Longer Life Foundation – Final Report
Project name: CD36 Variants and Stroke Risk Factors (2011-12)
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Abstract
An individual's risk of cardiovascular disease (CVD) and stroke reflects complex interactions between genetic, dietary and lifestyle influences. Chronically elevated cholesterol and triglyceride levels after a meal are risk factors for CVD and stroke. Current therapeutic guidelines focus on reducing blood triglyceride and low-density lipoprotein cholesterol concentrations to reduce cardiovascular events. However, there is need for the development of early-risk assessment tools to identify individuals at high disease risk to improve long-term health outcomes. Recent genetic studies associated CD36, a protein that functions in the utilization of dietary fats, with the risk of stroke. In previous studies, we identified common variations in the CD36 gene that associated with increased fasting blood lipid concentrations and with risk for metabolic syndrome (MetS).

The overall purpose of this proposal was to determine whether common single nucleotide polymorphisms (SNPs) on the CD36 gene influence postprandial (after a meal) hyperlipidemia and associate with markers of inflammation. We found that the stroke-associated and additional SNPs increase postprandial hyperlipidemia in response to a high-fat meal, reflecting a potential effect of SNPs on CD36 expression. Subsequently, we determined that these genetic variations in turn associate with CD36 expression in a tissue-specific manner. We are now evaluating whether the effect of SNPs on CD36 expression involves regulation of gene activity. The findings suggest that the association between CD36 SNPs and postprandial lipids and inflammatory markers may reflect the impact of SNPs on CD36 regulation in response to a high-fat meal, which would promote abnormalities of blood lipids. Moreover, these CD36 SNPs may be useful for early assessment of stroke risk and for designing approaches that target CD36 to reduce stroke risk or prevent disease for long-term health.

Lay Summary
In industrialized countries individuals spend most of the day in a postprandial state, which can induce production of proinflammatory factors that lead to atherosclerosis-related diseases such as stroke. The purpose of this study was to determine the functional relevance of variations in the CD36 gene, a gene that encodes a protein that facilitates tissue uptake of dietary fat and is involved in inflammatory signaling. Recent studies in humans and in rodent models implicate the transmembrane fatty acid transporter CD36, as an important link between fasting lipids concentrations, insulin resistance and atherosclerotic processes. Common variants in the CD36 gene associate with variability in fasting lipid levels and with the risk of metabolic syndrome (MetS) and stroke. However the functional relevance of CD36 genetic variations to postprandial lipid levels and associated metabolic inflammatory markers is not known. We evaluated the effect of common genetic variations in the human CD36 gene on lipid levels and markers of inflammation in response to a high-fat meal and found that the SNPs previously shown to influence risk of stroke and metabolic syndrome similarly associate with higher blood lipids after a high-fat meal. Characterization of identified variants in genes involved in lipid absorption and processing is important for developing sensitive and early biomarkers for susceptibility to dyslipidemia, cardiovascular disease and stroke.

Introduction
Chronically elevated postprandial (after a meal) lipid levels are linked to the progression of atherosclerosis and are a clinically significant risk factor for cardiovascular disease (CVD) and stroke. Complex interactions between diet, lifestyle and genetic factors influence fat absorption and clearance, thus impacting postprandial serum lipid concentrations. Elevated postprandial triglycerides and delayed

chylomicron clearance induce systemic inflammation and are risk factors for coronary artery disease (CAD) [1, 2]. The transmembrane fatty acid transporter, CD36, functions in the uptake of fatty acids and plays an important but incompletely understood role in lipid metabolism in humans, particularly in dietary fat intake, lipid utilization, and inflammatory signaling. In addition, CD36 is also involved in prostaglandin formation from arachidonic acid [3]. Rodent models of CD36 deficiency show elevated triglycerides after a meal and increased intestinal output of small chylomicrons [4, 5]. Findings in CD36-null mice on a 12-week western diet, however, showed reduced expression of proinflammatory cytokines supportive of a proatherogenic role of CD36 [6] under hyperlipidemic conditions.

Common genetic variations in the CD36 gene have been consistently shown to associate with fasting lipid levels, MetS and, more recently, with stroke in humans [7, 8]. Our published studies on humans identified associations between CD36 SNPs and blood VLDL and HDL [9]. However, whether common CD36 SNPs that impact CD36 expression or fasting lipid levels also influence postprandial lipids (meaning the absorption and handling of fat by tissues) remains unknown. Furthermore, these disease associations support pleiotropic effects (one gene having effects on multiple traits) of CD36 in lipid metabolism and inflammatory signaling.

Methods

Subjects: Selected CD36 SNPs from subjects participating in the Genetics of Lipid-Lowering and Diet Network (GOLDN) Study, an NIH-funded study in which Washington University in St. Louis takes part, were analyzed to examine the relationship between SNPs in CD36 on postprandial lipids and inflammatory markers. The GOLDN Study is unique because to date, it is the largest (n=1072) postprandial lipid study to use a standardized acute fat load followed by three weeks of fenofibrate treatment, and the use of nuclear magnetic resonance (NMR) technology to characterize lipoprotein particle size subclasses [10]. Briefly, metabolic responses were measured before and after three weeks of subject fenofibrate treatment to the ingestion of a high-fat milk shake containing 700 calories/m2 of body surface area (3% derived from protein, 14% from carbohydrate, and 83% from fat), with a cholesterol content of 240 mg and a ratio of polyunsaturated to saturated fat of 0.06. Participants were instructed to consume the milk shake within 15 minutes.

Statistical Analysis: The association tests between common CD36 haplotype-tag SNPs and lipid-loading factors followed by selected single trait analysis were implemented in SAS data analytics software and performed using linear mixed effect models. Analyses were tested for population stratification and corrected for familial relationships and covariates (age and gender). Additive effects of each SNP were tested to determine whether it was significantly different from zero. Associations were considered significant at a threshold of $p=\leq 0.007$.

Results

Table 1 shows the characteristics of the GOLDN subjects (n=1072) and the postprandial lipid levels. To determine potential pleiotropic effects of CD36 SNPs on NMR-derived lipoprotein concentrations and particle characteristics, SNPs were tested for association with factor scores [11]. Several of the SNPs support pleiotropic effects of CD36 on lipoprotein concentrations and particle size (Table 2). As shown in Table 2, multiple common SNPs (minor allele frequency >5% in the general population) were associated with latent lipid loading factors at fasting, 3.5 and 6.0 hour time points. Subsequently, selected SNPs were tested for single-trait analyses (Table 3). The SNP rs7755 A-allele associated with increased total and LDL cholesterol at 0, 3.5 and 6.0 hours, and showed an effect on chylomicron levels and VLDL size at 6.0 hours (Table 3). SNP rs7755 is located in an alternative 3'UTR of the CD36 gene and resides in a predicted miRNA recognition element. SNP rs1761665 showed similar effects on blood lipids at baseline,

3.5 and 6.0 hours after the fat challenge. SNP rs1761665 is strongly linked with SNP rs1761667 (r2 >0.80)] in Caucasian populations.

Rs1761667 was previously reported to associate with free FA levels and CD36 expression in platelets and monocytes [9, 12]. The three-week fenofibrate treatment blunted these effects (data not shown), suggesting potential regulatory effects of SNPs on CD36 in response to high-fat meal conditions. We also identified multiple SNPs proximal to the most upstream 5'UTR (data not shown) that were associated with triglyceride uptake and clearance after the milkshake was given.

Tests for association with circulating pro-inflammatory cytokines (hsCRP, TNFA, MCP1, IL2, IL6, adiponectin) in 250 subjects participating in the GOLDN Study showed only a relationship between a SNP (rs17246831, minor allele frequency 8%; p=0.0001) near the most upstream alternative 5'UTR and serum IL-2. The most upstream 5'UTR of CD36 (i.e. Exon 1D) is abundantly expressed in endothelial cells.

Table 1. Demographics and Mean (SD) lipid profile of the GOLDN Study population	
N	1117
Age (yrs)	48.5
Gender (% F)	52.7
BMI (kg/m^2)	28.3 ± 5.6
LDL (mg/dL)	
Fasting	116.5 ± 31.9
3.5 hr postprandial	118.6 ± 32.7
6.0 hr postprandial	121.4 ± 32.7
HDL	
Fasting	45.5 ± 13.5
3.5 hr postprandial	45.6 ± 14.1
6.0 hr postprandial	48.2 ± 14.3
Cholesterol	
Fasting	183.7 ± 38.6
3.5 hr postprandial	18.5 ± 40
6.0 hr postprandial	192.4 ± 41.2
Triglycerides	
Fasting	143.2 ± 31.9
3.5 hr postprandial	223.3 ± 139.4
6.0 hr postprandial	205.0 ± 156.6
Insulin (mU/L)	13.7 ± 8.2
Glucose (mg/dL)	101.5 ± 19

Table 2. Association between representative CD36 SNPs with Pre and Postprandial latent lipid factor loading in response to a high-fat meal challenge

SNP	Alleles	MAF (%)	β (se)	*p-value*	Gene Context
Fasting Factor 2: Triglycerides, VLDL size, VLDL, Large VLDL, Medium VLDL					
rs11983803	A/G	4	0.35 (0.12)	4.30E-03	
rs3211869	A/T	5	0.29 (0.11)	5.20E-03	intron
rs41364549	C/T	5	0.29 (0.11)	6.50E-03	intron
Fasting Factor 3: LDL-cholesterol, Total Cholesterol, small VLDL					
rs7755	A/G	46	-0.16 (0.05)	1.80E-03	utr-3
rs1761665	C/T	44	-0.20 (0.05)	9.00E-05	intron
rs1722507	C/T	39	-0.17 (0.05)	1.20E-03	intron
Fasting Factor 4: Small HDL, Small VLDL, reduced VLDL size, Medium HDL					
rs799940	A/G	13	0.25 (0.08)	1.10E-03	
3.5-hr Factor 2: Triglycerides, total VLDL, chylomicrons, Large VLDL, Medium VLDL, HDL size, Large HDL					
rs10081383	C/T	24	0.18 (0.06)	3.30E-03	
3.5-hr Factor3: Small VLDL, Total LDL, Total Cholesterol					
rs1761665	C/T	44	-0.17 (0.05)	1.60E-03	intron
rs6969989	A/G	37	-0.15 (0.05)	3.80E-03	intron
6-hr Factor 3: small VLDL, Total Cholesterol, Total Cholesterol					
rs1761665	C/T	44	-0.15 (0.05)	4.30E-03	intron
rs6969989	A/G	37	-0.15 (0.05)	3.30E-03	intron
6-hr Factor 4: VLDL size, small VLDL					
rs573316	C/T	6	-0.35 (0.11)	1.50E-03	
rs496617	A/G	6	-0.36 (0.12)	2.10E-03	
rs601671	G/T	37	-0.17 (0.05)	9.00E-04	
rs6467158	C/T	50	0.15 (0.05)	3.80E-03	

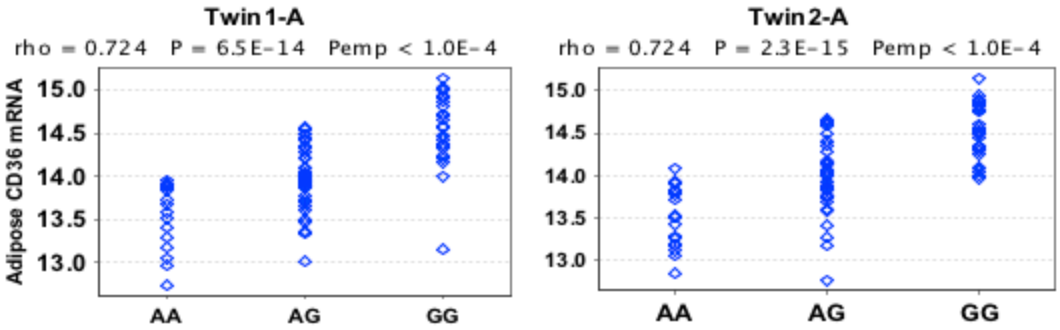
Table 3. SNP effect on single trait postprandial lipid concentrations

SNP	**rs1761665 (T/C)**			**rs7755 (G/A)**		
Time after fat challenge (hr)	**0**	**3.5**	**6**	**0**	**3.5**	**6**
Cholesterol (mg/dL)	2.00E-04	2.40E-03	1.70E-03	5.00E-04	8.30E-03	7.50E-03
LDL (mg/dL)	1.00E-04	5.00E-04	1.20E-03	2.00E-03	8.40E-03	5.50E-03
VLDL size (nm)			1.74E-02			4.60E-03
Chylomicrons (mg/dL)						8.60E-03

Values in the table are the resulting linear regression p-values. The associated allele, listed 2nd, positively associated with the corresponding lipid value.

The human CD36 gene has several alternative transcripts that are regulated by independent promoters and use alternative 3' untranslated regions (a short and long 3'UTR), which are important for transcript stabilization and cytoplasmic mRNA localization. These transcripts are differentially expressed in metabolically-active tissue and presumably specific confer distinctive regulation of CD36. We measured CD36 mRNA levels in abdominal subcutaneous fat from Caucasian female twin cohorts and found that SNP rs7755 selectively influenced expression of the CD36 long 3'UTR in adipose tissue (*Rho=0.724, corrected p<0.0001,* Figure 1), while SNP rs1761667 (a promoter SNP) associated with select 5'UTR transcript levels in adipose tissue and skeletal muscle (data not shown).

Figure 1. CD36 alternative 3'UTR (mRNA) expression by rs7755 genotype groups.



CD36 mRNA levels in abdominal subcutaneous fat from Caucasian female twin cohorts shows that individuals with the AA genotype have lower expression of the alternative untranslated exon compared to noncarriers in two cohorts (Twin 1-A, n=79 and Twin 2-A, n=87). Differences in CD36 expression between the genotypes levels were examined using Spearman's rank correlation analysis, Rho=0.724, corrected p<0.0001. Expression data from the Multiple Tissue Human Expression Resource (MuTHER) project retrieved from the GENEVAR database for this analysis.

Discussion, including implications and potential long-term extensions

These studies show that multiple common CD36 SNPs not only influence fasting lipid levels as previously reported, but influence absorption and clearance of serum lipids after a high-fat meal and associate with circulating IL-2, a proinflammatory immune response molecule. We found that several of these SNPs reside in sequences recognized by pre- and post-transcriptional regulatory factors. Based on these findings, we hypothesize that these SNPs impact CD36 expression and regulation in response to dietary fat. For example, SNPs rs7755 (previously reported to associate with the risk of stroke and metabolic syndrome) lies within a microRNA recognition site (microRNAs repress translation of its target genes) and a predicted RNA-editing element (molecular process which can lead to modified protein sequences) within an alternative 3'UTR. Presumably individuals with the rs7755 A-allele respond differently to a high-fat meal compared to individuals who do not carry the A-allele, as a result of altered CD36 levels and/or regulation.

Our findings with IL-2 support a pleiotropic role for CD36 in lipid handling and immune response after a high-fat meal challenge. IL-2 is a natural proinflammatory cytokine that is important for immune cell (T- and B-cell) proliferation. Adipose tissue in obesity is characterized by inflammation, including the recruitment and infiltration of macrophages and lymphocytes [13]. CD36 is abundantly expressed on macrophages in humans. In a recent mouse model study, CD36-deficient mice compared to wild-type

mice fed a high-fat diet had reduced adipose tissue inflammation with decreased pro-inflammatory cytokine expression and macrophage and T-cell accumulation [14].

In summary, our novel findings support influence of human CD36 SNPs on blood lipids, the handling of dietary fat and on the proinflammatory effects of high fat intake. Further studies are needed to replicate the association between CD36 SNPs and IL-2 in a larger cohort. We are currently examining the regulatory effects of these SNPs on CD36 via microRNA regulation. Based on our data, it could be proposed that down regulation of CD36 might be a viable therapeutic target for dyslipidemia and to reduce the associated disease risk factors.

Future plans including planned grant submissions

There is increasing awareness that the development of effective strategies for assessing disease risk or for treatment of disease will require an understanding of the influence of genetic variants. However, functional characterization of genetic disease susceptibility loci is often unavailable despite being necessary for translating their clinical significance. As a direct result of the studies described in this report, we have established collaborations with external investigators to further determine the impact of CD36 SNPs on cardiac endothelial function. Data from these studies will be included in a manuscript currently in preparation for submission to the journal *Diabetes.* Additionally, the data will be included in an investigator-initiated NIH-R01 application to be submitted February 2014 to further examine tissue-specific transcriptional and posttranscriptional regulation of the human CD36 gene and the functional impact of the disease-associated SNPs identified in this study. We hypothesize that some of these SNPs might alter tissue-specific expression and/or regulation of CD36 transcripts and impair crosstalk between various tissues active in FA metabolism resulting in abnormal lipid handling, inflammation and subsequent insulin resistance.

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