ORIGINAL ARTICLE

RARE *ATGL* HAPLOTYPES ARE ASSOCIATED WITH INCREASED PLASMA TRIGLYCERIDE CONCENTRATIONS IN THE GREENLAND INUIT

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ABSTRACT

Objectives. To genotype common genetic variants found in the adipose triglyceride lipase (*ATGL*) gene and test them for association with cardiovascular disease risk factors in the Greenland Inuit.
Study design. Candidate gene association study of discrete and quantitative traits related to cardiovascular health.
Methods. *ATGL* was sequenced in 10 European subjects to identify DNA sequence variants. The identified polymorphisms were subsequently genotyped in a population-based cohort of 1,218 unrelated Greenland Inuit subjects, ascertained from the Greenland Population Study. Genotypes and reconstructed haplotypes were tested for association with cardiovascular disease risk factors using additive, dominant or recessive models, corrected for age, sex and body mass index.
Results. Five single nucleotide polymorphisms and one 4-base pair deletion were identified in the European sample and were similarly polymorphic in the Greenland Inuit. Independently, variants were not associated with any cardiovascular traits. However, reconstructed rare *ATGL* haplotypes were associated with increased plasma triglyceride (TG) concentrations compared to the major haplotype under a dominant model (1.21±0.7 mmol/L and 1.11±0.6 mmol/L, respectively, p=0.006).
Conclusions. Rare *ATGL* haplotypes are associated with increased plasma TG concentrations in the Greenland Inuit.
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Keywords: adipose triglyceride lipase, cardiovascular disease, genetic association study, haplotype analysis, single nucleotide polymorphisms, triglyceride-rich lipoproteins

INTRODUCTION

Elevated plasma triglyceride (TG) concentration is re-emerging as a significant risk factor for cardiovascular disease (CVD) (1–3). Furthermore, elevated TG is a defining component of the metabolic syndrome (MetS) (4), a cluster of subphenotypes accompanied by increased risk of CVD and type 2 diabetes (T2D). Abnormal TG metabolism can lead to other disease phenotypes such as obesity, hepatic steatosis and hepatic insulin resistance, all conditions that are exacerbated by continued TG accumulation (5). It is known that common genetic variants in candidate metabolic genes contribute to the expression of diseases such as MetS (6–8). Similarly, genetic determinants of TG metabolism may contribute to the dysregulation of energy homeostasis and thus other common CVD-associated phenotypes.

Plasma TG-containing lipoproteins are produced from fatty acids (FA) obtained from diet, *de novo* hepatic FA synthesis and FA mobilized from adipose tissue TG stores (9). Therefore, common variants in genes required for FA synthesis and mobilization may contribute downstream to increased TG concentrations. A recently discovered enzyme required for FA mobilization in adipose tissue is adipose triglyceride lipase (ATGL), now known to be the rate-limiting enzyme responsible for initiating TG hydrolysis, rather than hormone-sensitive lipase, as previously thought (10–12). ATGL catabolizes TG found in cellular lipid droplets of adipose and non-adipose tissues into diacylglycerol and 1 FA molecule (13). Interestingly, ATGL also has transacylase activity, allowing the re-esterification of free FA to produce TG, suggesting an important role of ATGL in basal TG cycling and energy homeostasis (14).

ATGL activity is subject to complex endocrine regulation, multiple protein-protein interactions and different functions in adipose and non-adipose tissue, leaving its precise role in metabolism incompletely understood (15). Experiments in mice have demonstrated that abolishing ATGL impairs TG mobilization, causing increased adiposity, greater glucose utilization and insulin sensitivity and decreased plasma FA and TG (14). Conversely, ATGL overexpression causes TG mobilization, increased FA oxidation, accumulation of lipotoxic DAG molecules and subsequent insulin resistance (16–17). In humans, rare mutations in *ATGL* cause neural lipid storage disease (MIM 610717), an autosomal recessive condition marked by systemic TG deposition in multiple tissues (18).

Considering the importance of *ATGL* in energy homeostasis, we chose to investigate the association of genetic variation in *ATGL* with CVD risk factors in the Greenland Inuit. Aboriginal communities have historically proved valuable for genetic association studies of complex traits, given their relatively low background genetic and environmental variability (19). Furthermore, genetic variation in candidate lipoprotein metabolism genes has previously been associated with lipid traits in the Greenland Inuit (20–21), but this understanding is far from complete. We sought to (1) genotype genetic variants in *ATGL*; and (2) test these genotypes for association with traits related to cardiovascular health in the Greenland Inuit. We hypothesized that common genetic variants in *ATGL* would be associated with risk factors for CVD.

MATERIAL AND METHODS

Study subjects

This study was approved by the ethics boards at both Canadian institutions and by the Commission for Scientific Research in Greenland. Sequencing was conducted to identify genetic variants in 10 unrelated subjects of European descent, ascertained from normal population controls. Genotyping was subsequently conducted in 1,218 unrelated Greenland Inuit ascertained from 1997 to 2001 as part of the Greenland Population Study (22). This study was an epidemiological, cross-sectional survey of health and disease in Inuit subjects from Greenland and Denmark. Inclusion criteria were age above 18 years and self-declared Greenlandic ethnicity. In Denmark, subjects were ascertained via random sampling of the Greenlandic Population Registries. In Greenland, subjects were ascertained from Nuuk (population 14,000), Qasigiannguit (population 1,400) and 4 villages in the Uummannaq district (population 240–275 each), facilitated by Population Registries and random household sampling. Collection of baseline clinical attributes included body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), glucose, waist circumference, waist to hip ratio, fasting plasma concentrations of glucose, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and TG. MetS was diagnosed using criteria of the National Cholesterol Education Program's Adult Treatment Panel III (23). Type 2 diabetes prevalence was determined by quantifying subjects with fasting plasma glucose concentration >7.0 mmol/L.

Biochemical and genetic analyses

Genomic DNA was isolated from whole blood using Puregene DNA extraction kits (Gentra Systems, Minneapolis, MN). The *ATGL* locus (also known by Human Genome Organisation nomenclature as patatin-like phospholipase domain containing 2, abbreviated *PNPLA2*) was sequenced in 10 unrelated subjects of European ancestry to identify genetic variation at the DNA level, including single nucleotide polymorphisms (SNPs), and small insertions and deletions. Such genetic variation may affect protein structure and function; for example, non-synonymous SNPs cause amino acid substitutions in the protein sequence, while intronic and synonymous SNPs do not change protein sequence but may affect exon splicing, and insertions and deletions may disrupt the correct reading frame of the protein. All transcribed regions of the gene, including >100 bp at each intron-exon boundary, were amplified by PCR and purified using QIAquick Gel Extraction Kits (Qiagen, Valencia, CA). Purified DNA fragments were bidirectionally sequenced once per subject using the original amplification primers, and sequenced using a 3730 Automated DNA Sequencer (Applied Biosystems, Foster City, CA). The polymorphisms identified by sequencing were subsequently genotyped in the Greenland Inuit population cohort by SNaPshot using a 3730 Automated DNA Sequencer, or by validated TaqMan assays using an ABI Prism 7900HT Sequence Detection System (SDS) and automated software (Applied Biosystems, Foster City, CA). Primer and probe sequences used for genotyping are available upon request.

Statistical analyses

TG concentrations were log transformed to obtain a normal distribution prior to analysis, but are presented as untransformed values. Initial statistical associations for individual polymorphisms were tested using an additive model in PLINK (24). These included logistic and linear regression to evaluate discrete and quantitative traits, respectively, entering age, sex and body mass index as covariates. Pairwise linkage disequilibrium (that is the non-random association of variants that are physically close) and significant deviations of observed genotype frequencies from those expected by Hardy-Weinberg equilibrium (HWE) were evaluated using Haploview v4.1 (25), with a significance threshold set at $p<0.001$. Maximal likelihood haplotypes were generated using PHASE (26), and were tested for statistical association using dominant and recessive models in SAS v9.1 (SAS Institute, Cary, NC). Chi-square analysis was used for discrete traits, and ANOVA was used for quantitative traits, each entering age, sex and BMI as covariates. Nominal statistical significance of $p<0.05$ was adjusted using a Bonferroni multiple testing correction, setting a new statistical significance threshold at $p<0.01$.

RESULTS

Baseline attributes

Baseline clinical attributes were collected for 1,218 unrelated Greenland Inuit living in Denmark and Greenland (Table I). Subjects were 18 to 86 years of age, and 55.3% were female. Mean trait values were mostly similar between genders, except lipid profiles which were less favourable in men, and MetS prevalence, which was increased in women.

Identification of genetic variation in ATGL

Sequencing identified 6 polymorphisms within transcribed regions of the *ATGL* gene in the European subjects (Table II). These included 5 SNPs (2 intronic, 2 synonymous

Table I. Baseline attributes of study subjects (mean±standard deviation).

	Overall	Male	Female
Participants	1,218	544 (44.7%)	674 (55.3%)
Age (years)	43.4±14.1	43.4±14.2	43.4±14.1
BMI (kg/m^2)	26.2±5.0	26.0±4.5	26.4±5.4
Systolic blood pressure (mmHg)	118.7±18.6	119.8±16.9	117.8±19.8
Diastolic blood pressure (mmHg)	72.8±11.3	74.3±11.6	71.5±10.9
Waist circumference (cm)	88.5±12.9	90.4±12.1	87.0±13.3
Waist-to-hip ratio	0.90±0.08	0.93±0.08	0.88±0.08
Glucose (mmol/L)	5.78±1.15	5.83±0.89	5.74±1.31
Total cholesterol (mmol/L)	5.90±1.17	5.89±1.17	5.90±1.17
LDL-C (mmol/L)	3.80±1.07	3.82±1.08	3.80±1.08
HDL-C (mmol/L)	1.57±0.44	1.53±0.47	1.60±0.42
TG (mmol/L)	1.16±0.66	1.20±0.69	1.12±0.65
Metabolic syndrome (%)	14.4	12.2	16.2
Type 2 diabetes* (%)	7.0	7.6	6.1

*Based on data for 508 males and 637 females. BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

and 1 non-synonymous) and 1 4-base pair deletion (Fig. 1). The non-synonymous SNP was most likely to affect protein function, as the 4-bp deletion was found in the 3' untranslated region, not affecting the reading frame of the protein. Among these polymorphisms, the intronic SNP C5595G was not previously reported.

Association between ATGL and cardiovascular disease-related traits in the Greenland Inuit

Variants identified in the European subjects were subsequently genotyped in the cohort of Greenland Inuit, and similarly found to be polymorphic. Pairwise linkage disequilibrium was variable between the polymorphisms, ranging from no linkage to r^2=0.78 (Fig. 1). Minor allele frequencies also varied, ranging from 3.5%–23.3% (Fig. 1). The non-synonymous SNP C5888T, causing a leucine to proline substitution at residue 481, deviated significantly from genotype frequencies expected by HWE and was thus removed from subsequent analyses.

Each genotype was tested for association with traits related to CVD using an additive model. However, no significant associations

Table II. Polymorphisms identified in *ATGL*.

Polymorphism	dbSNP	Type of variation	Position
C3721T	rs7942159	Intronic SNP	c.3705+16 A>G
C4908G	rs1135628	Synonymous SNP	c.4908 C>G (p.291 Pro>Pro)
C5595G	-	Intronic SNP	c.5535+60 C>G
T5888C	rs1138693	Non-synonymous SNP	c.5888 T>C (p.481 Leu>Pro)
C5904T	rs1138694	Synonymous SNP	c.5904 C>T (p.486 Pro>Pro)
del6091	-	Deletion	c.*del129_132

SNP, single nucleotide polymorphism.

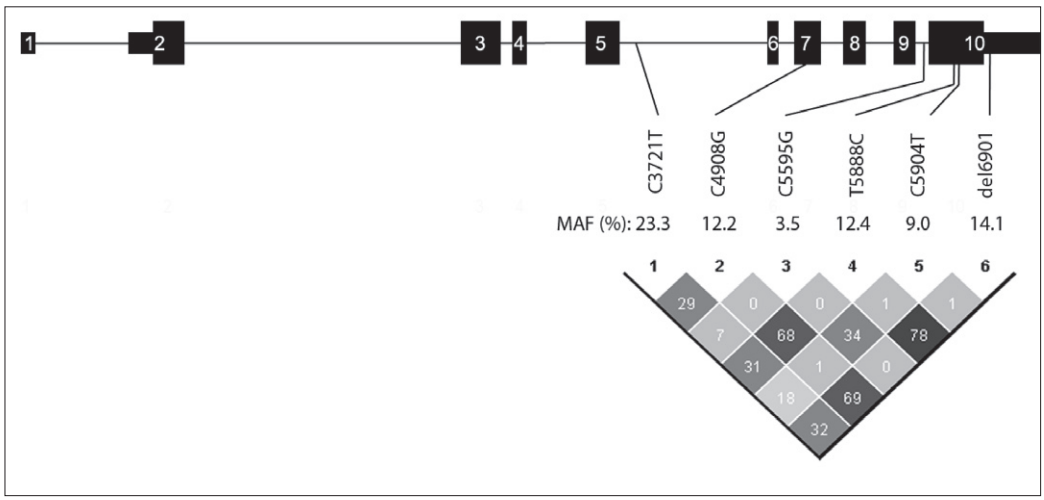

Figure 1. Structure of the *ATGL* gene. Positions of genotyped polymorphisms are indicated, including minor allele frequencies (MAF) and pair wise measures of linkage disequilibrium (r^2). Exons are indicated by thick black boxes, with exon number written in white; introns are indicated by a thin black line.

Table III. Association between *ATGL* genotypes and cardiovascular disease risk factors.

	C3721T		C4908G		C5595G		C5904T		6091del	
	Effect	p	Effect	p	Effect	p	Effect	p	Effect	p
Diastolic BP (mm Hg)	-0.28	0.58	-0.22	0.73	1.30	0.28	1.31	0.08	-0.51	0.39
Systolic BP (mm Hg)	-0.23	0.76	0.40	0.68	1.37	0.46	1.15	0.33	-0.46	0.62
Waist circumference (cm)	0.00	1.00	0.00	0.90	0.01	0.26	0.00	0.37	0.00	0.77
Waist-hip ratio (cm)	-0.10	0.72	-0.28	0.42	0.51	0.45	0.03	0.94	-0.33	0.32
Glucose (mmol/L)	0.01	0.81	0.04	0.56	0.16	0.25	0.07	0.43	0.07	0.29
TC (mmol/L)	-0.01	0.91	-0.02	0.71	-0.05	0.67	0.00	0.98	-0.06	0.31
HDL-C (mmol/L)	-0.03	0.18	-0.02	0.49	-0.09	0.06	-0.03	0.34	-0.03	0.22
LDL-C (mmol/L)	0.01	0.79	-0.03	0.67	0.00	0.98	0.03	0.67	-0.05	0.40
TG (mmol/L)	0.01	0.53	0.02	0.16	0.04	0.08	0.01	0.48	0.02	0.12
MetS (%)	0.97	0.86	0.94	0.79	1.64	0.26	0.92	0.74	0.90	0.62
T2D (%)	1.38	0.09	1.42	0.13	1.58	0.29	1.49	0.14	1.49	0.07

Tests for association were conducted in PLINK (24) using an additive model, entering age, sex and body mass index as covariates. Effect is the regression coefficient (ß) from linear regression of quantitative traits, or the odds ratio (OR) from logistic regression of dichotomous traits (MetS and T2D). BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; MetS, metabolic syndrome; T2D, type 2 diabetes.

Table IV. Associations between *ATGL* haplotypes and cardiovascular disease risk factors under dominant and recessive models.

	Characteristics	Major haplotype	Rare haplotypes	p-Value
Dominant model	Number	645 (47% male)	573 (41% male)	-
	Age (years)	43.4±14.2	43.4±14.1	-
	BMI (kg/m2)	26.2±4.7	26.2±5.3	-
	Diastolic BP (mm Hg)	72.8±11.3	72.7±11.4	0.948
	Systolic BP (mm Hg)	118.6±18.1	118.7±19.1	0.855
	Waist circumference (cm)	88.7±12.7	88.3±13.2	0.489
	Waist to hip ratio (cm)	0.90±0.08	0.90±0.09	0.825
	Glucose (mmol/L)	5.74±1.27	5.82±1.04	0.219
	Total cholesterol (mmol/L)	5.87±1.12	5.92±1.22	0.486
	HDL-C (mmol/L)	1.57±0.44	1.55±0.45	0.058
	LDL-C (mmol/L)	3.78±1.03	3.84±1.12	0.320
	TG (mmol/L)	1.11±0.62	1.21±0.71	0.006
	MetS (%)	11.8	16.7	0.505
	T2D (%)	5.4	8.4	0.055
Recessive model	Number	1123 (45% male)	95 (42% male)	-
	Age (years)	43.4±14.1	44.0±14.3	-
	BMI (kg/m2)	26.2±5.0	26.5±5.2	-
	Diastolic BP (mm Hg)	72.7±11.3	74.2±12.0	0.223
	Systolic BP (mm Hg)	118.5±18.4	120.5±20.0	0.383
	Waist circumference (cm)	88.4±12.9	89.4±13.2	0.487
	Waist to hip ratio (cm)	0.90±0.08	0.90±0.86	0.964
	Glucose (mmol/L)	5.77±1.20	5.81±0.78	0.841
	Total cholesterol (mmol/L)	5.90±1.17	5.80±1.20	0.252
	HDL-C (mmol/L)	1.60±0.44	1.58±0.47	0.944
	LDL-C (mmol/L)	3.81±1.08	3.70±0.10	0.189
	TG (mmol/L)	1.15±0.66	1.20±0.73	0.580
	MetS (%)	13.9	20.7	0.103
	T2D (%)	6.7	8.1	0.633

Quantitative traits are shown as mean ± standard deviation, dichotomous traits are shown as percent. Age, sex and body mass index were entered as covariates when testing for association using both models. BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; MetS, metabolic syndrome; T2D, type 2 diabetes.

were found between any specific polymorphisms and quantitative or discrete traits (Table III). Haplotype analysis was subsequently undertaken to determine if multiple polymorphisms could influence traits related to cardiovascular disease. Maximal likelihood haplotype generation yielded 21 different haplotypes. The major haplotype (3721C-4908C-5595C-5904C-6091ins) had a frequency of 72.4%, while the next most frequent haplotypes had frequencies of 4.5% (3721T-4908C-5595C-5904T-6091ins) and 3.5% (3721T-4908C-5595C-5904C-6091ins). No discernable pattern could be identified from the remaining haplotypes, each having frequencies <1.5%. All non-major haplotypes were grouped as "rare" *ATGL* haplotypes, and tested for associations with traits using dominant and recessive models, corrected for age, sex and BMI (Table IV). A significant association was identified between rare *ATGL* haplotypes and serum TG concentrations under a dominant model. Subjects with rare haplotypes had higher serum TG concentrations (mean±standard deviation) than subjects with the dominant haplotype (1.21±0.7 mmol/L and 1.11±0.6 mmol/L; p=0.006). Even though the 2 most frequent non-major haplotypes carried a similar SNP, these haplotypes together did not appear to be driving the observed association (p=0.076). No other traits related to cardiovascular health were associated with *ATGL* haplotypes.

DISCUSSION

The principal finding of this study is that *ATGL* haplotypes are associated with plasma TG concentrations in the Greenland Inuit. Subjects carrying rare *ATGL* haplotypes had higher plasma TG concentrations than subjects carrying the major haplotype. However, no specific combination of haplotypes was found to underlie the observed association. Trends towards decreased HDL-cholesterol and increase T2D prevalence were also observed in subjects with rare *ATGL* haplotypes; however, these were not statistically significant. To our knowledge, this study is the first to demonstrate any such association in an Aboriginal population.

Our observations are relevant to the study of *ATGL*, as they suggest that functional variants carried on rare *ATGL* haplotypes may modify *ATGL* expression or function. However, such functional variants were not directly genotyped by our study. These variants may exist in non-coding sequence flanking the 5' or 3' ends of the gene, in promoter, enhancer or regulatory elements which could modulate ATGL expression. Similarly, sequence variants specific to the Greenland Inuit that could be carried on rare *ATGL* haplotypes would have been missed by our sequencing approach. While these are limitations of our study, it is important to identify those functional variants through subsequent analyses, as we have clearly demonstrated that a significant association exists between *ATGL* and plasma TG concentrations.

Our observations suggest that the effect of a functional variant would likely increase ATGL activity. Under normal conditions, TG stored in adipose tissue is mobilized by ATGL, releasing FA for transport to the liver, where TG is resynthesized, packaged into very low-density lipoprotein (VLDL) and delivered to peripheral tissues for energy metabolism (Fig. 2, top). Increased ATGL activity would increase flux of FA to the liver, translating into increased circulating VLDL and thus increased plasma TG

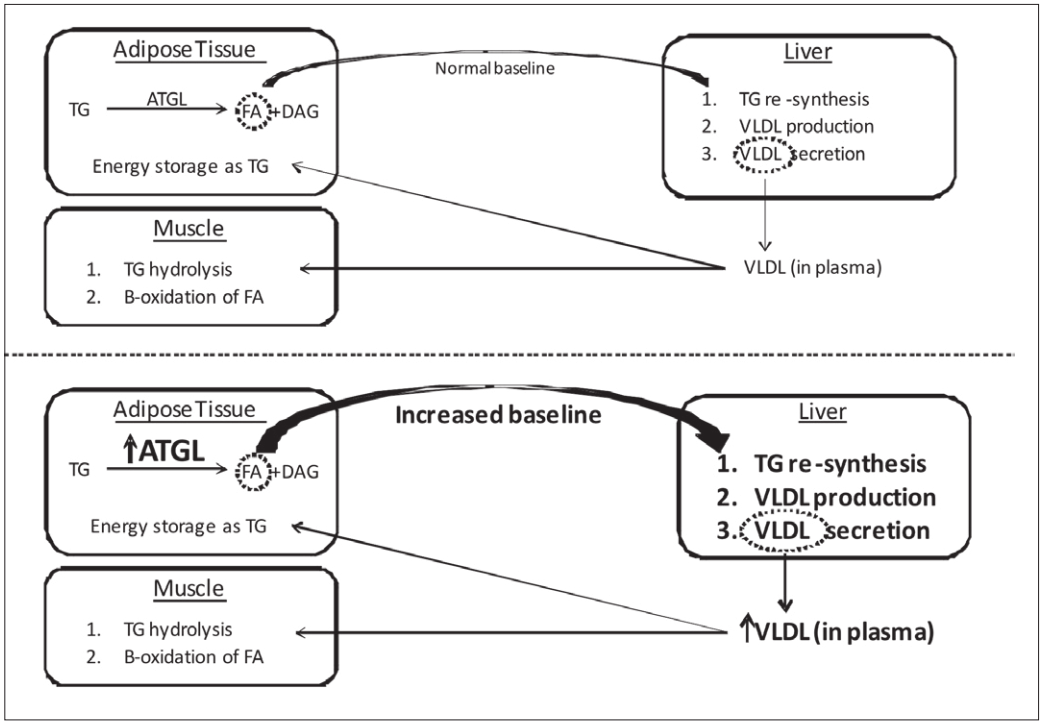

Figure 2. Contribution of ATGL activity to plasma triglyceride concentrations.
Top: At a normal baseline of TG hydrolysis, ATGL hydrolyzes triglycerides (TG) to provide fatty acid (FA) transport to the liver, releasing diacylglycerol (DAG) for subsequent enzymatic hydrolysis. In the liver, FA are resynthesized into TG, packaged into very low-density lipoprotein (VLDL), and VLDL is secreted for distribution of TG to peripheral tissues. When energy is required by tissues such as muscle (fasted state), VLDL delivers TG as a source of FA for ß-oxidation. When energy is available (fed state), TG is returned to adipose for storage. Energy obtained from feeding is also stored as TG in adipose tissue.
Bottom: We hypothesize that historically, increased *ATGL* activity may have provided an increased baseline of FA transport to increase energy availability to tissues, a metabolic advantage in the fasted state. Adoption of a Westernized lifestyle removes the need for such rapid energy metabolism, resulting in futile cycling of TG, an increased baseline of TG hydrolysis and thus increased plasma TG concentrations.

concentrations (Fig. 2, bottom). It is tempting to speculate that increased *ATGL* activity could have once represented an environmental adaptation to a traditional feast or famine lifestyle. Increased adipose tissue TG hydrolysis would have provided a constant flux of FA for hepatic VLDL delivery to peripheral tissues, allowing rapid energy metabolism in the fasted state (Fig. 2, bottom). In times of abundance, the transacylase activity of *ATGL* could have assisted in the re-esterification of FA into TG for storage, maximizing metabolic efficiency through accelerated energy storage. However, in the absence of starvation, an increased baseline of TG hydrolysis would similarly increase plasma TG concentrations through constant production of VLDL. Interestingly, VLDL overproduction is a hallmark of MetS (27), a disease prevalent in the Greenland Inuit (28). Further, a complication of MetS is insulin resistance, also prevalent in the Greenland Inuit (29), which could be stimulated by accumulation of lipotoxic diacylglycerol species caused by increased TG hydrolysis (30). Other factors such a Westernized

diet and sedentary lifestyle undoubtedly also contribute to the observed disease prevalence among Greenland Inuit (31–32).

Only 1 candidate gene study has interrogated *ATGL* for its association with plasma TG concentrations (33). An association was found between common variants in *ATGL* and decreased plasma TG concentrations, a direction of effect opposite to that observed by our study. It is difficult to compare studies as our sample was smaller, population-based and had less variation in TG values. Further, our study identified relatively fewer SNPs, with minor allele frequencies decreased by 11%–18%, perhaps limiting our ability to detect an individual effect of each SNP. Further analyses will be required to determine the exact nature of the association between *ATGL* and plasma TG concentrations. However, our results support that a physiologically relevant association does exist.

Our study complements the growing body of knowledge surrounding *ATGL* and its involvement in TG metabolism. Our findings also support the continued observation that common genetic determinants contribute to variation in quantitative metabolic traits. We have shown that a significant association between rare *ATGL* haplotypes and plasma TG concentration exists in a sample of Greenland Inuit, with observed trends towards other aspects of unfavourable lipid metabolism. Further validation is required to elucidate the mechanism by which *ATGL* modulates plasma TG concentration.

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Conflict of interest disclosures

The authors disclose no conflicts of interest.

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