

Article

ANGPTL3 Variants Associate with Lower Levels of Irisin and C-Peptide in a Cohort of Arab Individuals

Muath Alanbaei ^{1,†}, Mohamed Abu-Farha ^{2,3,†}, Prashantha Hebbar ^{4,†}, Motasem Melhem ³, Betty S. Chandy ³, Emil Anoop ³, Preethi Cherian ², Irina Al-Khairi ², Fadi Alkayal ⁴, Fahd Al-Mulla ^{5,*}, Jehad Abubaker ^{2,*} and Thangavel Alphonse Thanaraj ^{4,*}

¹ Department of Medicine, Faculty of Medicine, Kuwait University, Kuwait

² Biochemistry and Molecular Biology Department, Dasman Diabetes Institute, Kuwait

³ Special Services Department, Dasman Diabetes Institute, Kuwait

⁴ Genetics and Bioinformatics Department, Dasman Diabetes Institute, Kuwait

⁵ Chief Scientific Officer, Research Division, Dasman Diabetes Institute, Kuwait

[†] These authors contributed equally to this work

* Correspondence: jehad.abubaker@dasmaninstitute.org (J.A.); Tel.: +965 2224 2999 Ext. 3563; (J.A.); fahd.al-mulla@dasmaninstitute.org (F.A.-M.); Tel.: +965 2224 2999 Ext. 3563 (F.A.-M.); alphonse.thangavel@dasmaninstitute.org (T.A.T.); Tel.: +965 2224 2999 Ext. 3563 (T.A.T.)

Abstract: ANGPTL3 is an important regulator of lipid metabolism. Its inhibition in people with hypercholesterolemia reduces plasma lipid levels dramatically. Genome-wide association studies have associated ANGPTL3 variants with lipid traits. Irisin, an exercise modulated protein, has been associated with lipid metabolism. Intracellular accumulation of lipids impairs insulin action and contributes to metabolic disorders. In this study, we evaluate the impact of ANGPTL3 variants on levels of irisin and markers associated with lipid metabolism and insulin resistance. ANGPTL3 rs1748197 and rs12130333 variants were genotyped in a cohort of 278 Arab individuals from Kuwait. Levels of irisin and other metabolic markers were measured by ELISA. Significance of association signals was assessed using Bonferroni-corrected P-values and empirical P-values. The study variants were significantly associated with low levels of c-peptide and irisin. Levels of c-peptide and irisin were mediated by interaction between carrier genotypes (GA+AA) at rs1748197 and measures of IL13 and TG, respectively. While levels of c-peptide and IL13 were directly correlated in individuals with reference genotype, they were inversely correlated in individuals with carrier genotype. Irisin correlated positively with TG which is strong in individuals with carrier genotypes. These observations illustrate ANGPTL3 as a potential link connecting lipid metabolism, insulin resistance and cardioprotection.

Keywords: ANGPTL3-DOCK7; irisin; c-peptide; triglyceride; interleukin 13; lipid metabolism; insulin resistance; Arab population

1. Introduction

Dyslipidemia is a major metabolic disease that is affecting more people due to the increased trend in obesity rates worldwide. As a response to positive energy balance, the excess energy is stored as triglyceride (TG) in adipose tissue as well as ectopically. While such a mechanism in humans helps in coping with fluctuating energy supplies, it predisposes persistently over-nourished individuals to weight gain leading to obesity. This unfavorable lipid accumulation happens in “ectopic sites” such as the liver and skeletal muscle, and even in pancreatic β -cells [1]. Such “ectopic” lipid accumulation and disorders of fatty acid-lipid metabolism lead to metabolic dysregulation including increased insulin resistance, inflammation and oxidative stress [2]. Active research is pursued to delineate the mechanistic details of the connection between lipid metabolism and insulin resistance and their modulation by physical exercise.

Angeopietin-like3 (ANGPTL3) protein plays an important role in controlling lipoprotein metabolism and it has been proposed that it may be a novel and effective target for the treatment of dyslipidemia and CVD [3-5]. In coordination with other ANGPTL proteins mainly ANGPTL4 and 8, they collectively regulate lipoprotein lipase activity (LPL). LPL is an important regulator of lipid partitioning to different organs and its tight regulation is critical. ANGPTL3 can directly inhibit LPL or in combination with ANGPTL8 under feeding condition directing triglyceride to adipocytes for storage and away from ectopic sites.

ANGPTL3 gene lies on the forward strand of chromosome 1p31 within an intronic region of *DOCK7* gene, located on the reverse strand. It has been previously reported that a lead SNP namely rs2131925 identifies *ANGPTL3* as TG-associated locus. The rs2131925 is an intronic SNP in the *DOCK7* gene corresponding to a location upstream of *ANGPTL3*. The NHGRI-EBI GWAS Catalog [6], which is a curated collection of all published genome-wide association studies, revealed two more SNPs rs1748197 and rs12130333 from *ANGPTL3* having strong effects on lipid metabolism. Both these two variants are in strong linkage-disequilibrium (LD) with the lead SNP – the first one at $r^2=0.995$ and the second one at $r^2=0.5$. The rs1748197 is an intronic SNP from *ANGPTL3* gene and the rs12130333 is a regulatory region variant downstream of *ANGPTL3* gene. A number of reports in literature presents rs12130333 as located within the *ANGPTL3* locus (i.e. *ANGPTL3* SNP) or as near *ANGPTL3* gene [e.g.[4,7-10]]. Several annotations for trait associations were observed for these two variants. For instance, the rs1748197 was shown to be associated with serum metabolite levels of high-density lipoprotein (HDL) triglyceride (HDL-TG), Monounsaturated fatty acid (MUFA), triglyceride in medium HDL particles (M-HDL-TG), Phosphatidylcholine (PC), Polyunsaturated fatty acid (PUFA), total choline (TotCho), total fatty acid (TotFA), extremely large very low density lipoprotein (XXL_VLDL_C) [11]. While many epidemiological studies have shown that high plasma HDL-C concentrations are associated with lower cardiovascular risk [12,13], recent studies have shown that high HDL-TG is a factor that plays a role in increased cardiovascular risk associated with high TG and that this might be due to the dysfunctional HDL caused by TG overload that in turn could alter vascular healing mechanisms [14,15]. It is also concluded that HDL-TG was positively related to glucose and insulin levels (not to HbA1c) underlining its direct association to glucose metabolic disturbances, opposite to HDL-C and total HDL particles (HDL-P) [15]. On the other hand, the trait annotations observed for the study variant rs12130333 were its association with levels of TG and total cholesterol. Interestingly, the two study variants as well as the leading SNP at the *ANGPTL3* locus for TG were associated with a decrease in LDL-C levels [16,17].

Based on data emerging from various GWA studies on *ANGPTL3* and its role in regulating plasma lipid levels, *ANGPTL3* has been targeted as a treatment for people with elevated plasma lipids, especially for people diagnosed with homozygous familial hypercholesterolemia. Evinacumab, the *ANGPTL3* inhibitor, is a human monoclonal antibody that has been recently approved by United States Food and Drug Administration (FDA). In the Phase 3 clinical trial, patients receiving Evinacumab showed a 49% reduction in LDL-C levels from baseline relative to those receiving placebo at week 24 after treatment [18]. Indeed, this result is very promising since LDL reduction in patients with homozygous familial hypercholesterolemia is very difficult [19]. However, the trial assessed only a small number of patients for a limited period where long term safety and cardiovascular outcomes risk have not been fully examined [18].

Given the above reported link between the *ANGPTL3* SNP variants and lipid metabolism, our aim in this study was to identify associations between these variants and markers associated with lipid metabolism and insulin resistance, such as irisin - a recent marker that has been associated with insulin resistance and cardiovascular risk. Irisin was also shown to be primarily an exercise induced protein. Irisin is a novel myokine product from cleaving a transmembrane protein named Fibronectin type III domain-containing protein 5 (FNDC5). This cleaved form of FNDC5 is released into blood circulation and has been

shown to play an important role in modulating browning of white adipose tissue during exercise [11]. We have previously reported an increased level of this protein in the plasma from obese and type 2 diabetic (T2D) people [20]. There has been considerable interest in the recent past on delineating the physiological role of irisin in processes such as glucose homeostasis and metabolic disorders [21–23]. Further, irisin is now termed as “*exercise-induced myokine*” and the role of physical exercise in inducing the level of irisin and thereby promoting healthy systematic metabolism is now actively examined [24–28]. Irisin was also shown to be involved in fatty acid oxidation. Based on its involvement in various metabolic pathways including lipid metabolism, we were interested in investigating the interaction between ANGPTL3 variants and plasma levels of irisin.

2. Materials and Methods

2.1. Recruitment of Participants and Study Cohort

The Ethical Review Committee of Dasman Diabetes Institute reviewed and approved the study protocol as per the guidelines of the Declaration of Helsinki and of the US Federal Policy for the Protection of Human Subjects (Study number RA2010-003). Native adult Kuwaiti individuals of Arab ethnicity were recruited as study subjects. Pregnant women were excluded. The cohort comprised 278 subjects. For each participant upon enrolment, we recorded data on age, sex, health disorders (e.g. diabetes and hypertension) and baseline characteristics such as height, weight, waist circumference and blood pressure. Information on whether the participant takes lipid lowering or diabetes and antihypertensive medication was recorded and was subsequently used to adjust the models for genotype-trait association tests. Every participant signed the informed consent form before participating in the study.

2.2. Blood sample Collection and Processing

After confirming that the participant had fasted overnight, blood samples were collected in EDTA-treated tubes. Gentra Puregene[®] kit (Qiagen, Valencia, CA, USA) was used to extract DNA, which was quantified using Quant-iT[™] PicoGreen[®] dsDNA Assay Kit (Life Technologies, Grand Island, NY, USA) and Epoch Microplate Spectrophotometer (BioTek Instruments). Absorbance values at 260–280 nm were checked for adherence to an optical density range of 1.8–2.1.

2.3. Estimation of Plasma Levels of Various Biomarkers

Plasma was separated from blood samples by centrifugation, was aliquoted and stored at -80°C .

Plasma level of irisin was detected using the irisin recombinant enzyme immunoassay kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA. Cat #EK-067-29). For the assay, kit instructions were followed: briefly, plasma samples were thawed on ice and centrifuged for 5 min at $10,000 \times g$ at 4°C to remove any remaining cells or platelets. Then samples were diluted $40\times$ with the $1\times$ assay buffer (provided in the kit). The intra-assay coefficient for this ELISA assay was 1.0%–7.0%, while the inter-assay coefficient was $< 20\%$.

Plasma level of ANGPTL3 was detected using the Quantikine Human Angiopoietin-like 3 (ANGPTL3) ELISA (R&D systems, Minneapolis, MN, USA. Cat# DANL30). Blood samples from participants were collected using EDTA tubes and plasma was obtained following the centrifugation of the blood tubes at $400\times g$ for 10 min. Plasma was then transferred into new tubes and stored at -80°C for later use. For the assay, kit instructions were followed. In brief, plasma samples were thawed on ice and centrifuged for 5 min at $10,000 \times g$ at 4°C , to remove any remaining cells or platelets. For the assay samples were diluted $50\times$ with calibrator diluent RD6Q provided in the kit. Standards were prepared in calibrator diluent RD6Q using the recombinant human ANGPTL3 standard provided in the kit (diluted to the following concentrations (ng/mL): 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156).

Then 100µl of the assay diluent RD1-76 was transferred into the wells of the plate followed by 50µl of sample and standards into respective wells. The plate was then incubated for 2hrs at room temperature (RT) with gentle shaking. Following incubation, the plate was washed 4X with 1X wash buffer provided in the kit and 200µl of ANGPTL3 conjugated antibody was added into each well. The plate was then incubated for 1hr at RT on shaker, followed by the wash step. The substrate (200µl) was then added into each well and incubated for 30min at RT on benchtop protected from light. Finally, the reaction was stopped using the stop solution provided in the kit and the absorbance of each well was read at 450nm using the Synergy H4 plate reader. Concentration of the unknown samples was determined using the standard curve equation obtained from plotting the standard concentration in relation to the O.D. reading. The intra-assay coefficient for this ELISA assay was 2.0%–5.0%, while the inter-assay coefficient was < 10%.

C-peptide plasma level was detected using Mercodia C-peptide ELISA (Mercodia AB, Sylveniusgatan, Sweden, Cat# 10-1136-01). Blood samples from participants were collected, processed, and stored as described in the protocol above. For the assay, kit instructions were followed. Briefly, for the assay samples were centrifuged as described above and used as is. Calibrators and controls used for the assay are provided with the kit in lyophilized form and were reconstituted using distilled water (Calibrator concentrations used were (pmol/l): 95.2, 328, 1270, 2510, 4060). For the assay 25µl of the calibrator, control and sample was transferred into the respective wells of the plate followed by 50µl of assay buffer provided in the kit. The plate was then incubated for 1hr at room temperature (RT) with shaking. Following the incubation, the plate was washed 6X with 1X wash buffer provided in the kit and 100µl of the enzyme conjugate 1X solution, provided in the kit, was added into each well. The plate was then incubated for 1hr at RT on shaker, followed by the wash step. The substrate TMB (200µl) was then added into each well and incubated for 15min at RT on benchtop protected from light. Finally, the reaction was stopped using the stop solution provided in the kit and the absorbance of each well was read at 450nm using the Synergy H4 plate reader. Concentration of the unknown samples was determined using the standard curve equation obtained from plotting the standard concentration in relation to the O.D. reading. The intra-assay coefficient for this ELISA assay was 2.0%–5.0%, while the inter-assay coefficient was < 10%.

2.4. Targeted Genotyping of the ANGPTL3 study variants rs1748197 and rs12130333

We performed candidate SNP genotyping using TaqMan® Genotyping Assay kit on ABI 7500 Real-Time PCR System from Applied Biosystems (Foster City, CA, USA). Each polymerase chain reaction sample was composed of 10 ng of DNA, 5× FIREPol® Master Mix (Solis BioDyne, Estonia), and 1 µl of 20× TaqMan® SNP Genotyping Assay. We set the thermal cycling conditions at 60°C for 1 min and at 95°C for 15 min followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. We performed Sanger sequencing, using the BigDye™ Terminator v3.1 Cycle Sequencing on an Applied Biosystems 3730xl DNA Analyzer, for selected cases of homozygotes and heterozygotes to validate genotypes determined by the above techniques.

2.5. Quality procedures for SNP and Trait Measurements

We used PLINK (version1.9) [29] to assess SNP quality and statistical associations with traits. We calculated minor allele frequency (MAF) and Hardy–Weinberg equilibrium for the two study variants. Any quantitative trait value < Q1- 1.5 * IQR or any value > Q3+1.5 * IQR was considered as an outlier and was excluded from further statistical analysis.

2.6. Allele-based association tests and thresholds for ascertaining statistical significance

Allele-based statistical association tests for the two study variants with 28 quantitative traits and biomarker levels were performed using linear regression adjusting for

regular corrections toward age and sex. We also adjusted for diabetes medication and lipid-lowering medication. Of the 28 quantitative traits, the matSpD tool [30] (available at <https://sites.google.com/site/qutsgel/software/matspd-local-version>) identified only 17 as effective independent traits. Correction for multiple testing was assessed by adjusting the P-value threshold for the 17 independent traits (0.05/effective number of independent traits), which was (0.05/17 = 0.003). Empirical P-values (P_{emp} -value) were generated using the max(T) permutation procedure available in PLINK, based on 10,000 permutations. A threshold of <0.05 was set for P_{emp} -value.

2.7. Assessing the interaction of correlations between study variants and traits

Interaction of association signal (involving a SNP and associating trait) with another trait (example: Irisin~ rs1748197+age+sex+TG+rs1748197*TG) was evaluated using multi-variable linear regression tests using R statistical environment [31]. Summary statistics on the relationships among the traits/biomarkers included percentage variation in response variable (r^2), estimates denoting standardized beta-coefficients, standard error, and significance of test (P) for the genotype distributions. P-value threshold was set at 0.05.

3. RESULTS

3.1. Characteristics of the two ANGPTL3 variants

In our study cohort, comprising 278 participants, the minor allele (A) of rs1748197_G_A occurred at a frequency of 0.355 and the minor allele (T) of rs12130333_C_T occurred at 0.146. Both the SNPs passed the tests for Hardy–Weinberg equilibrium (HWE). Summary statistics on the two study variants are presented in Supplementary Table S1.

3.2. Characteristics of the study cohort

The mean age of the participants in the study cohort was 46.25 ± 12.38 years, with the ratio of males to females as 1:1.22 (Table 1). The cohort comprised mostly class I obese people with a mean body mass index (BMI) of 29.93 ± 5.17 kg/m² and mean waist circumference (WC) of 99.36 ± 13.36 cm. Up to 48.6% of the participants were obese and 43.16% were afflicted with type 2 diabetes. Mean values for HbA1c ($6.31 \pm 1.3\%$), LDL (3.13 ± 0.96 mmol/l), HDL (1.2 ± 0.32 mmol/l), total cholesterol (TC) (5.02 ± 1.09 mmol/l) and TG (1.22 ± 0.6 mmol/l) were normal or near normal. Of the 278 people, 101 were taking T2D medication and 88 were taking medications to control the level of lipid in blood circulation.

Table 1. Clinical characteristics of the study cohort. A total of 28 traits and biomarkers were considered in the study.

Traits	Number of participants measured	All participants (Mean±SD)	Non-diabetic participants (Mean±SD)	Diabetic participants (Mean±SD)	P-value ^s (for differences in mean values between diabetic and non-diabetic participants)
Male:Female	278	125:153	65:93	60:60	0.1772
Age (in years)	278	46.25±12.38	42.04±12.57	51.8±9.70	3.03e-12
Height (in meter)	278	1.64±0.09	1.64±0.09	1.65±0.09	0.516
Weight (in kg)	278	81.40±16.23	77.65±16.49	86.27±14.57	7.07e-06
BMI (kg/m ²)	278	29.93±5.17	28.81±5.46	31.41±4.37	1.36e-05
WC (in cm)	177	99.36±13.36	93.95±13.43	105.22±10.57	3.58e-09
HDL (in mmol/l)	260	1.20±0.32	1.25±0.31	1.13±0.31	0.0018
TC [®] (in mmol/l)	272	5.27±1.04	5.56±1.42	5.40±1.22	0.068
LDL [®] (in mmol/l)	269	3.38±0.94	3.68±1.39	3.51±1.16	0.049

Non-HDL* (in mmol/l)	258	4.018±1.05	4.34±1.31	4.16±1.18	0.031
TG (in mmol/l)	260	1.22±0.59	1.07±0.57	1.43±0.56	7.09e-7
FPG (in mmol/l)	241	5.77±1.24	5.21±0.63	6.73±1.42	5.19e-16
HbA1c (%)	254	6.31±1.29	5.61±0.60	7.30±1.36	<2.2e-16
Irisin (ng/ml)	219	556.95±192.26	507.96±170.77	620.89±200.67	1.83e-05
IL7 (pg/ml)*	163	13.23±5.75	12.09±5.44	14.86±5.82	0.0026
IL13 (pg/ml)	158	9.67±4.84	9.21±4.95	10.33±4.63	0.1491
Insulin (pg/ml)	195	14.90±11.97	13.41±11.19	16.83±12.71	0.0511
c-peptide (pg/ml)	161	2.66±1.73	2.76±1.71	2.56±1.75	0.4636
ANGPTL3 (ng/ml)	195	37.42±10.29	36.77±10.33	38.21±10.24	0.3337
TNFα (pg/ml)	167	127.67±32.16	125.31±32.97	131.19±30.81	0.2417
Obese status	278	135:143	62:96	73:47	0.00056
Diabetes status	278	120:158	0:158	120:0	-
Anti-diabetic medication	278	101(med):177 (no med)	158 (no med):0	19 (No med):101 (med)	0.001
Lipid lowering medication	278	88(med):190	21(med):137	67(med):53	1.43e-13

*, Pearson's Chi-square test was used for categorical variables and Student's t-test was used for quantitative variables. *, Levels of the following 11 interleukins were considered: IL1b, IL1ra, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL13 and IL17. A total of 28 traits and biomarkers were considered in association tests.

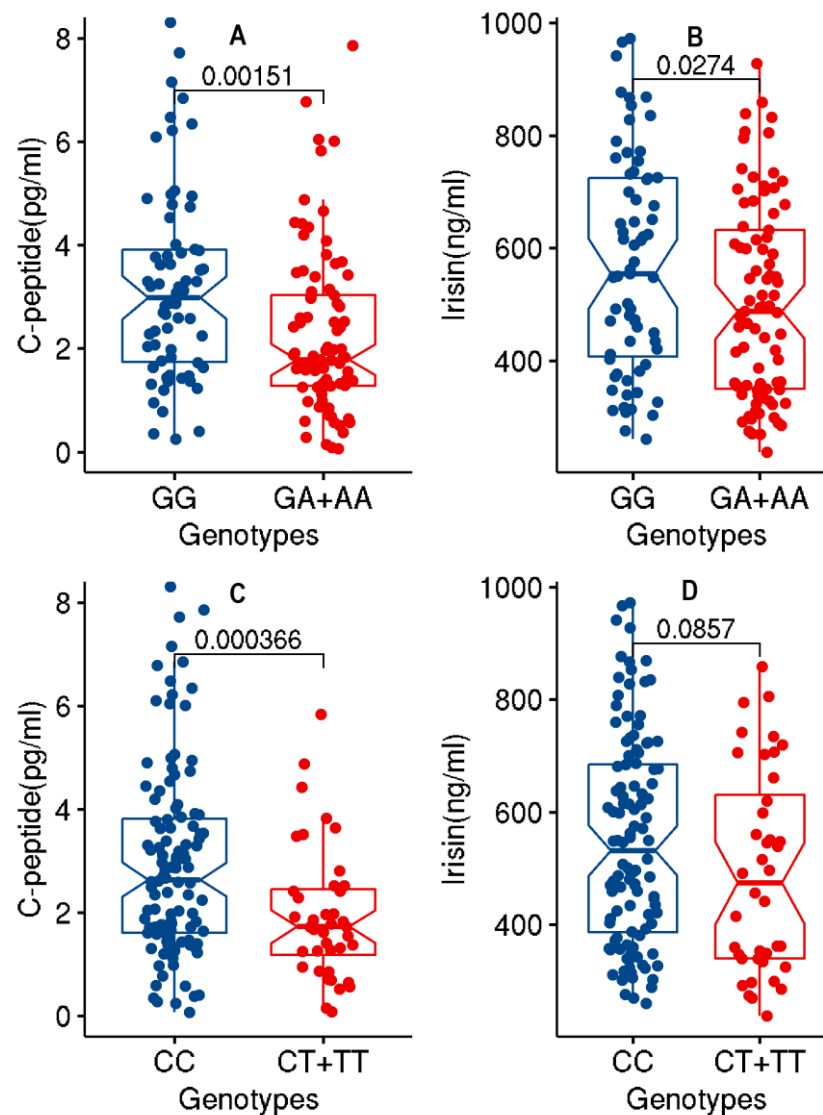
*, The values for TC, and LDL were adjusted for lipid lowering medication by adopting procedures used in [32]: TC_{adjusted}=TC/0.8; and LDL_{adjusted}=LDL/0.7. The nonHDL was calculated by subtracting HDL from adjusted TC.

Participants in our study cohort afflicted with diabetes differed significantly from those without diabetes in anthropometric trait measurements (age [P-value=3.03E-12], weight [P-value=7.07E-06], BMI [P-value=1.36E-05] and WC [P-value=3.58E-09]), lipid trait measurements (HDL [P-value=0.0018] and TG [P-value=7.09E-07]), glycemic indices (FPG [P-value=5.19E-16] and HbA1c [P-value=2.2E-16]), in obesity status and insulin [P-value=0.051], irisin [P-value=1.83E-05].

Examination of the clinical characteristics with participants distributed gender-wise (Supplementary Table S2) showed significant differences in the traits of height, weight, WC, HDL, TG and FPG.

Upon partitioning the cohort based on genotypes (GG versus GA+AA) at the rs1748197 variant, it was found that the sub-cohorts exhibited significant differences (P-value ≤ 0.05) in the mean values for irisin and c-peptide. In a similar manner, upon partitioning the cohort based on genotypes (CC versus CT+TT) at the rs12130333 variant, the sub-cohorts exhibited significant differences (P-value ≤ 0.05) in the mean values for c-peptide and irisin (**Figure 1 and Supplementary Table S3**). Significant differences were also seen in the levels of ANGPTL3 but only with the rs1748197 variant (40.077±10.23 versus 35.65±9.85; P-value=0.004).

Figure 1. Box-plots displaying data distribution for the phenotype traits of c-peptide and irisin in individuals with genotypes (GA+AA) containing the effect allele or genotypes (GG) homozygous for reference allele at variant rs1748197 (Figure 1.A, B); and in individuals with genotypes (CT+TT) containing the effect allele or genotypes (CC) homozygous for reference allele at variant rs12130333 (Figure 1.C, D).



3.3. Association of the two ANGPTL3 study variants with lower levels of c-peptide and Irisin at significant P-values

Statistically significant associations (P-value threshold corrected for multiple testing ≤ 0.0019 and empirical P-value, $P_{emp}\text{-value} \leq 0.05$) between the variants (with the minor allele as the effect allele) and two traits (c-peptide and irisin) were observed (**Table 2**): (rs1748197:c-peptide [P-value=0.0001; $P_{emp}\text{-value} = 0.006$; effect size=-0.6976]; rs1748197: irisin [P-value=0.0002; $P_{emp}\text{-value} = 0.01$; effect size=-63.1]; rs12130333:c-peptide [P-value=0.0003; $P_{emp}\text{-value} = 0.015$; effect size=-0.9002]; rs12130333: irisin [P-value=0.002; $P_{emp}\text{-value} = 0.098$; effect size=-72.61]; though the $P_{emp}\text{-value}$ is >0.05 for the last association (rs12130333: irisin), the P-value is significant. Both the variants were seen associated with lower levels of TG at P-value threshold not adjusted for multiple testing (≤ 0.05) (Supplementary Table S4).

Table 2. Results of association tests for the two study variants with the levels of c-peptide and irisin, using genetic model based on additive mode of inheritance. Of the 28 traits studied for association with the two variants, only 17 were found independent of one another. Significant P-values passing the threshold for multiple testing ($P\text{-value} \leq 0.003 = 0.05/17$) and significant $P_{emp}\text{-value} \leq 0.05$ are high-lighted in bold & italics font. Association tests were adjusted for age, sex (Regular correction) and further confounders of diabetes medication (DM) and lipid lowering medication (LLM). Association signals with other traits not meeting the significance dictated by P-value threshold adjusted for multiple testing are given in Supplementary Table S4.

Traits	SNP with effect allele	Correction	Sample size [®]	Beta	P-value	Empirical P-value (P_{emp} -value)
c-peptide	rs1748197	R	160	-0.6976	<i>0.000127</i>	<i>0.00679</i>
		DM	160	-0.6944	<i>0.000161</i>	<i>0.00939</i>
		LLM	160	-0.6964	<i>0.000138</i>	<i>0.00739</i>
	rs12130333	R	161	-0.9002	<i>0.00032</i>	<i>0.0154</i>
		R+DM	161	-0.8991	<i>0.000335</i>	<i>0.0174</i>
		R+LLM	161	-0.9117	<i>0.000288</i>	<i>0.0167</i>
Irisin	rs1748197	R	217	-63.1	<i>0.000299</i>	<i>0.0149</i>
		DM	216	-67.78	<i>9.58E-05</i>	<i>0.0047</i>
		LLM	216	-63	<i>0.000357</i>	<i>0.0184</i>
	rs12130333	R	218	-72.61	<i>0.002135</i>	0.0979
		R+DM	217	-70.87	<i>0.002436</i>	0.1184
		R+LLM	217	-74.3	<i>0.001806</i>	0.0898

[®], Sample size differs for each of the traits - examination of outliers and missing information for each trait measurement, through quality control steps, led to differing sample sizes for different traits.

Evaluation of the identified association signals from these two variants with the levels of irisin and c-peptide revealed that these two association signals were consistently significant (P -value ≤ 0.05) in the sub-cohorts of individuals afflicted with diabetes (Supplementary Table S5).

3.4. Associations of the haplotype of the ANGPTL3 study variants with the levels of c-peptide, and irisin

Upon considering the two study variants as a haplotype (rs1748197-rs12130333), we observed significant associations between the haplotype with irisin and c-peptide when the haplotype contains effect alleles at both the variants (rs1748197_A-rs12130333_T) or reference alleles at both the variants (rs1748197_G-rs12130333_C) (Table 3). The association with irisin was particularly significant as both the P -values and P_{emp} -values were significant. While the reference haplotype (G-C) was associated with increased levels of irisin and c-peptide, the haplotype with effect alleles (A-T) was associated with decreased levels of these biomarkers.

Table 3. Results of association tests for the haplotype of rs1748197-rs12130333 with the levels of c-peptide and irisin. Effect allele at rs1748197_G_A is A and at rs12130333_C_T is T. Significant P -values (≤ 0.05) and significant P_{emp} -value ≤ 0.05 are high-lighted in bold & italic font. Significant P -values are highlighted in bold font.

Trait	Haplotype (GC forms the reference haplotype)	Frequency	Beta	P-value	Empirical P-value (P_{emp} -value)
Irisin	AT	0.134	-74.9	<i>0.00313</i>	<i>0.0122</i>
	GT	0.013	-110	0.262	0.6224
	AC	0.221	-36.4	0.0951	0.2853
	GC	0.632	68.2	<i>0.000162</i>	<i>0.00059</i>
c-peptide	AT	0.134	-1.45	<i>0.0368</i>	0.1363
	GT	0.013	-2.92	0.304	0.6326
	AC	0.221	-0.49	0.413	0.7832

GC	0.632	1.17	0.0182	0.0891
----	-------	------	---------------	--------

3.5. Interactions between genotypes at the study variants and correlations among the levels of c-peptide or irisin and other traits

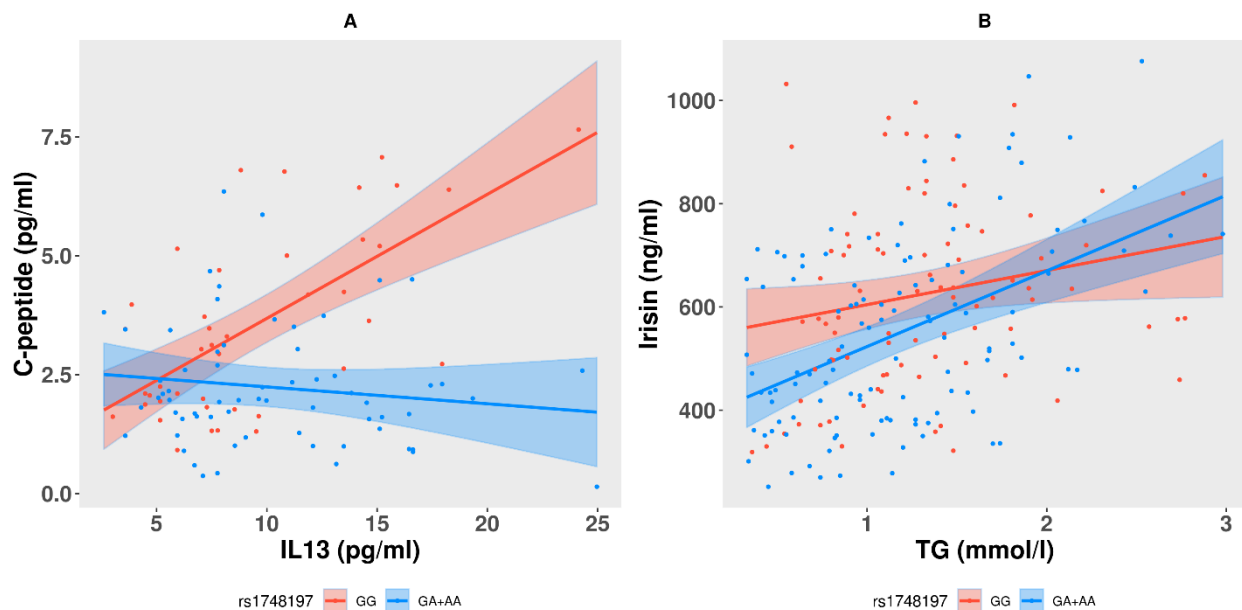
Results of examining correlations amongst biomarkers and their interaction with genotypes at the study variants are presented in **Table 4**.

Table 4. Linear regression model illustrating the link between the rs1748197 and interaction of c-peptide with IL13 or of Irisin with TG. Results are shown for carrier genotypes (GA and AA) regressed against the reference GG genotype.

Trait (response variable)	Genotype and interacting trait (Predict variable)	Estimate	Std. Error	P-value	Adj. R-square	Model P-value
Model:c-peptide~rs1748197+age+sex+IL13+rs1748197*IL13						
c-peptide	(Intercept)	-0.2927	0.8453	0.7299	0.3416	4.84E-09
	rs1748197GA+AA	1.5210	0.6446	0.0202		
	IL13	0.261	0.046	1.96E-07		
	rs1748197GA+AA:IL13	-0.2961	0.0584	1.88E-06		
Model:Irisin~ rs1748197+age+sex+TG+rs1748197*TG						
Irisin	(Intercept)	296.583	74.581	9.78E-05	0.2163	2.27E-10
	rs1748197GA+AA	-161.75	57.993	0.0058		
	TG	66.04	32.31	0.042		
	rs1748197GA+AA:TG	80.551	41.507	0.053		

The results revealed that (i) measures of c-peptide was mediated by interaction between carrier genotypes (GA+AA) at rs1748197 and measures of IL13; and (ii) measures of irisin was mediated by interaction between carrier genotypes (GA+AA) at rs1748197 and measures of TG. No interaction between the genotypes at the other variant rs12130333 with any of the biomarkers was observed. The model for the interaction of c-peptide with IL13 at the genotypes GG (with intercept coefficient -0.292 and slope 0.26) and (GA+AA) (with mean change 1.52 pg/ml in c-peptide and slope -0.036 [indicated by the sum of 0.261 and -0.2961]) suggested that one unit increase in IL13 would result in 0.036 pg/ml decrease in c-peptide. However, exactly the opposite effect was observed with regard to the reference genotype of GG as evident from the slopes of plot (**Figure 2.A**). From the interaction analysis of irisin with TG level at genotypes GG (with intercept 296.58 and slope 66.04) and (GA+AA) (with mean change in irisin -161.75 ng/ml and slope 146.59 [indicated by the sum of 66.04+80.55] suggested that one unit increase in TG would result in 146.59 ng/ml increase in irisin (see Table 4). Though similar effect was observed with genotype GG, steepness of the slope (i.e. effect) at the carrier genotypes (GA+AA) is much higher than that at the reference genotype GG suggesting that reduction in irisin with increase in TG is more rigorous in individuals carrying the (GA+AA) genotypes (**Figure 2.B**).

Figure 2. Interactions observed in (A) c-peptide with IL13 (negative correlation) and in (B) Irisin with TG (positive correlation) in interaction with the GA+AA carrier genotype at the study variant of rs1748197.



3.6. Disease status of the cohort participants and the impact of the effect alleles at the study variants on the levels of c-peptide, irisin and TG

We performed allele-based logistic regression analysis to evaluate whether the disease status (obesity, diabetes, and hypertension) of the study participants is due to the variants (**Supplementary Table S6**). Though the odds-ratio (OR) values were notable, the P-values were not significant. This was done to confirm that the impact of the effect alleles at the two variants on the levels of c-peptide, irisin and TG is not due to associated diseases in the study cohort.

3.7. Power calculation

Power calculation results suggest slight differences between observed (Table 2) and expected (**Supplementary Table S7**) effect sizes for the sample size used for c-peptide (n=161) and irisin (n=217). The observed and expected effect sizes for the variants rs1748197 (MAF=0.35) and rs12130333 (MAF=0.15) at closest sample size of c-peptide (n=167) were [observed=-0.69, expected=-0.54] for rs1748197 and [observed=-0.90, expected=-0.73] for rs12130333; similarly, the effect sizes considering the closest sample size of irisin (n=214) were [observed=-63.1, expected=-54.07] for rs1748197 and [observed=-72.61, expected=-72.23] for rs12130333. Similarly, the observed (and expected) beta-values for association of rs1748197 with irisin interacting with TG at sample size of 218 were 80.551 (and 91.63). Also, the observed (and expected) beta-values for association rs1748197 with c-peptide interacting with IL13 at sample size of 160 were -0.296 (and -0.113). At these sample sizes, upon considering type 1 error at >0.05 and population mean \pm standard deviation of each of the traits, the observed genetic effect accounts approximately for 3.6% variance in irisin level and 4.6% variance in c-peptide level.

4. DISCUSSION

In our study we investigated two *ANGPTL3* gene variants (rs1748197_A and rs12130333_T) that are previously associated with metabolite levels and metabolic traits. The rs1748197 variant has been shown to be associated with the metabolites of lipids, particularly those of VLDL and HDL, in Finnish population [11] while rs12130333 has been associated with TG in cohorts of European and mixed populations [33-35] as well as with total cholesterol in a cohort of mixed ancestries [35]. Further, rs1748195 which is in strong Linkage disequilibrium with rs1748197 ($r^2 = 0.96$) has been shown to be associated with TG at genome-wide significance in individuals of European ancestry [36] and at

suggestive P-value in a meta-analysis from Kuwait [37]. We report in this study, for the first time, association of these two variants with irisin and c-peptide at significant P-values and empirical P-values; participants with carrier genotypes at either of the two study variants have significantly lower levels of irisin and c-peptide. Further, haplotype comprising minor alleles of both the SNPs showed significant association with lower levels of Irisin.

Furthermore, we observe suggestive evidence for both the variants to associate with lower levels of TG. Participants with carrier genotypes at rs1748197 have significantly lower levels of ANGPTL3 protein. It is possible that this reduction in TG level is associated mainly with increased LPL activity since ANGPTL3 levels were also reduced. This could be a main driver in reducing the level of TG seen in people carrying this variant as it has been reported in many other studies that loss of function or oligonucleotide targeting of ANGPTL3 was associated with reduced plasma lipoproteins [38,39]. Oligonucleotide targeting of ANGPTL3 also resulted in reduction of atherogenic lipoprotein levels including LDL-C, VLDL and ApoB amongst others [38]. Given the fact that ANGPTL3 is a potent inhibitor of LPL [40], it is expected that reducing its level will result in LPL activation. However, interestingly in this paper irisin could also play a role in this pathway. Even though, irisin has been initially labelled as a muscle produced protein, its expression has been also detected in the liver where it played a role in hepatic glucose and lipid metabolism [41]. Additionally, ANGPTL3 is a main regulator of lipid uptake into skeletal muscles through its attenuation of LPL activity adding another potential mechanism of regulation [42]. As a result, it can be proposed that a crosstalk between the two proteins might mediate their regulation of lipid metabolism potentially through regulating LPL activity. However, further work is needed to validate these findings and shed more light on the potential mechanism of action.

When we tested the effect of type 2 diabetes (T2D) disease status in the study cohort, we found that the levels of irisin as well as other clinical traits, such as TG, FPG, HbA1c, BMI and waist circumference were significantly higher in individuals with T2D when compared to non-diabetic individuals. Levels of irisin are known to be elevated in T2D and are positively correlated with BMI while negatively correlated with visceral adiposity [43]. Irisin levels are positively associated with insulin resistance as assessed by HOMA-IR [44]. While the higher levels of the above-mentioned biomarkers in the study participants with T2D are as expected, our results that the effect alleles at the two study variants lead to lower levels of these biomarkers make us inquisitive about the physiological impact of the effect alleles. This is of particular interest especially since our observations indicated that (i) the effect alleles at the two variants do not contribute to the risk of T2D, obesity or hypertension in our cohort; (ii) we do not find any significant differences in the genotype distributions (carrier versus reference) between diabetic and non-diabetic individuals; (iii) the measurements of anthropometry traits, glycaemia traits or lipid traits do not differ significantly between the participants with carrier genotypes and those with reference genotypes; and iv) the effect allele and carrier genotypes at the two study variants are associated with lower levels of irisin and c-peptide in our cohort.

Our study results suggest that TG mediates the levels of irisin via interaction with the carrier genotypes at rs1748197 and that IL-13 mediates the levels of c-peptide via interaction with the carrier genotypes at rs1748197. As regards c-peptide, increased levels are observed with the (GA+AA) carrier genotype at rs1748197, while the opposite effect was observed with the reference GG genotype. A reduction in irisin was observed accompanied with an increase in the level of TG. This effect was more evident in individuals carrying the carrier genotypes (GA+AA). Similar results have been previously reported, where serum irisin level was shown to be inversely associated with liver TG contents in obese adults [45]. Irisin is thought to improve hepatic glucose and lipid metabolism by way of positively impacting the liver and pancreatic cells through reducing endoplasmic

reticulum stress and thereby to act as insulin sensitizing hormone [22]. Studies have demonstrated that high plasma TG are associated with insulin resistance and T2D [46]. This was also observed among individuals who only show insulin resistance and have not yet developed T2D [47]. Levels of c-peptide are known to be indicators for MetS and Coronary artery disease (CAD) [48-52] and they positively correlate with HOMA-IR [53,54]. Increased level of IL13 has been observed in subjects with insulin resistance [55]. It has been also demonstrated, using mice models, that IL13 plays a key role in the regulation of hepatic glucose production [56] and in controlling blood sugar [57]. Based on the current knowledge as mentioned above on the role of irisin, TG, c-peptide and IL13 and our observation that the effect alleles present at the two study variants lead to lower levels of irisin and c-peptide, we speculate that these two variants provides protection against insulin resistance in people who carry these carrier genotypes.

Irisin has remained an interesting molecule since its identification as an endurance exercise induced molecule [25], which was further validated by other studies [58,59]. Nonetheless, it was further reported by Timmons et al that muscle FNDC5 (the parent protein from which irisin is cleaved) was not changing after exercise [27] which was further corroborated in a human clinical trial where no difference was observed in the level of irisin in a group of young healthy males after exercise [59]. These conflicting observations led many people to suggest a problem in the method of quantification [60] while others suggested an alternative mode of regulation for FNDC5/irisin expression in lipid metabolism [41]. Irrespective of the effect of exercise on the irisin level, our data further supports a role for Irisin in lipid metabolism potentially through ANGPTL3 and LPL.

We understand that the low sample size of the study cohort as well as the variation in sample size between c-peptide and irisin association studies may slightly inflate the observed effect size of the association analysis. Nevertheless, the strength of these associations was testified by means of empirical P-value calculations. Therefore, it is not necessary to test these associations in cohorts of larger sample size.

5. CONCLUSIONS

The *ANGPTL3* variants are associated with lower levels of irisin and c-peptide and thereby act as protective variants for diabetes and metabolic disorders. The associations between *ANGPTL3* variants and these markers shed light on the possible impact of these variants on metabolism and explain the cardioprotective impact of *ANGPTL3* variants by lowering the level of irisin. Our findings illustrate the key role that the *ANGPTL3* plays in connection between lipid metabolism and insulin resistance.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Supplementary Table S1: SNP quality assessment tests for Hardy-Weinberg equilibrium (HWE); Supplementary Table S2: Clinical characteristics of the study cohort as per gender-wise distribution.; Supplementary Table S3: Differences in the levels of c-peptide and irisin between individuals with reference homozygous genotypes and those with carrier genotypes at each of the two study variants.; Supplementary Table S4: Results of association tests for the two study variants with phenotype traits, using genetic model based on additive mode of inheritance. Association tests were adjusted for age, sex (Regular correction) and further confounders of diabetes medication (DM) and lipid lowering medication (LLM). Significant P-values passing the threshold for multiple testing (P-value $\leq 0.003 = 0.05/17$) and significant $P_{emp\text{-value}} \leq 0.05$ are high-lighted in bold & italics font. Associations with P-values ≤ 0.05 are highlighted in bold font.; Supplementary Table S5: Evaluation of the association signals relating to c-peptide and irisin in the sub-cohorts of entirely diabetic individuals and of entirely non-diabetic individuals.; Supplementary Table S6: Logistic regression analysis for impact of the two study variants on the disease status of the study participants.; Supplementary Table S7: Power Calculation for the association of the rs1748197_A variant (MAF=0.35) and rs12130333_T variant (MAF=0.15) with c-peptide and Irisin. Calculations for “gene only” hypothesis were performed using additive genetic model (AA versus AG versus GG for rs1748197; TT versus TC versus CC for rs12130333); calculations for “gene-environment GxE” hypothesis were performed

using dominant genetic model (AA versus (AG+GG) for rs1748197; TT versus TC+CC for rs12130333).

Author Contributions: Conceptualization, M.A., M.A.-F. J.A., F.A.M. and T.A.T.; Data curation, M.M.; Formal analysis, M.A.-F., P.H. and T.A.T.; Investigation, M.A., F.A.-M., J.A. and T.A.T.; Methodology, M.M., B.S.C., E.A., P.C., I.A.K. and F.A.; Software, P.H.; Resources, M.A.-F.; Software, P.H.; Supervision, M.A.-F and J.A.; Validation, P.H.; Writing – Original Draft Preparation, P.H., T.A.T., M.A.-F., M.A. and J.A.; Writing – Review & Editing, M.A.-F, P.H., T.A.T., J.A. and F.A.M. All authors read and approved the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and of the US Federal Policy for the Protection of Human Subjects, and was approved by the Ethical Review Committee of Dasman Diabetes Institute (Study number RA2010-003).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section “MDPI Research Data Policies” at <https://www.mdpi.com/ethics>. You might choose to exclude this statement if the study did not report any data.

Acknowledgments: Kuwait Foundation for the Advancement of Sciences (KFAS) is acknowledged for institutional funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Unger, R.H. Minireview: Weapons of lean body mass destruction: The role of ectopic lipids in the metabolic syndrome. *Endocrinology* **2003**, *144*, 5159-5165.
2. Savage, D.B.; Petersen, K.F.; Shulman, G.I. Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiol Rev* **2007**, *87*, 507-520.
3. Abu-Farha, M.; Abubaker, J.; Tuomilehto, J. Angptl8 (betatrophin) role in diabetes and metabolic diseases. *Diabetes Metab Res Rev* **2017**, *33*.
4. Su, X.; Peng, D.Q. New insights into angptl3 in controlling lipoprotein metabolism and risk of cardiovascular diseases. *Lipids Health Dis* **2018**, *17*, 12.
5. Graham, M.J.; Lee, R.G.; Brandt, T.A.; Tai, L.J.; Fu, W.; Peralta, R.; Yu, R.; Hurh, E.; Paz, E.; McEvoy, B.W., *et al.* Cardiovascular and metabolic effects of angptl3 antisense oligonucleotides. *N Engl J Med* **2017**, *377*, 222-232.
6. Buniello, A.; MacArthur, J.A.L.; Cerezo, M.; Harris, L.W.; Hayhurst, J.; Malangone, C.; McMahon, A.; Morales, J.; Mountjoy, E.; Sollis, E., *et al.* The nhgri-ebi gwas catalog of published genome-wide association studies, targeted arrays and summary statistics 2019. *Nucleic acids research* **2019**, *47*, D1005-D1012.
7. Ma, L.; Ballantyne, C.M.; Belmont, J.W.; Keinan, A.; Brautbar, A. Interaction between snps in the rxra and near angptl3 gene region inhibits apob reduction after statin-fenofibric acid therapy in individuals with mixed dyslipidemia. *J Lipid Res* **2012**, *53*, 2425-2428.
8. Wang, J.; Ban, M.R.; Zou, G.Y.; Cao, H.; Lin, T.; Kennedy, B.A.; Anand, S.; Yusuf, S.; Huff, M.W.; Pollex, R.L., *et al.* Polygenic determinants of severe hypertriglyceridemia. *Hum Mol Genet* **2008**, *17*, 2894-2899.
9. Jaromi, L.; Csongei, V.; Polgar, N.; Rappai, G.; Szolnoki, Z.; Maasz, A.; Horvatovich, K.; Safrany, E.; Sipeky, C.; Magyari, L., *et al.* Triglyceride level-influencing functional variants of the angptl3, cilp2, and trib1 loci in ischemic stroke. *Neuromolecular Med* **2011**, *13*, 179-186.

10. Minicocci, I.; Montali, A.; Robciuc, M.R.; Quagliarini, F.; Censi, V.; Labbadia, G.; Gabiati, C.; Pigna, G.; Sepe, M.L.; Pannozzo, F., *et al.* Mutations in the *angptl3* gene and familial combined hypolipidemia: A clinical and biochemical characterization. *The Journal of clinical endocrinology and metabolism* **2012**, *97*, E1266-1275.
11. Gallois, A.; Mefford, J.; Ko, A.; Vaysse, A.; Julienne, H.; Ala-Korpela, M.; Laakso, M.; Zaitlen, N.; Pajukanta, P.; Aschard, H. A comprehensive study of metabolite genetics reveals strong pleiotropy and heterogeneity across time and context. *Nature communications* **2019**, *10*, 4788.
12. Rader, D.J.; Hovingh, G.K. Hdl and cardiovascular disease. *Lancet* **2014**, *384*, 618-625.
13. Emerging Risk Factors, C.; Di Angelantonio, E.; Sarwar, N.; Perry, P.; Kaptoge, S.; Ray, K.K.; Thompson, A.; Wood, A.M.; Lewington, S.; Sattar, N., *et al.* Major lipids, apolipoproteins, and risk of vascular disease. *JAMA* **2009**, *302*, 1993-2000.
14. Holmes, M.V.; Millwood, I.Y.; Kartsonaki, C.; Hill, M.R.; Bennett, D.A.; Boxall, R.; Guo, Y.; Xu, X.; Bian, Z.; Hu, R., *et al.* Lipids, lipoproteins, and metabolites and risk of myocardial infarction and stroke. *J Am Coll Cardiol* **2018**, *71*, 620-632.
15. Girona, J.; Amigo, N.; Ibarretxe, D.; Plana, N.; Rodriguez-Borjabad, C.; Heras, M.; Ferre, R.; Gil, M.; Correig, X.; Masana, L. Hdl triglycerides: A new marker of metabolic and cardiovascular risk. *Int J Mol Sci* **2019**, *20*.
16. Teslovich, T.M.; Musunuru, K.; Smith, A.V.; Edmondson, A.C.; Stylianou, I.M.; Koseki, M.; Pirruccello, J.P.; Ripatti, S.; Chasman, D.I.; Willer, C.J., *et al.* Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* **2010**, *466*, 707-713.
17. Oldoni, F.; Palmen, J.; Giambartolomei, C.; Howard, P.; Drenos, F.; Plagnol, V.; Humphries, S.E.; Talmud, P.J.; Smith, A.J. Post-gwas methodologies for localisation of functional non-coding variants: *Angptl3*. *Atherosclerosis* **2016**, *246*, 193-201.
18. Raal, F.J.; Rosenson, R.S.; Reeskamp, L.F.; Hovingh, G.K.; Kastelein, J.J.P.; Rubba, P.; Ali, S.; Banerjee, P.; Chan, K.C.; Gipe, D.A., *et al.* Evinacumab for homozygous familial hypercholesterolemia. *N Engl J Med* **2020**, *383*, 711-720.
19. Grundy, S.M.; Stone, N.J.; Bailey, A.L.; Beam, C.; Birtcher, K.K.; Blumenthal, R.S.; Braun, L.T.; de Ferranti, S.; Faiella-Tommasino, J.; Forman, D.E., *et al.* 2018 aha/acc/aacvpr/aapa/abc/acpm/ada/ags/apha/aspc/nla/pcna guideline on the management of blood cholesterol: A report of the american college of cardiology/american heart association task force on clinical practice guidelines. *Circulation* **2019**, *139*, e1082-e1143.
20. AlKhairi, I.; Cherian, P.; Abu-Farha, M.; Madhoun, A.A.; Nizam, R.; Melhem, M.; Jamal, M.; Al-Sabah, S.; Ali, H.; Tuomilehto, J., *et al.* Increased expression of meteorin-like hormone in type 2 diabetes and obesity and its association with irisin. *Cells* **2019**, *8*.
21. Perakakis, N.; Triantafyllou, G.A.; Fernandez-Real, J.M.; Huh, J.Y.; Park, K.H.; Seufert, J.; Mantzoros, C.S. Physiology and role of irisin in glucose homeostasis. *Nat Rev Endocrinol* **2017**, *13*, 324-337.
22. Arhire, L.I.; Mihalache, L.; Covasa, M. Irisin: A hope in understanding and managing obesity and metabolic syndrome. *Front Endocrinol (Lausanne)* **2019**, *10*, 524.
23. Mahgoub, M.O.; D'Souza, C.; Al Darmaki, R.; Baniyas, M.; Adeghate, E. An update on the role of irisin in the regulation of endocrine and metabolic functions. *Peptides* **2018**, *104*, 15-23.
24. Tine Kartinah, N.; Rosalyn Sianipar, I.; Nafi'ah; Rabia. The effects of exercise regimens on irisin levels in obese rats model: Comparing high-intensity intermittent with continuous moderate-intensity training. *BioMed research international* **2018**, *2018*, 4708287.
25. Bostrom, P.; Wu, J.; Jedrychowski, M.P.; Korde, A.; Ye, L.; Lo, J.C.; Rasbach, K.A.; Bostrom, E.A.; Choi, J.H.; Long, J.Z., *et al.* A *pgc1-alpha*-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* **2012**, *481*, 463-468.
26. Villarroya, F. Irisin, turning up the heat. *Cell Metab* **2012**, *15*, 277-278.
27. Timmons, J.A.; Baar, K.; Davidsen, P.K.; Atherton, P.J. Is irisin a human exercise gene? *Nature* **2012**, *488*, E9-10; discussion E10-11.

28. Maalouf, G.E.; El Khoury, D. Exercise-induced irisin, the fat browning myokine, as a potential anticancer agent. *Journal of obesity* **2019**, *2019*, 6561726.
29. Chang, C.C.; Chow, C.C.; Tellier, L.C.; Vattikuti, S.; Purcell, S.M.; Lee, J.J. Second-generation plink: Rising to the challenge of larger and richer datasets. *Gigascience* **2015**, *4*, 7.
30. Li, J.; Ji, L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity (Edinb)* **2005**, *95*, 221-227.
31. Team, R.C. R: A language and environment for statistical computing. **2020**.
32. Liu, D.J.; Peloso, G.M.; Yu, H.; Butterworth, A.S.; Wang, X.; Mahajan, A.; Saleheen, D.; Emdin, C.; Alam, D.; Alves, A.C., et al. Exome-wide association study of plasma lipids in >300,000 individuals. *Nat Genet* **2017**, *49*, 1758-1766.
33. Kathiresan, S.; Melander, O.; Guiducci, C.; Surti, A.; Burt, N.P.; Rieder, M.J.; Cooper, G.M.; Roos, C.; Voight, B.F.; Havulinna, A.S., et al. Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. *Nat Genet* **2008**, *40*, 189-197.
34. Kulminski, A.M.; Huang, J.; Loika, Y.; Arbeev, K.G.; Bagley, O.; Yashkin, A.; Duan, M.; Culminskaya, I. Strong impact of natural-selection-free heterogeneity in genetics of age-related phenotypes. *Aging (Albany NY)* **2018**, *10*, 492-514.
35. Wojcik, G.L.; Graff, M.; Nishimura, K.K.; Tao, R.; Haessler, J.; Gignoux, C.R.; Highland, H.M.; Patel, Y.M.; Sorokin, E.P.; Avery, C.L., et al. Genetic analyses of diverse populations improves discovery for complex traits. *Nature* **2019**, *570*, 514-518.
36. Willer, C.J.; Sanna, S.; Jackson, A.U.; Scuteri, A.; Bonnycastle, L.L.; Clarke, R.; Heath, S.C.; Timpson, N.J.; Najjar, S.S.; Stringham, H.M., et al. Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat Genet* **2008**, *40*, 161-169.
37. Hebbar, P.; Abubaker, J.A.; Abu-Farha, M.; Alsmadi, O.; Elkum, N.; Alkayal, F.; John, S.E.; Channanath, A.; Iqbal, R.; Pitkanieni, J., et al. Genome-wide landscape establishes novel association signals for metabolic traits in the arab population. *Human genetics* **2020**.
38. Katzmann, J.L.; Packard, C.J.; Chapman, M.J.; Katzmann, I.; Laufs, U. Targeting rna with antisense oligonucleotides and small interfering rna: Jacc state-of-the-art review. *J Am Coll Cardiol* **2020**, *76*, 563-579.
39. Musunuru, K.; Pirruccello, J.P.; Do, R.; Peloso, G.M.; Guiducci, C.; Sougnez, C.; Garimella, K.V.; Fisher, S.; Abreu, J.; Barry, A.J., et al. Exome sequencing, angptl3 mutations, and familial combined hypolipidemia. *N Engl J Med* **2010**, *363*, 2220-2227.
40. Abu-Farha, M.; Ghosh, A.; Al-Khairi, I.; Madiraju, S.R.M.; Abubaker, J.; Prentki, M. The multi-faces of angptl8 in health and disease: Novel functions beyond lipoprotein lipase modulation. *Prog Lipid Res* **2020**, *80*, 101067.
41. Mo, L.; Shen, J.; Liu, Q.; Zhang, Y.; Kuang, J.; Pu, S.; Cheng, S.; Zou, M.; Jiang, W.; Jiang, C., et al. Irisin is regulated by car in liver and is a mediator of hepatic glucose and lipid metabolism. *Molecular endocrinology* **2016**, *30*, 533-542.
42. Zhang, R. The angptl3-4-8 model, a molecular mechanism for triglyceride trafficking. *Open Biol* **2016**, *6*, 150272.
43. Rana, K.S.; Pararasa, C.; Afzal, I.; Nagel, D.A.; Hill, E.J.; Bailey, C.J.; Griffiths, H.R.; Kyrou, I.; Randevara, H.S.; Bellary, S., et al. Plasma irisin is elevated in type 2 diabetes and is associated with increased e-selectin levels. *Cardiovascular diabetology* **2017**, *16*, 147.
44. Park, K.H.; Zaichenko, L.; Brinkoetter, M.; Thakkar, B.; Sahin-Efe, A.; Joung, K.E.; Tsoukas, M.A.; Geladari, E.V.; Huh, J.Y.; Dincer, F., et al. Circulating irisin in relation to insulin resistance and the metabolic syndrome. *The Journal of clinical endocrinology and metabolism* **2013**, *98*, 4899-4907.
45. Zhang, H.J.; Zhang, X.F.; Ma, Z.M.; Pan, L.L.; Chen, Z.; Han, H.W.; Han, C.K.; Zhuang, X.J.; Lu, Y.; Li, X.J., et al. Irisin is inversely associated with intrahepatic triglyceride contents in obese adults. *J Hepatol* **2013**, *59*, 557-562.
46. Avramoglu, R.K.; Basciano, H.; Adeli, K. Lipid and lipoprotein dysregulation in insulin resistant states. *Clin Chim Acta* **2006**, *368*, 1-19.

47. Ginsberg, H.N.; Zhang, Y.L.; Hernandez-Ono, A. Regulation of plasma triglycerides in insulin resistance and diabetes. *Archives of medical research* **2005**, *36*, 232-240.
48. Forst, T.; Kunt, T.; Pflutzner, A.; Beyer, J.; Wahren, J. New aspects on biological activity of c-peptide in iddm patients. *Exp Clin Endocrinol Diabetes* **1998**, *106*, 270-276.
49. Cardellini, M.; Farcomeni, A.; Ballanti, M.; Morelli, M.; Davato, F.; Cardolini, I.; Grappasonni, G.; Rizza, S.; Guglielmi, V.; Porzio, O., et al. C-peptide: A predictor of cardiovascular mortality in subjects with established atherosclerotic disease. *Diab Vasc Dis Res* **2017**, *14*, 395-399.
50. Becht, F.S.; Walther, K.; Martin, E.; Nauck, M.A. Fasting c-peptide and related parameters characterizing insulin secretory capacity for correctly classifying diabetes type and for predicting insulin requirement in patients with type 2 diabetes. *Exp Clin Endocrinol Diabetes* **2016**, *124*, 148-156.
51. Cabrera de Leon, A.; Oliva Garcia, J.G.; Marcelino Rodriguez, I.; Almeida Gonzalez, D.; Aleman Sanchez, J.J.; Brito Diaz, B.; Dominguez Coello, S.; Bertomeu Martinez, V.; Aguirre Jaime, A.; Rodriguez Perez Mdel, C. C-peptide as a risk factor of coronary artery disease in the general population. *Diab Vasc Dis Res* **2015**, *12*, 199-207.
52. Williams, G.V.; Gambhir, K.K.; Nunlee-Bland, G.; Abrams, C.K.; Ganta, V.; Odonkor, W. Significance of plasma c-peptide in obese african american adolescents. *J Natl Med Assoc* **2011**, *103*, 907-916.
53. Khan, H.A.; Sobki, S.H.; Ekhzaimy, A.; Khan, I.; Almusawi, M.A. Biomarker potential of c-peptide for screening of insulin resistance in diabetic and non-diabetic individuals. *Saudi J Biol Sci* **2018**, *25*, 1729-1732.
54. Yanai, H.; Hirowatari, Y. Fasting serum c-peptide levels (>1.6ng/ml) can predict the presence of insulin resistance in japanese patients with type 2 diabetes. *Diabetes & metabolism* **2017**, *43*, 97-98.
55. Martinez-Reyes, C.P.; Gomez-Arauz, A.Y.; Torres-Castro, I.; Manjarrez-Reyna, A.N.; Palomera, L.F.; Olivos-Garcia, A.; Mendoza-Tenorio, E.; Sanchez-Medina, G.A.; Islas-Andrade, S.; Melendez-Mier, G., et al. Serum levels of interleukin-13 increase in subjects with insulin resistance but do not correlate with markers of low-grade systemic inflammation. *Journal of diabetes research* **2018**, *2018*, 7209872.
56. Stanya, K.J.; Jacobi, D.; Liu, S.; Bhargava, P.; Dai, L.; Gangl, M.R.; Inouye, K.; Barlow, J.L.; Ji, Y.; Mizgerd, J.P., et al. Direct control of hepatic glucose production by interleukin-13 in mice. *J Clin Invest* **2013**, *123*, 261-271.
57. López, J.C. Il-13 controls blood sugar. *Nature Medicine* **2013**, *19*, 142.
58. Zhang, J.; Valverde, P.; Zhu, X.; Murray, D.; Wu, Y.; Yu, L.; Jiang, H.; Dard, M.M.; Huang, J.; Xu, Z., et al. Exercise-induced irisin in bone and systemic irisin administration reveal new regulatory mechanisms of bone metabolism. *Bone Res* **2017**, *5*, 16056.
59. Huh, J.Y.; Panagiotou, G.; Mougios, V.; Brinkoetter, M.; Vamvini, M.T.; Schneider, B.E.; Mantzoros, C.S. Fndc5 and irisin in humans: I. Predictors of circulating concentrations in serum and plasma and ii. Mrna expression and circulating concentrations in response to weight loss and exercise. *Metabolism* **2012**, *61*, 1725-1738.
60. Albrecht, E.; Schering, L.; Buck, F.; Vlach, K.; Schober, H.C.; Drevon, C.A.; Maak, S. Irisin: Still chasing shadows. *Molecular metabolism* **2020**, *34*, 124-135.