA1 show increased cell surface expression of TLR4 and cytokine expression in response to LPS (Yvan-Charvet et al., 2008), suggesting communication between the two. This pathway provides a potential mechanism by which TLR4 could affect HDL levels. In individuals carrying the intronic polymorphism rs5030728, activation of TLR4 by LPS and SFAs could be affected thereby making TLR4 less capable of signaling PPARγ down regulation. If such is the case, these individuals would not respond the same to TLR4 agonists as those who are homozygous for the major allele.

While the ability for SFAs to directly activate TLR4 signaling has been debated (Lee et al., 2001; Lee et al., 2003b; Erridge & Samani, 2009), TLR4 clearly responds to dietary lipids (Shi et al., 2006; Poggi et al., 2007). After 22 weeks on a high fat diet, mice lacking functional TLR4 were found to have increased PPARγ expression in subcutaneous adipose tissue compared to control mice fed the same diet (Poggi et al., 2007). Numerous studies have also linked TLR4 to the effects of dietary fat on insulin resistance (Shi et al., 2006; Poggi et al., 2007; Tsukumo et al., 2007) and found mice that lack this receptor to be protected despite a high fat diet. The effects of a high SFA diet on cholesterol metabolism has not yet been investigated in TLR4 null animals.
Current recommendations from the World Health Organization indicate that dietary SFA should be restricted to no more than 10% of total energy intake because of potentially adverse effects on lipid metabolism and subsequent cardiovascular risk (Mann, 2006b). The effects of decreasing SFA intake on HDL cholesterol show a high degree of inter-individual variability (Schaefer et al., 1997). Our results suggest that genotype may be an important factor with regards to the effects of SFAs on HDL cholesterol and account, at least in part, for some of this individual variation regarding dietary SFA and HDL cholesterol. In the past, our lab has also shown polymorphisms in TNF-α and NFκB to affect the relationship between dietary PUFA and HDL cholesterol (Fontaine-Bisson et al., 2007a; Fontaine-Bisson et al., 2007b; Fontaine-Bisson et al., 2009). All these polymorphisms affect proteins involved in immunity, and in particular the inflammatory response, implicating a role for inflammation in the relationship between dietary fat and HDL-cholesterol. Future studies investigating the effects of combined polymorphisms are needed in order to clarify potential clinical implications of these genotype association studies in the relationships between dietary fats and HDL-cholesterol.

In summary, we found that polymorphisms in TLR4 affect factors related to the metabolic syndrome in a young, healthy population. The Asp299Gly polymorphism was associated with increased insulin, HOMA-IR, HOMA-β and family history of diabetes, while an intronic polymorphism (rs5030728) modified the relationship between dietary SFA and HDL-cholesterol. Further studies are needed to confirm these results in other populations as well as to establish mechanisms by which they may be occurring.
CHAPTER FIVE:
Genetic polymorphisms in Nucleotide binding oligomerization domains 1 and 2
5.1 Abstract

**Background:** The innate immune receptor Toll-like receptor 4 (TLR4) has been implicated in mediating some of the effects of dietary lipids on inflammation and type 2 diabetes. Similar to TLR4, the nucleotide binding oligomerization domains (Nods) 1 and 2 are also proteins of innate immunity, which can respond to lipids and initiate pro-inflammatory signaling. Because of the similarities between these proteins, it is possible that the Nod proteins can also affect factors related to type 2 diabetes and the metabolic syndrome.

**Objectives:** To determine the effect of Nods 1 and 2 genotypes (Nod1 Glu266Lys and Nod2 Ser268Pro) on factors associated with the metabolic syndrome (MetS). We also sought to determine if these genotypes interact with diet to modify the relationship between dietary lipids and factors associated with the MetS.

**Design:** Young, healthy men and women (n= 1274) between the ages of 20-29 were genotyped for both polymorphisms. They also completed a 1 month, semi-quantitative food frequency questionnaire and gave fasting blood samples to measure biomarkers associated with the metabolic syndrome.

**Results:** The Glu266Lys polymorphism in Nod1 was not associated with any of the biomarkers of the metabolic syndrome. However, the polymorphism did modify the association between dietary saturated fat (SFA) and insulin sensitivity, as measured by HOMA-IR (p for interaction = 0.04). Individuals who were Glu/Glu homozygous or Glu/Lys heterozygotes showed no significant relationship between dietary SFA and
HOMA-IR, while those who were Lys/Lys homozygotes showed a positive relationship between dietary SFA and HOMA-IR ($\beta = 0.033 \pm 0.02$, $p=0.03$). No additional effects were found with other dietary fatty acids or other factors associated with the metabolic syndrome. The Nod2 Ser268Pro polymorphism was not associated with components of the metabolic syndrome and did not modify the relationship between dietary lipids and any of the metabolic syndrome biomarkers.

**Conclusions:** The Nod1 Glu266Lys polymorphism modified the relationship between dietary SFA and HOMA-IR, suggesting that Nod1 may act as an intracellular lipid sensor affecting insulin sensitivity.

### 5.2 Introduction

Although the mechanisms by which the metabolic syndrome (MetS) and type 2 diabetes (T2D) develop are still unknown, low-grade inflammation seems to play a role in promoting the development of these conditions (Wellen & Hotamisligil, 2005; Pradhan, 2007). Dietary fat and genetics can both influence one’s inflammatory status and contribute to developments of these disorders (Mann, 2006a; Peeters *et al.*, 2008), suggesting common pathways which may involve these factors.

Toll-like receptor 4 (TLR4) is a receptor of innate immunity that initiates NFkB mediated inflammatory signaling in response to lipopolysaccharide (LPS) (Medzhitov, 2001). TLR4 plays a role in high fat diet induced insulin resistance as mice lacking a functional TLR4 show blunted inflammation and do not develop insulin resistance despite a high fat diet (Shi *et al.*, 2006; Tsukumo *et al.*, 2007). This effect may also be
specific to dietary saturated fatty acids (SFAs), as mice deficient in TLR4 did not
develop obesity or increased insulin levels on a diet high in SFAs, but did on an
isocaloric diet high in polyunsaturated fatty acids (PUFAs) (Davis et al., 2008). We have
also observed that polymorphisms in TLR4 affect components of the metabolic
syndrome and also modify the association between dietary SFA and HDL-cholesterol
levels (Chapter 4). However, the absence of TLR4 has been found to not be entirely
sufficient to completely block the effects of lipids on insulin action, suggesting that TLR4
is not the sole pathway by which lipids affect insulin sensitivity (Radin et al., 2008).

In addition to toll-like receptors, the nucleotide-binding oligomerization domains
(Nods) 1 and 2 are cytosolic proteins of innate immunity (Girardin et al., 2003a). The
Nod proteins play a role in innate immunity detecting peptidoglycan structures. Nod1
detects structures unique to gram negative bacteria, while Nod2 detects structures
found in both gram negative and positive bacteria (Girardin et al., 2003a; Girardin et al.,
2003b). Similar to TLR4, Nods recognize these structures through the leucine rich
repeat domain at the carboxy terminus (Chamaillard et al., 2003; Inohara & Nunez,
2003), and activate common NFκB pathways (Inohara et al., 2000; Abbott et al., 2004).
Both types of innate immune proteins have been shown to be modulated by fatty acids
(Lee et al., 2003b; Zhao et al., 2007), although this has recently been debated (Erridge
& Samani, 2009). Using colonic epithelial cells free of TLR4, Zhao et al. (Zhao et al.,
2007), showed that SFAs dose dependently activated NFκB and increased IL-8
expression through the Nod proteins. Conversely, PUFAs inhibited these effects also
through the Nod proteins.
Polymorphisms in the Nod proteins have been implicated in a number of inflammatory diseases. Single nucleotide polymorphisms (SNPs) in Nod1 have been associated with increased risk for atopic eczema, asthma, elevated levels of immunoglobulin E (Hysi et al., 2005; Weidinger et al., 2005) and inflammatory bowel disease in some populations (McGovern, 2005). SNPs in Nod2 have been associated with Crohn’s disease and ulcerative colitis (Hugot et al., 2001; Ogura et al., 2001a), Blau disease (Miceli-Richard et al., 2001) and early onset sarcoidosis (Kanazawa et al., 2005). Despite the emerging role of innate immunity in the MetS and T2D, the relationship between polymorphisms in Nod proteins and risk of these conditions has not yet been studied.

The objective of the present study was to investigate whether SNPs in the Nod proteins could affect risk factors for the metabolic syndrome and whether they modified the relationship between diet and factors associated with the metabolic syndrome. Polymorphisms chosen had a relatively high frequency in the general population and exonic SNPs were chosen as they result in amino acids changes that could affect function of the protein. The Glu266Lys polymorphism in Nod1 (rs2075820) replaces a guanine with an adenine at position 1319 in exon 6 of the Nod1 gene, while the Pro268Ser polymorphism in Nod2 (rs2066842) replaces a thymine with a cytosine at position 907 in exon 4 of the Nod2 gene. The effects of these SNPs were investigated in a young, healthy population.
5.3 Methods

Please see Chapters 3 and 4 for details regarding methods.

5.3.1 Genotyping

DNA was isolated from whole blood using the GenomicPrep Blood DNA Isolation kit (Amersham Pharmacia Biotech, Piscataway, NJ). The two polymorphisms were detected using TaqMan® allelic discrimination assays (Glu266Lys ABI no. C___2641989_10; Pro268Ser ABI no. C__11717470_20) from Applied Biosystems (Foster City, CA), with real-time PCR on an ABI 7000 Sequence Detection System. PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.

5.4 Statistical Analysis

All statistical analyses were performed using SAS version 9.11 (SAS Institute Inc, Cary, NC). The SAS procedure GLM was used to perform a one-way analysis of variance to test for differences in the characteristics between genotypes (Glu/Glu, Glu/Lys and Lys/Lys; Pro/Pro and Ser carriers for the Nod1 and Nod2 SNPs, respectively) and the CONTRAST statement used for pair-wise group comparisons. The Chi-squared test was used to analyze categorical variables (ethnicity, gender, smoking status, family history of diabetes). Non-normally distributed variables (BMI, waist circumference, free fatty acids, insulin, HOMA-IR, HOMA-β, triglycerides (TG), monounsaturated fat intake (MUFA) and polyunsaturated fat intake (PUFA)) were loge
transformed for analysis and their antilogs are reported. Hs-CRP was transformed following a gamma distribution using the GENMOD procedure, and the median and interquartile range values for this variable are given. Any anthropometric, dietary and lifestyle variables that were significantly different across genotypes were adjusted for in analysis of metabolic characteristics and all subject characteristics are presented in Table 2.

The Nod1 Glu266Lys polymorphism was common and present in all ethnocultural groups and was analyzed by its three genotypes without exclusion of any cultural groups. The frequency of the Ser allele in Nod2 was low in East Asians (n=8, 2%) so they were excluded from Nod2 Ser268Pro analysis. The Pro/Pro genotype was also rare in the entire population (n=22) so they were combined with the Ser/Pro heterozygotes, creating a carrier group, in order to facilitate analysis.

The GLM procedure was also used to test whether the effect of dietary total, SFA, MUFA or PUFA as a continuous or categorical variable on different components of the MetS varied across the three genotypes for each polymorphism. A diet-gene interaction was found for dietary SFA and the Nod1 Glu266Lys (rs2075820) on HOMA-IR. The model included HOMA-IR as the response variable and genotype, SFA and their interaction as the predictor variables. Covariates that were significantly associated with the outcome and reduced the variance of the relationship were included in the model (Fibre intake, HDL:Total cholesterol ratio, CRP, waist circumference). Potential covariates that were associated with the outcome or differed between genotypes were added individually to the model to test whether the interaction changed (age, alcohol intake, ethnocultural group, energy intake, MUFA intake, triglycerides and free fatty
acids). These variables were not included in the final model. Dietary SFA was adjusted for total energy intake by using the nutrient density method (% of energy from dietary fatty acids) (Willett, 1998). Slopes of the three lines were estimated using the GLM procedure on a fully adjusted model including the genotype and genotype × SFA interaction terms. No interactions were found between Nod1 genotype and any of these potential confounders. Departure of genotype distributions from Hardy–Weinberg equilibrium was assessed using a Chi-square test with 1 d.f. Significant p-values are two-sided and less than 0.05. Tukey’s post-hoc test was used to correct for multiple comparisons when appropriate.

5.5 Results

5.5.1 Nod1

The allele frequencies for the Nod1 Gly266Lys SNP were 48% for the Glu/Glu, 43% for the Glu/Lys and 9% for the Lys/Lys. The minor allele (Lys) was present in 30% of the total population. Distribution of the polymorphism was significantly different between ethnicities (p=0.003, Chi-squared test) as the polymorphism was less frequent in Caucasians when compared to the other groups (Table 5.1). Genotype frequency in the total population did not deviate from Hardy-Weinberg equilibrium (p=0.87).

The Glu266Lys polymorphism was associated with differences in total cholesterol levels, which was mediated through LDL (Table 5.2). Carriers of the Lys allele had significantly lower total and LDL-cholesterol levels compared to the Glu/Glu
homozygotes (p=0.004 and p=0.003, respectively), although the difference was small and probably not biologically significant in this population. None of the other metabolic characteristics differed between genotypes.

The polymorphism in Nod1 modified the association between dietary SFA intake and HOMA-IR, a calculated measurement of insulin sensitivity. A significant diet x gene interaction between SFA intake and the Glu266Lys polymorphism on HOMA-IR (p=0.041, adjusted for dietary fibre, total:HDL cholesterol, waist circumference and hs-CRP). Adjustment for alcohol intake and ethnicity, which differed significantly across the genotypes, did not change the interaction (p=0.041). Further adjustment for variables that were associated with the outcome did not alter the results. A positive association between SFA intake (% Energy) and HOMA-IR was only seen in individuals who were homozygous for the Lys allele (Table 5.3), while no significant relationship was present in the other genotypes. The slope of the relationship between SFA and HOMA-IR in Lys/Lys individuals was 0.033 ± 0.02 (p=0.03) and was significantly different from the slope of the other two genotypes (p=0.03 for Glu/Glu and p=0.02 for Glu/Lys). The slopes of this relationship in the other two genotypes (Glu/Glu and Glu/Lys) were not significantly different from each other (p=0.81). This effect did not differ between the different ethnocultural groups (p=0.22 for the three way interaction between genotype x SFA x ethnocultural group). No diet x gene interaction was found for any of the other factors associated with the metabolic syndrome (waist circumference, TG, HDL, glucose, systolic and diastolic blood pressure and hs-CRP).

SFA intake was divided into a binary variable according to recommended intake levels (≤ 10% energy intake) in order to bypass assumptions of linearity (Figure 5.1).
The interaction approached significance when this method was used (p= 0.08, adjusted for dietary fibre, total:HDL cholesterol, waist circumference and hs-CRP). Contrasting the highest and lowest intakes of SFA by genotype, mean HOMA-IR was significantly higher in individuals consuming above recommended levels of SFA in the Lys/Lys genotype (p=0.02). There were no difference in HOMA-IR levels according to SFA intake in the other two genotypes (p= 0.55 for Glu/Glu and p=0.55 for Glu/Lys).

5.5.2 Nod2

The frequency of the Ser268Pro genotypes in this population were 75% for Ser/Ser, 22% Ser/Pro and 3% Pro/Pro. Because of the rarity of the Pro/Pro genotype, these individuals were combined with the Ser/Pro genotype to create a Pro carrier group. When analyzed by ethnocultural group (table 5.1), the frequency the Pro allele was very low in East Asians (2%) so this group was omitted from the population to avoid cultural confounding in the Ser/Ser genotype. When this was done, the frequency of the Ser allele was 20% in the total population. The frequency of this polymorphism was in Hardy-Weinberg equilibrium when East Asians were excluded (p=0.29).

The Ser268Pro polymorphism in Nod2 was not associated with differences in any factors related to the metabolic syndrome. It also did not modify the relationship between any dietary fat and these factors (waist circumference, TG, HDL, glucose, systolic and diastolic blood pressure, hs-CRP and HOMA-IR).
**TABLE 5.1** Nod 1 and Nod2 genotype frequencies by ethnicity.

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Nod1 Glu266Lys</th>
<th></th>
<th></th>
<th>Nod2 Pro268Ser</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glu/Glu</td>
<td>Glu/Lys</td>
<td>Lys/Lys</td>
<td>Pro/Pro</td>
<td>Pro/Ser +</td>
<td>Ser/Ser</td>
</tr>
<tr>
<td>Caucasians, n (%)</td>
<td>314 (55)</td>
<td>210 (37)</td>
<td>44 (8)</td>
<td>211 (57)</td>
<td>244 (43)</td>
<td></td>
</tr>
<tr>
<td>East Asians, n (%)</td>
<td>178 (44)</td>
<td>186 (46)</td>
<td>44 (10)</td>
<td>398 (98)</td>
<td>9 (2)</td>
<td></td>
</tr>
<tr>
<td>South Asians, n (%)</td>
<td>49 (41)</td>
<td>57 (47)</td>
<td>15 (12)</td>
<td>95 (79)</td>
<td>26 (21)</td>
<td></td>
</tr>
<tr>
<td>Other, n (%)</td>
<td>34 (38)</td>
<td>42 (47)</td>
<td>13 (15)</td>
<td>67 (75)</td>
<td>22 (25)</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 5.2 Clinical and metabolic characteristics and dietary intake by Nod1 and Nod2 genotypes\(^1,2\)

<table>
<thead>
<tr>
<th></th>
<th>Nod1 Glu266Lys</th>
<th>Nod2 Ser268Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glu/Glu (n=478)</td>
<td>Glu/Lys (n=427)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>22.8 ± 0.1</td>
<td>22.3 ± 0.1</td>
</tr>
<tr>
<td>Gender (% Female)</td>
<td>70.7%</td>
<td>67.9%</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>22.7 ± 0.1</td>
<td>22.5 ± 0.2</td>
</tr>
<tr>
<td>Smoking Status (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>83.1%</td>
<td>85.7%</td>
</tr>
<tr>
<td>Past</td>
<td>9.8%</td>
<td>7.0%</td>
</tr>
<tr>
<td>Present</td>
<td>7.1%</td>
<td>7.3%</td>
</tr>
<tr>
<td>Physical Activity (MET(\cdot)h/week)</td>
<td>7.7 ± 0.1</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>74.1 ± 0.5</td>
<td>73.9 ± 0.5</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>113.5 ± 0.6</td>
<td>115.1 ± 0.6</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>68.5 ± 0.4</td>
<td>69.7 ± 0.4</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>4.3 ± 0.04(^a)</td>
<td>4.2 ± 0.04(^b)</td>
</tr>
<tr>
<td>Variable</td>
<td>Group 1</td>
<td>Group 2</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/L)</td>
<td>2.3 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/L)</td>
<td>1.5 ± 0.02</td>
<td>1.5 ± 0.02</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.95 ± 0.02</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td>Free Fatty Acids (µmol/L)</td>
<td>477.8 ± 13.3</td>
<td>470.0 ± 13.5</td>
</tr>
<tr>
<td>hs-CRP (mg/L)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.4 ± 0.7</td>
<td>0.3 ± 0.7</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.8 ± 0.02</td>
<td>4.8 ± 0.02</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>50.5 ± 1.5</td>
<td>51.6 ± 1.5</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.5 ± 0.05</td>
<td>1.6 ± 0.05</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>116.4 ± 3.8</td>
<td>115.2 ± 3.8</td>
</tr>
<tr>
<td>Family History Diabetes (%)</td>
<td>11.5%</td>
<td>11.5%</td>
</tr>
<tr>
<td>Energy Intake (kcal)</td>
<td>1915 ± 29.3</td>
<td>1989 ± 31.0</td>
</tr>
<tr>
<td>Total Fat (% Energy)</td>
<td>30.0 ± 0.3</td>
<td>29.5 ± 0.3</td>
</tr>
<tr>
<td>SFA (% Energy)</td>
<td>9.8 ± 0.1</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>MUFA (% Energy)</td>
<td>11.8 ± 0.2</td>
<td>11.5 ± 0.2</td>
</tr>
<tr>
<td>PUFA (% Energy)</td>
<td>5.6 ± 0.08</td>
<td>5.6 ± 0.08</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
<td>241.4 ± 5.9</td>
<td>253.7 ± 6.3</td>
</tr>
<tr>
<td>Carbohydrates (% Energy)</td>
<td>52.9 ± 0.4</td>
<td>53.2 ± 0.4</td>
</tr>
</tbody>
</table>
Fibre (g/100g carbohydrates)  | 9.1 ± 0.1 | 8.9 ± 0.2 | 8.9 ± 0.3 | 0.58 | 9.1 ± 0.2 | 9.3 ± 0.2 | 0.52
Protein (% Energy)        | 17.1 ± 0.2 | 17.5 ± 0.2 | 17.4 ± 0.4 | 0.28 | 16.8 ± 0.2 | 16.8 ± 0.3 | 0.87
Alcohol (g/d)             | 6.0 ± 0.4<sup>a</sup> | 5.2 ± 0.4<sup>b</sup> | 5.5 ± 0.95<sup>ab</sup> | 0.05 | 6.7 ± 0.6 | 8.1 ± 0.8 | 0.03

1 hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of beta cell function; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

2 Metabolic characteristics are adjusted for subject characteristics and dietary variables that were different between each genotype (ethnicity and alcohol intake for Nod1 or ethnicity alcohol intake and smoking status for Nod2). Metabolic characteristics include: waist circumference, systolic and diastolic blood pressures, total, LDL and HDL cholesterol, triglycerides, free fatty acids, hs-CRP, glucose, insulin, HOMA-IR and HOMA-β.

3 <i>P</i> values for differences between genotypes were obtained by using a one-way ANOVA for continuous variables with Tukey’s post-hoc test when appropriate. The chi-square test was used to test for differences between genotypes in categorical variables.

4 median ± interquartile range given for hs-CRP.
TABLE 5.3

Association of SFA intake (% energy) and HOMA-IR for Nod1 Glu266Lys (rs2075820) genotypes in a young, healthy population (n= 998)

<table>
<thead>
<tr>
<th>Genotypes (n)</th>
<th>β ± SE</th>
<th>P for Slope</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu/Glu (478)</td>
<td>-0.002 ± 0.006</td>
<td>0.77</td>
<td>0.11 0.07 0.041</td>
</tr>
<tr>
<td>Glu/Lys (427)</td>
<td>-0.003 ± 0.006</td>
<td>0.61</td>
<td>0.03</td>
</tr>
<tr>
<td>Lys/Lys (93)</td>
<td>0.033 ± 0.02</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

Values of β regression coefficients ± S.E. and were obtained using a general linear model adjusted for fibre intake (% carbohydrates), Total:HDL cholesterol ratio, waist circumference and hs-CRP. p-value for the slope between SFA intake and HOMA-IR for each Nod1 genotype, and the effect of genotype, SFA intake and the interaction between the two in the model.
FIGURE 5.1

Nod1 Glu266Lys polymorphism (rs2075820) modifies the association between dietary SFA intake and HOMA-IR in a young, healthy population.

Values are means ± S.E.M. adjusted for fibre intake (% carbohydrates), Total:HDL cholesterol ratio, waist circumference and hs-CRP

1.1
5.6 Discussion

The objective of this study was to investigate whether polymorphisms in Nod1 (Glu266Lys) or Nod2 (Ser268Pro) were associated with differences in factors of the MetS and whether it modified the association between diet and these factors. The Glu266Lys SNP in Nod1 was found to modify the association between dietary SFA and insulin sensitivity, measured through HOMA-IR. There was a positive relationship between dietary SFA and HOMA-IR in individuals who were homozygous (Lys/Lys) for the polymorphism, while no relationship existed in the other genotypes.

The effect of the Glu266Lys polymorphism on Nod1 functionality is not known, however it appears as though it may affect lipid response. Nod1 has been found to respond to lipids (Zhao et al., 2007), eliciting a pro-inflammatory response to SFAs. Higher intakes of SFA could be promoting Nod1 signaling, either directly or indirectly, which would lead to an NFκB mediated inflammatory response. Another innate immune protein, Toll-like receptor 4 (TLR4), has been implicated in development of T2D as a result of a high fat diet (Shi et al., 2006). Both Nod1 and TLR4 share similar structures and signaling pathways. Cross-talk between TLRs and Nod proteins has also been observed, as expression of Nod1 and Nod2 mRNA is induced by TLR activation (Rosenstiel et al., 2003; Takahashi et al., 2006). While TLR4 provides a possible role of innate immunity in linking dietary fat to insulin resistance, it does not consider intracellular fatty acids, which strongly correlate with insulin resistance (Pan et al., 1997).
Epidemiological evidence suggests that the consumption of a Western diet, high in SFAs, correlates with the development of insulin resistance (Maron et al., 1991; Parker et al., 1993) and in vitro research in myocytes has indicated that SFAs can cause insulin resistance (Montell et al., 2001; Chavez & Summers, 2003). Animal studies have also shown that a high SFA diet can promote the accumulation of intramuscular lipids, which have been highly associated with insulin resistance (Lee et al., 2006). In humans, insulin resistance directly correlates with increased SFAs in skeletal muscle triglycerides (Manco et al., 2000). Mechanisms by which this may be occurring include the inhibitory effects of these lipid metabolites on the insulin receptor, however, skeletal muscles exposed to SFAs can also produce TNF-α and IL-6 (Jove et al., 2005; Jove et al., 2006), but no mechanism has been found to explain this occurrence.

Nod1 is found in glucose metabolizing tissues, including muscle cells (Inohara et al., 1999), liver and adipose (Dharancy et al., 2009). Thus, there is a potential that in these cells, particularly muscle cells, Nod1 could act as sensors for intracellular fats and lead to impaired insulin sensitivity. In order to affect Nod1, SFAs must be transported into the cell or incorporated into cell membrane phospholipids. Dietary fat intake can change the lipid constituents of the plasma membrane to reflect fat intake (Clandinin et al., 1991), Nod1 associated with the plasma membrane might be able to sense these lipids upon their release by phospholipases. Additionally, transport of fatty acids from the circulation into the cell by fatty acid transporters could allow cytosolic Nod1 to be exposed to lipids as well. However, whether the pro-inflammatory response from Nod1 affects insulin sensitivity remains to be determined.
The Ser268Pro polymorphism in Nod2 was not associated with any of the metabolic characteristics of this population and did not interact with any dietary lipids to affect the relationship between these dietary components and factors of the metabolic syndrome. Contrary to Nod1, which is fairly ubiquitous, Nod2 is expressed in very specific cell types. These cell types include macrophages (Ogura et al., 2001b), dendritic cells (Tada et al., 2005) and colonic epithelial cells (Hisamatsu et al., 2003). These results indicate that either the particular polymorphism studied does not affect the response of Nod2 to lipids, or that this protein does not play as big of a role as others in mediating inflammation in response to diet in healthy individuals. In obesity, there is infiltration of the adipose tissue by macrophages which can mediate an inflammatory response. It’s possible that Nod2 could play a more significant role in individuals with higher a BMI. However, there were not enough obese individuals in the population (n=27) to measure this effect appropriately.

In summary, the Nod1 Glu266Lys polymorphism modifies the association between dietary SFA and insulin sensitivity, measured through HOMA-IR, in a young, healthy population. We also showed that the Nod2 Ser268Pro polymorphism had no effect on factors of the metabolic syndrome in response to dietary fat. These results support the role of innate immunity, particularly Nod1, in mediating the effects of diet on the MetS. This is the first time these SNPs have been investigated in this regard and Nod1 appears to be important to promoting a pro-inflammatory response to lipids, intracellularly. Future research should investigate this relationship further to elucidate mechanisms by which this effect may be occurring.
CHAPTER SIX:
Limitations, Implications and Future Directions
6.1 Overview

The overall objective of this thesis was to determine whether genetic polymorphisms in three proteins of innate immunity (TLR4 rs4986790 (Asp299Gly) and rs5030728, Nod1 rs2075820 (Glu266Lys) and Nod2 rs2066842 (Ser268Pro)) modify the relationship between dietary fat and biomarkers of the metabolic syndrome (MetS). We also investigated whether any of these factors differed between genotypes of these polymorphisms.

**Objective 1 (Chapter 4):** To determine whether the TLR4 Asp299Gly and intronic SNP rs5030728 are associated with biomarkers the MetS.

**Results:** Individuals who carried the Gly allele at amino acid position 299 had significantly higher insulin levels and were also less insulin sensitive compared to Asp/Asp individuals. They also had a higher prevalence of a family history of diabetes. The intronic SNP was not associated with any factors of the MetS.

**Objective 2 (Chapter 4):** To determine whether the TLR4 Asp299Gly and intronic SNP rs5030728 modify the relationship between dietary fat and biomarkers of the MetS.
Results: The TLR4 intronic SNP rs5030728 modified the association between dietary SFA and fasting HDL-cholesterol concentrations. An increase in SFA was inversely associated with HDL-cholesterol in the G/G genotype; however SFA was positively associated with HDL-cholesterol in the G/A genotype. There was no association between HDL-cholesterol and SFA intake in the A/A genotype. The Asp299Gly polymorphism did not modify the association between dietary fat and any risk factors of the MetS.

Objective 3 (Chapter 5): To determine whether the Nod1 Glu266Lys and Nod2 Ser268Pro polymorphisms are associated with biomarkers of the MetS.

Results: Individuals who carried the 266Lys allele had lower total and LDL-cholesterol compared to Glu/Glu individuals, however, this difference was small and probably not biologically significant. The Nod2 Ser268Pro polymorphism was not associated with any components of the MetS.
Objective 4 (Chapter 5): To determine whether the Nod1 Glu266Lys and Nod2 Ser268Pro polymorphisms modify the relationship between dietary fat and biomarkers of MetS.

Results: The Nod1 Glu266Lys polymorphism modified the association between dietary SFA and fasting HOMA-IR. There was no association between dietary SFA and HOMA-IR in individuals carrying the 266Glu allele. In Lys/Lys individuals, there was a positive association between dietary fat and HOMA-IR. The Nod2 Ser268Pro polymorphism did not modify the association between dietary fat and any risk factors of the MetS.

6.2 Conclusion

To summarize, this thesis investigated whether genetic variation in proteins of innate immunity responsible for initiation of pro-inflammatory signaling modify the association between dietary fat and components of the MetS. Specifically, two polymorphisms in TLR4 (rs4986790 (Asp299Gly) and rs5030728) and one in each of Nod1 (Glu266Lys) and Nod2 (Ser268Pro) were investigated. The findings presented in this thesis suggest that TLR4 may be involved in the etiology of the MetS as the Asp299Gly polymorphism in TLR4 was associated with decreased insulin sensitivity while the intronic SNP in TLR4 modified the association between dietary SFA and HDL-
cholesterol. The intracellular protein, Nod1 may also be playing a role as we found the Glu266Lys polymorphism to be associated with a small decrease in total and LDL cholesterol and the SNP also modifies the relationship between dietary SFA and HOMA-IR. The Ser268Pro polymorphism in Nod2 did not affect any of the metabolic outcomes we measured. These results suggest that innate immunity and dietary fat are important to the development of the MetS and that genetic variation in proteins of innate immunity affect how diet influences risk for this condition.

6.3 Limitations

There are always advantages and disadvantages to a particular study design, and it is important to note the limitations of a study when evaluating its results. The studies presented in this thesis were observational in design and used an FFQ to evaluate dietary intake. Assessment of dietary intake is always difficult in nutritional research, and dietary fat in particular is difficult to measure. Advantages of using an FFQ are that it does not require a lot of work on behalf of study subjects compared to other methods. However, asking subjects to recall food and its portion size for meals they’ve eaten a month ago can introduce recall bias. There may also be some foods which individuals eat regularly that were not listed on the FFQ, such as traditional foods from different ethnocultural groups. Missing responses on the FFQ can also lead to underestimation of caloric and nutrient intakes. Our study population was comprised of young adults in good health, with no known reason to have impaired ability to recall food intake. The FFQ also gave subjects an opportunity to list foods they eat regularly and
specify an amount at the end of the survey in order to capture ethnic foods. Finally, the FFQ was reviewed by the study coordinator prior to being analyzed and subjects are contacted if any information was missing. FFQs are to be used only to rank individuals for different nutrient intakes. The inability of FFQs to determine absolute intake of a nutrient is another limitation of this method of dietary analysis and we have recognized this limitation in our study.

The effects of these polymorphisms on gene expression and protein levels were not measured in this study and are not known for these polymorphisms. However, our studies were not intended to investigate this issue and further research into the effects of these polymorphisms on protein function and expression are now warranted because of our results.

The results of this study also do not give insight into lipid metabolism during inflammation. Although it appears as though dietary fat is detected by these proteins of innate immunity, this study does not give confirmation or mechanisms by which lipid metabolism is being altered. The origin of these lipids which may be detected is not revealed by this study. Again, these studies were not designed to investigate these questions but our results do support further research into this area.

Finally, as the study was observational in design, it does not allow for causal attribution of the effects of these genotypes on the biomarkers of the MetS in response to diet. However, we did detect significant interactions between dietary SFA and the TLR4 intronic SNP and the Nod1 Glu266Lys polymorphism on fasting HDL-cholesterol
and HOMA-IR, respectively. This would provide a good rationale for future studies to investigate potential causal mechanisms that may be occurring.

6.4 Future Directions

This study integrated genetic techniques in a traditional nutrition epidemiology design in order to assess whether genotype affected the metabolic response to dietary fat. This and other studies in nutrigenomics will contribute to the eventual goal of personalized nutrition. However, before this goal can be attained much research is still necessary to understand mechanisms by which these polymorphisms are affecting metabolism.

Ex-vivo studies using peripheral blood mononuclear cells (PBMC), adipocytes and myocytes should be completed with cells expressing each of these genotypes. These cells can be stimulated with the PAMPs for each protein in order to assess the effects of these polymorphisms on mRNA expression and protein function. These studies would provide insight into the functional consequences of these polymorphisms in cells relevant to T2D and MetS etiology.

It would also be beneficial to determine if the polymorphisms which affected MetS components and interacted with diet to affect metabolic biomarkers actually translated into differences in risk for these diseases. A prospective study could be implemented whereby individuals are matched based on environmental factors known to affect MetS risk and the only difference between matched individuals would be
genotype. Prospective incidence of T2D and MetS in each genotype could then be measured and the three genotype groups compared to assess relative risk. Studies of this type could be done with individual polymorphisms or in haplotype blocks.

Dietary intervention would also be a future study that could be completed once the results on the polymorphisms in this thesis and others in these genes have been replicated. Given the costs and ethical considerations of clinical trials, they should be completed only when mechanisms have been deciphered. Healthy male subjects would be recruited by genotype for either of the TLR4 polymorphisms or Nod1 polymorphisms studied. Male subjects would be chosen as the different phases of the menstrual cycle in women affects inflammatory cytokine levels. For the TLR4 intronic polymorphism studied, subjects could be given either a high (>10% of energy) or low (≤10% of energy) dose of SFAs and then their response in plasma cholesterol levels could be measured. For the Nod1 SNP the SFA dose would be administered and then measurement of fasting glucose and insulin levels and an oral glucose tolerance test (OGTT) would be completed. These studies could be completed in an acute or chronic manner. In a chronic study, the same level of SFAs would be ingested over a one month period and the glucose and insulin levels and OGTT could be completed on the first and last day of study. Response in cytokine levels would also be measured in order to assess a potential mechanism based on inflammatory status. This type of study would allow for evaluation of the biological impact of these polymorphisms in humans.
6.5 Implications

A growing body of evidence suggests that there are individuals and population subgroups that have different levels of susceptibility to T2D and the MetS, which are likely related to genetic factors. It is possible that some of these factors may open up new approaches for treatment. There is some indication that genetic susceptibility to certain sub-types of T2D may be influenced by a range of dietary factors (e.g., fatty acids) or alleviated by various micronutrients (e.g., vitamin D).

The MetS, including insulin resistance, T2D, obesity and atherosclerosis have been linked to genetic variations leading to altered production or function of circulating innate immune proteins, cellular pattern-recognition receptors and inflammatory cytokines. This relationship may be further influenced by numerous nutritional factors known to be associated with the development of T2D (e.g., dietary fat). The studies presented in this thesis were initiated in an effort to identify the genetic determinants of the MetS and to elucidate how nutritional factors modify their effect. This study serves as an example of the complexity in treatment and control of metabolic disease, illustrating how the same dietary treatment could result in different individual responses due to genetics. The ultimate goal of this research was to contribute new strategies to reduce the rising incidence of the MetS and its associated diseases in Canadians.


