UNIVERDIDAD NACIONAL AUTOMONA DE MEXICO
DOCTORADO EN CIENCIAS BIOMEDICAS
FACULTAD DE MEDICINA

LOS POLIMORFISMOS DE LOS GENES QUE CODIFICAN PARA LAS SUBUNIDADES ALFA Y BETA DE LAS INTERLEUCINAS 27 Y 35 SE ASOCIAN CON ENFERMEDAD ARTERIAL CORONARIA PREMATURA Y PARÁMETROS METABÓLICOS EN POBLACIÓN MEXICANA.

T ES I S

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INDICE

1. **RESUMEN**

1.1 Español……………………………………………………………………………………9

1.2 Inglés………………………………………………………………………….................10

2. **INTRODUCCIÓN**

2.1 MARCO TEORICO……………………………………………………………………..12

2.1.1 Epidemiología de la enfermedad cardiovascular……………………………………12

2.1.2 Historia familiar y genética de la enfermedad arterial coronaria…………………..16

2.1.3 Estrategias genéticas y genómicas en la búsqueda de genes involucrados en el desarrollo de las enfermedades cardiovasculares.…………………………………20

2.1.4 Análisis de ligamiento…………………………………………………………………..20

2.1.5 Análisis de asociación………………………………………………………………….23

2.1.6 Sistema inmune en la aterosclerosis…………………………………………………26

2.1.7 Lipoproteína de baja densidad e inflamación……….………………………………28

2.1.8 Familia de la interleucina 12…………….……………………………………………..30

2.2 ANTECEDENTES……………………………………………………………………..…..34

2.2.1 Interleucina 27……………………………………………………………………………..35

2.2.2 Interleucina 35……………………………………………………………………………..38

2.2.3 Genes que codifican para las subunidades α y β de las interleucinas 27 y 35 y su asociación con enfermedad cardiovascular………………….…………………………..…41

2.2.3.1 Gen *IL-27p28*……………………………………..……………………41

2.2.3.2 *Gen IL-12A*…………………………………………..…………………42

2.2.3.3 Gen *EBI3*……………………………………………………..……………..43

3. **PLANTAMIENTO DEL PROBLEMA**…………………….……………………………………45

4. **JUSTIFICACIÓN**…………………………………………………………………………………46

5. **PREGUNTA DE INVESTIGACIÓN**………………………………….…………………………47

6. **HIPÓTESIS**……………………………………………………………………………………47

7. **OBJETIVOS**…………………………………………………………………………………...47

7.1 Objetivo general………………………………………………………………………….47

7.2 Objetivos específicos…………………………………………………………………….47

8. **METODOLOGÍA**……………………………………………………………………….……...48

8.1 Población………………………………………………………………………...………48

8.2 Mediciones antropométricas y definición de factores de riesgo cardiovascular……………………………………………………..…….…….……49

8.3 Análisis de laboratorio……………..…………………………………………………...51

8.4 Estudio de tomografía axial computada……….……………………………………52

8.5 Estudio genético………………………….…….……………………………………52

8.5.1 Selección de los polimorfismos…………………….………………………….....52

8.5.2 Detección de los polimorfismos…………………..……………………………..53

8.5.3 Determinación de los genes de ancestría……………………………………...54

8.6 Aproximación funcional…………………………..…………………………………56

8.6.1 Determinación de las concentraciones de IL-27 e IL-35…….……………………56

8.7 Ensayo de luciferasa…………………………………………………...…………56

8.8 Análisis estadístico……………………………………………………………..……58

9. **RESULTADOS**……………………………...……………………………………………..59

9.1 Resultados de interleucina 27………………………….……………………...…...59

9.1.1 Características clínicas y parámetros metabólicos……….……………………….59

9.1.2 Asociación de los polimorfismos con la EAC prematura…………..……………………60

9.1.3 Análisis de los haplotipos del gen *IL27p28*……………………………….………….62

9.1.4 Análisis de los polimorfismos del gen *IL27p28* con parámetros clínicos y metabólicos…………………………………………………………………..………63

9.1.5 Concentraciones de IL-27 en pacientes con EAC prematura y controles…………………………………………………………………………….....65

9.1.6 Ensayos de luciferasa………………………………………….…………….…………......66

9.2 Resultados de interleucina 35……………………………………….…………..68

9.2.1 Características clínicas y parámetros metabólicos……………………………....68

9.2.2 Asociación de los polimorfismos de *EBI3 e IL-12A* con EAC prematura……………………………………………………………………......69

9.2.3 Análisis de los haplotipos del gen *EBI3*……………………………………….....71

9.2.4 Análisis de los haplotipos del gen *IL-12A*…………………………………………72

9.2.5 Asociación de los polimorfismos de los genes *EBI3* e *IL-12A* con parámetros metabólicos…………………………………………….............................73

9.2.6 Asociación de los polimorfismos de los genes *EBI3* e *IL-12A* con las concentraciones de IL-35………………………………………………………76

10. **DISCUSIÓN**…………………………………………………………………………....79

11. **CONCLUSIONES**……………………………………………………………………..86

12. **PERSPECTIVAS**………………………………………………………………............87

13. **BIBLIOGRAFÍA**………………………………………………………………………88

14. **ANEXOS**…………………………………………………………………………..……..96

14.1. Publicaciones generadas del proyecto de tesis de doctorado

14.2 Otras publicaciones derivadas del proyecto

14.2.1 Como primer autor o autor correspondiente

14.2.2 Como coautor

ABREVIATURAS

apoA Apolipoproteína A1

apoB Apolipoproteína B

apoB 100 Apolipoproteína B100

ALT Alanina aminotransferasa

AST Aspartato aminotransferasa

CAC Calcificación arterial coronaria

C-HDL Colesterol de la lipoproteína de alta densidad, por sus siglas en ingles

C-LDL Colesterol de la lipoproteína de baja densidad, por sus siglas en ingles

CnoHDL Colesterol no HDL

CNVs Variantes en el número de copias, por sus siglas en inglés.

DE Desviación estándar

DMT2 Diabetes mellitus tipo 2

EAC Enfermedad arterial coronaria

EAP Enfermedad arterial periférica

EBI3 Gen 3 inducido por virus de Epstein-Barr

ECV Enfermedad cardiovascular

EVC Enfermedad vascular cerebral

FR Factores de riesgo

GAS Grasa abdominal subcutánea

GAT Grasa abdominal total

GAV Grasa abdominal visceral

GEA Genética de la enfermedad aterosclerosa

GGT Gamma glutamiltranspeptidasa

gp 130 Glicoproteína 130

GWAS estudio de asociación del genoma completo, por sus siglas en inglés

GWLS Genome-wide linkage scan, por sus siglas en inglés

HOMA-RI Modelo homeostático para determinarla resistencia a la insulina

IC Intervalo de confianza

IL-2 Interleucina 2

IL-4 Interleucina 4

IL-6 Interleucina 6

IL-10 Interleucina 10

IL-17 Interleucina 17

IL-12 Interleucina 12

IL-23 Interleucina 23

IL-27 Interleucina 27

IL-35 Interleucina 35

IMC Indice de masa corporal

LD Desequilibrio de ligamiento, por sus siglas en inglés

LDL Lipoproteína de baja densidad

PCRas Proteína C reactiva de alta sensibilidad

REACH Reduction of atherothrombosis for continued health

RM Razón de momios

SM Síndrome metabólico

SNP Single nucleotide polymorphism, por sus siglas en inglés

STRs Short tandem repetitive, por sus siglas en inglés.

TDT Transmission disequilibrium test, por sus siglas en inglés

ÍNDICE DE FIGURAS

Figura 1. Mortalidad global y morbilidad atribuible a la enfermedad cardiovascular y los principales factores de riesgo en personas de más de 30 años de edad………...13

Figura 2. Distribución de la enfermedad cardiovascular por regiones en el registro REACH…..14

Figura 3. Diez principales causas de mortalidad en México por género en 2013……………....16

Figura 4. Componentes inmunes de la placa aterosclerosa……………………………………..27

Figura 5. Activación de células T en el vaso…………………………………………………….....30

Figura 6. Miembros de la familia de la interleucina 12…………………………………………....33

Figura 7. Modelo molecular de la interleucina 27………………………………………………....37

Figura 8. Modelo molecular de la interleucina 35………………………………………………...39

Figura 9. Posición en el cromosoma de los polimorfismos seleccionados en el gen *IL-27p28*...54

Figura 10. Posición en el cromosoma de los polimorfismos seleccionados en el gen *IL-12A*….55

Figura 11. Posición en el cromosoma de los polimorfismos seleccionados en el gen *EBI3*…....55

Figura 12. Mapa de DL del gen *IL27p28*…………………………………………………………...62

Figura 13. Concentraciones de interleucina 27 en plasma……………………………………....65

Figura 14. El polimorfismo rs40837 del gen *IL-27p28* afecta el nivel de expresión de luciferasa en presencia del miR-379-5p…………………………………………………………………67

Figura 15. Mapa de DL del gen *EBI3*………………………………………………………………71

Figura 16. Mapa de DL del gen *IL12A*……………………………………………………………..72

Figura 17. Concentraciones de interleucina 35 en plasma………………………………………76

INDICE DE TABLAS

Tabla 1. Principales causas de muerte en México, 1922-2013…………………………………....15

Tabla 2. Razón de momios para las combinaciones parentales de ataque al corazón…………18

Tabla 3. Heredabilidad de factores de riesgo cardiovascular a partir del estudio del corazón de Framingham………………………………………………………………………………………19

Tabla 4. Estudios y sus hallazgos de la participación de las interleucinas 27 y 35 en aterosclerosis……………………………………………………………………………………...40

Tabla 5. Polimorfismos seleccionados para el estudio…………………………………………..55

Tabla 6. Características clínicas y factores de riesgo cardiovascular en los grupos estudiados………………………………………………………………………………………59

Tabla 7. Asociación de los polimorfismos del gen *IL27p28* con enfermedad arterial coronaria prematura………………………………………………………………………………………61

Tabla 8. Frecuencias de haplotipos del gen *IL-27p28*…………………………………………...62

Tabla 9. Asociación de los polimorfismos del gen *IL27p28* con parámetros metabólicos en los sujetos control……………………………………………………………………………………..63

Tabla 10. Asociación de los polimorfismos del gen *IL27p28* con parámetros metabólicos en pacientes con EAC…………………………………………………………………………..……64

Tabla 11. Concentraciones de IL-27 en plasma en los grupos estudiados en función de los genotipos de *EBI3* e *IL-27p28*……………………………………………………………………...66

Tabla 12. Características clínicas y metabólicas de los grupos estudiados……………………...68

Tabla 13. Prevalencia de factores de riesgo cardiovascular en la población estudiada……………………………………………………………………………………….69

Tabla 14. Asociación entre los polimorfismos de *EBI3* e *IL-12A* y enfermedad arterial coronaria prematura………………………………………………………………………………………70

Tabla 15. Frecuencias de haplotipos del gen *EBI3*………………………………………………72

Tabla 16. Frecuencia de haplotipos del gen *IL-12A*……………………………………………..73

Tabla 17. Asociación entre los polimorfismos de *EBI3* e *IL-12A* y anormalidades metabólicas en pacientes con enfermedad arterial coronaria prematura……………………………………….74
Tablas 18. Asociación entre los polimorfismos de *EBI3* e *IL-12A* y anormalidades metabólicas en el grupo control……………………………………………………………………………......75
Tabla 19. Concentraciones de IL-35 en plasma en los grupos estudiados en función de los genotipos de *EBI3* e *IL-12A*………………………………………………………………………..77

1. RESUMEN

1.1 Resumen en español

Las interleucinas 27 (IL-27) y 35 (IL-35) tiene un papel importante en el desarrollo de la aterosclerosis. El objetivo del trabajo fue establecer si los polimorfismos de los genes que codifican para las subunidades de las IL-27 e IL-35 se asocian con la presencia de la enfermedad arterial coronaria (EAC) prematura y/o con algún parámetro clínico o metabólico y definir su efecto funcional. Utilizando sondas Taqman, se genotipificaron 11 polimorfismos con posible efecto funcional (4 presentes en el gen *IL-27p28* que codifica para la subunidad p28 de IL-27 [rs26528, rs17855750, rs181206 y rs40837], 4 en el gen *IL-12A* que codifica para la subunidad p35 de IL-35 [rs2243115, rs2243123, rs583911, y rs568408] y 3 en el gen *EBI3* que codifica para la subunidad EBI3 presente en ambas citocinas [rs428253, rs4740 y rs4905]) en 1107 sujetos control y 1162 pacientes con EAC prematura. Las asociaciones se evaluaron mediante análisis de regresión logística. Se determinaron las concentraciones plasmáticas de IL-27 e IL-35 en subgrupos de pacientes y controles. Las concentraciones de IL-27 e IL-35 fueron significativamente más altas en los pacientes en comparación con los controles. En el grupo control se observó que las concentraciones de IL-35 fueron significativamente diferentes dependiendo de los genotipos de *EBI3* rs4740 y rs4905. Los alelos rs26528*T* y rs40837*A* del gen *IL-27p28*, rs42853*C* y rs2243115*G* de los genes *EBI3* e *IL-12A,* respectivamente, se asociaron con menor riesgo de padecer EAC prematura. El alelo rs40837*A* en presencia del miR-379-5p disminuye significativamente la expresión del gen de la luciferasa. Algunos polimorfismos estudiados se asociaron con variables clínicas y metabólicas. Los datos sugieren que los alelos *IL-27p28* rs26528*T*, *IL-27p28* rs40837*A,*

EBI3 rs42853*C* e *IL-12A* rs2243115*G* pudieran ser considerados como marcadores genéticos de protección para EAC prematura en nuestra población**.**

1.2 Resumen en inglés

Interleukin-27 and interleukin-35 play an important role of in the development of atherosclerosis. The aim of this study was to esgtablish whether the polymorphisms of the genes that encoded for the subunits of these cytokines are associated with premature coronary artery disease (CAD) and/or clinic and metabolic parameters. Using Taqman assays, eleven polymorphisms were genotyped (4 of the *IL-27p28* gen that encodes for the interleukin-27 p28 subunit [rs26528, rs17855750, rs181206 y rs40837], 4 in the *IL-12A* gene that encodes for the interleukin-35 p35 subunit [rs2243115, rs2243123, rs583911, y rs568408], and 3 in the *EBI3* gene that encodes for the EBI3 subunit present in both cytokines [rs428253, rs4740 y rs4905]) in 1162 premature CAD patients and 1107 controls. Logistic regression analyses were performed to assess associations. Interleukin-27 and interleukin-35 plasma concentrations were measured in a subsample of patients and controls. Interleukin-27 and interleukin-35 plasma concentrations were significantly higher in patients compared to controls. Significant different concentrations of interleukin-35 were observed in EBI3 rs4740 and rs4905 genotypes only in the control group. The rs26528 *T* and rs40837 *A* alleles of the *IL-27p28* gen, rs42853*C* and rs2243115*G* of the *EBI3* e *IL-12A* genes*,* respectively, were associated with lower risk of premature CAD. Co-transfection of the rs40837*A* allele and miR-379-5p significantly decreased luciferase gene expression. Some of the studied polymorphisms were associated with clinic and metabolic parameters. The data suggest that the *IL-27p28* rs26528*T*, *IL-27p28* rs40837*A,*

EBI3 rs42853*C* and *IL-12A* rs2243115*G* alleles may be considered as protective genetic markers for premature CAD in our population.

2. INTRODUCCIÓN

2.1 MARCO TEÓRICO

2.1.1 Epidemiología de la enfermedad cardiovascular

La enfermedad cardiovascular (ECV) se puede concebir como un continuo, inicia con la presencia de factores de riesgo cardiovascular, prosigue vía la enfermedad vascular progresiva con su punto final en el daño del órgano, falla del órgano y la muerte.[1]

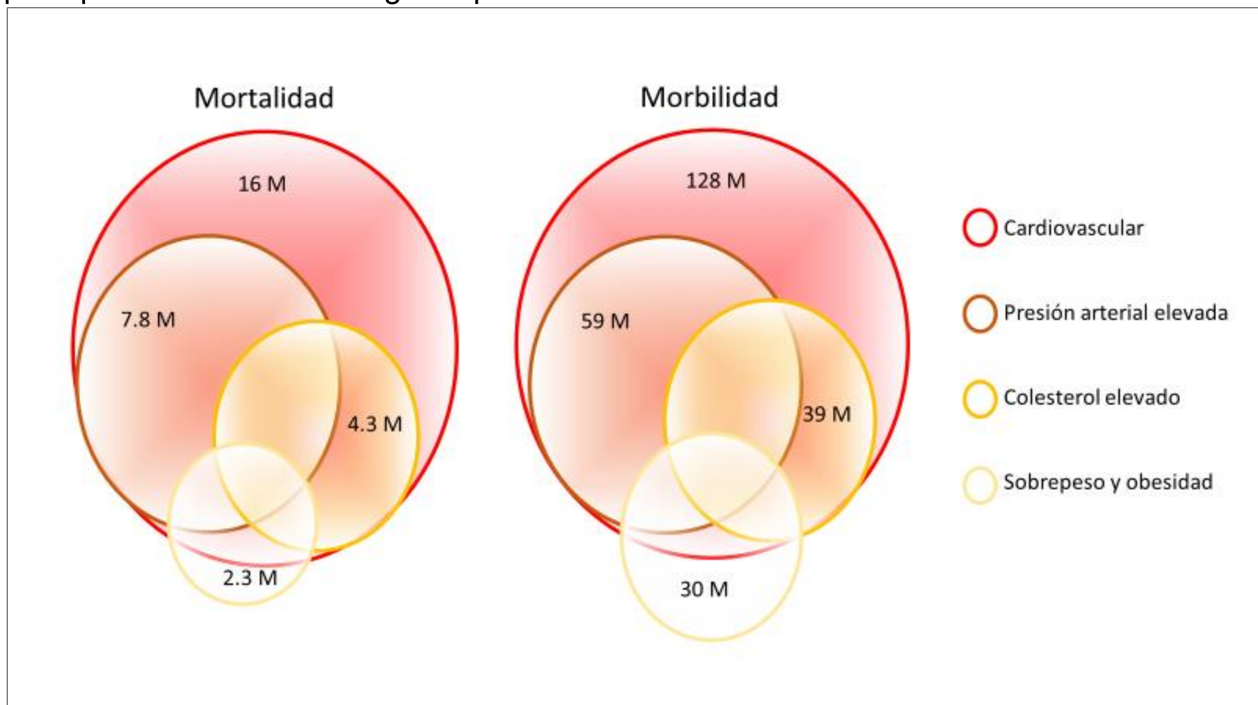
Los factores de riesgo modificables, entre los que se encuentran la hipertensión, tabaquismo, obesidad abdominal, dislipidemia, diabetes mellitus, estrés, disminución del consumo de frutas y vegetales y falta de ejercicio regular, son los principales causantes de la morbilidad y mortalidad cardiovascular, y en conjunto explican más del 90% de los infartos al miocardio.[1,2] Los factores de riesgo cardiovascular raramente se presentan de forma aislada, por el contrario, tienden a asociarse en un individuo.[3,4] El número de personas con múltiples factores de riesgo cardiovascular se está incrementando de manera alarmante. Aproximadamente, 70% de los individuos que desarrollarán ECV presentan múltiples factores de riesgo, los cuales interactúan de forma sinérgica e incrementan el riesgo total de ECV en el individuo.[5] La mayoría de los eventos cardiovasculares se presentan en personas con elevaciones modestas y, por tanto, desapercibidas de múltiples factores de riesgo, en lugar de un incremento importante en un factor de riesgo aislado y, consecuentemente, para muchos individuos la muerte es la única manifestación de ECV.

La prevalencia de los factores de riesgo cardiovascular está en aumento. El estimado de la mortalidad global total, muestra que las 3 principales causas de muerte en los países industrializados son la hipertensión, el tabaquismo y las cifras elevadas de colesterol.[6–8]

Tendencias similares se observan en la actualidad en los países de economías emergentes.

La ECV es la principal causa de muerte a nivel mundial y contribuye con 16.7 millones de muertes cada año, principalmente debidas a ataques del corazón y accidentes cerebro vasculares.[9] De continuar la tendencia actual, se espera que esta cifra se incremente aproximadamente a 25 millones de muertes para el 2020.[10] En 2004, la enfermedad coronaria isquémica provocó un estimado de 7.2 millones de muertes y la enfermedad vascular cerebral (EVC) resultó en 5.5 millones de muertes, representando el 22% de la mortalidad global.[7]

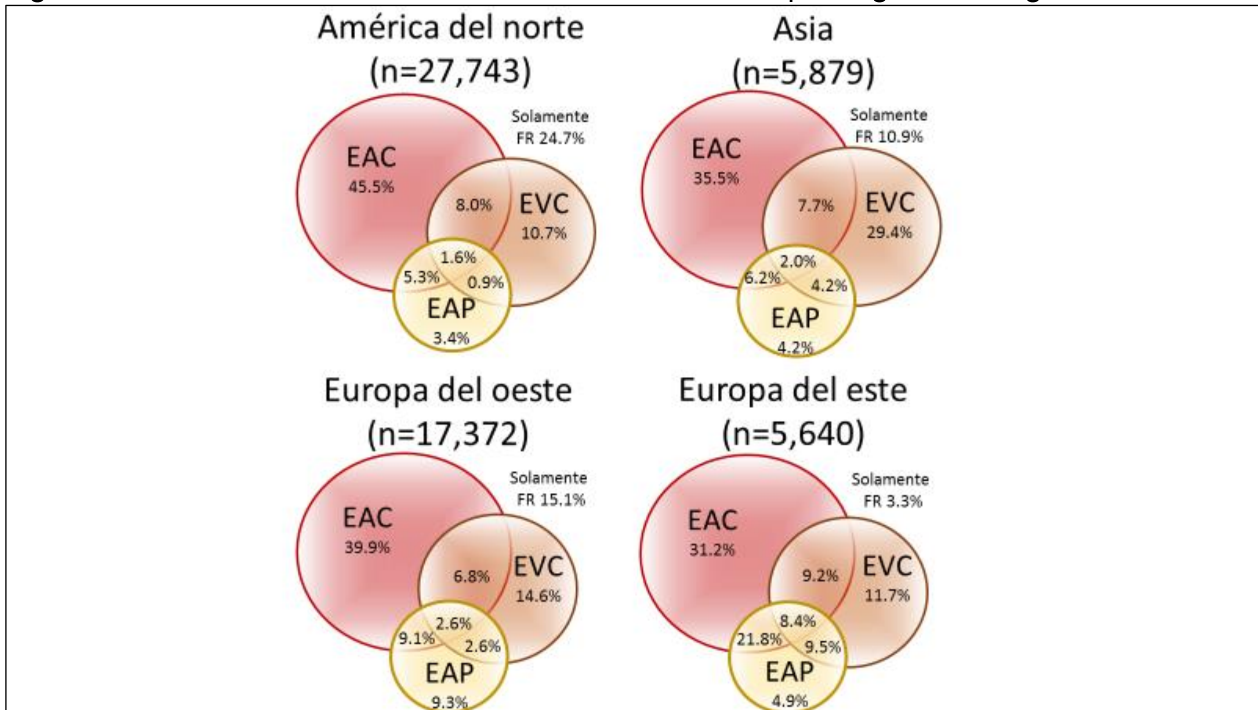
Figura 1. Mortalidad global y morbilidad atribuible a la enfermedad cardiovascular y los principales factores de riesgo en personas de más de 30 años de edad.



El tamaño de los círculos es proporcional al número de muertes (izquierda) o personas afectadas por enfermedad cardiovascular (derecha; medida en incapacidad ajustada por años de vida) en millones (M). Tomada y adaptada de Ezzati M, Vander Hoorn S, Lawes CMM, Leach R, James WPT, et al. (2005) Rethinking the "Diseases of Affluence" Paradigm: Global Patterns of Nutritional Risks in Relation to Economic Development. PLOS Medicine 2(5): e133. https://doi.org/10.1371/journal.pmed.0020133
http://journals.plos.org/plosmedicine/article?id=10.1371/journal.pmed.0020133

La ECV afecta a 128 millones de personas, lo que representa 8 veces el número de muertes por ECV (Figura 1).[8] El elevado costo de la enfermedad no se atribuye a la mortalidad, sino a los eventos cardiovasculares no fatales y a sus consecuencias de largo plazo. El costo futuro de la morbilidad cardiovascular, se incrementará probablemente debido no solo al incremento en la edad de la población, sino, además, como consecuencia de la epidemia de obesidad y factores de riesgo (FR) cardiovascular relacionados.

Figura 2. Distribución de la enfermedad cardiovascular por regiones. Registro REACH



Distribución de las características de la ECV por regiones de acuerdo al registro REACH, un registro internacional, prospectivo, observacional de pacientes con edad mayor o igual a 45 años y con enfermedad arterial coronaria (EAC) establecida, EVC o enfermedad arterial periférica (EAP), o con tres o más factores de riesgo (FR) para aterosclerosis. Tomada y adaptada de: Dahlöf B. Cardiovascular disease risk factors: epidemiology and risk assessment. Am J Cardiol. 2010 Jan 4;105(1 Suppl):3A-9A. **Licence number: 4216551158695**

El registro REACH (por sus siglas en inglés, Reduction of Atherothrombosis for Continued Health) publicado en 2006, contiene información global recolectada en 44 países y 67,888

pacientes con edad mayor o igual a 45 años, sobre factores de riesgo para aterosclerosis. Este registro mostró que los factores de riesgo cardiovascular clásicos (hipertensión, concentraciones elevadas de colesterol, diabetes, obesidad y tabaquismo) son consistentes y comunes en diversos grupos étnicos, además de estar sub-tratadas y no controladas en varias regiones del mundo.[11] El registro REACH también encontró diferencias regionales en la distribución de diferentes categorías de enfermedad cardiovascular (EAC, EVC y EAP) (Figura 2).

En México, en 1922 las primeras causas de mortalidad correspondían a enfermedades infecciosas o transmisibles, patrón que se conservó hasta los años cincuenta. A partir de esta fecha comenzó la transición en las causas de muerte, las enfermedades crónicas o no transmisibles comenzaron a posicionarse en los primeros lugares (Tabla 1).

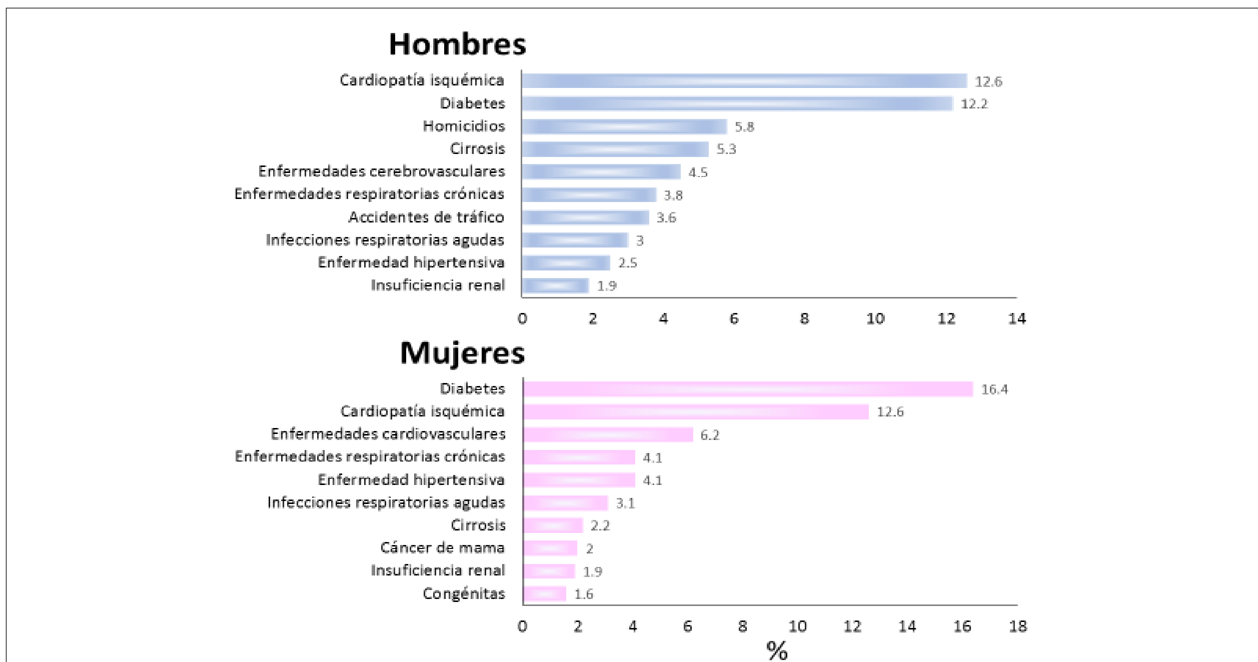
Tabla 1. Principales causas de muerte en México, 1922-2013

	1922	1950	1970	1990	2000	2013
1	Neumonía, influenza	Gastroenteritis, colitis	Neumonía, influenza	Enfermedades del corazón	Diabetes mellitus	Diabetes mellitus
2	Diarreas, enteritis	Gripe y neumonía	Enteritis y otras enfermedades	Tumores malignos	Enfermedades del corazón	Enfermedades isquémicas del corazón
3	Fiebre y caquexia palúdica	Ciertas enfermedades de la primera infancia	Enfermedades del corazón	Accidentes	Cirrosis y otras enfermedades del hígado	Tumores malignos
4	Tos ferina	Accidentes, envenenamientos y violencia	Ciertas causas de enfermedades perinatales	Diabetes mellitus	Enfermedades cerebrovasculares	Accidentes
5	Viruela	Paludismo	Tumores malignos	Ciertas afecciones originadas en el periodo perinatal	Ciertas afecciones originadas en el periodo perinatal	Enfermedades del hígado

Fuentes: Perdigón-Villaseñor G, Fernández-Cantón SB. Principales causas de muerte en la población general e infantil en México, 1922-2013. Bol Med Hosp Infant Mex. 2008;65:238-40. / INEGI. Estadísticas de mortalidad. Disponible en: http://www3.inegi.org.mx/sistemas/temas/default.aspx?s=est&c=17484

En la actualidad, el Sistema Nacional de Salud, afirma que la cardiopatía isquémica y la diabetes mellitus, son dos de los más grandes problemas de la salud pública, y constituyen las principales causas de mortalidad en nuestro país (Figura 3). La tendencia de la mortalidad por cardiopatía isquémica continúa siendo de ascenso gradual. Entre 2000 y 2013 el aumento fue de más de 45%, pasando de 43.5 muertes por 100,000 habitantes en 2000 a 63.3 en 2013.

Figura 3. Diez principales causas de mortalidad en México por género en 2013



Fuente: INEGI/SS Estadísticas vitales. Defunciones generales 2013

2.1.2 Historia familiar y genética de la enfermedad arterial coronaria

La presencia de ECV en varios miembros de una misma familia se asocia con un conjunto de factores de riesgo específicos para ECV, tanto tradicionales como de estilo de vida, cada uno de los cuales tiene componentes ambientales y genéticos. Pacientes con

historia familiar de EAC tienen prevalencias más altas de FR tradicionales para ECV, que disminuyen las oportunidades de prevención.[12]

La historia familiar positiva aumenta el riesgo de la mayoría de las condiciones cardiovasculares, incluyendo ECV (45% más frecuente en sujetos con hermanos afectados), EVC (50% más frecuente en individuos con familiares de primer grado afectados), fibrilación atrial (80% más probable en individuos con historia del padecimiento en los padres), falla del corazón (probabilidad 70% más alta en sujetos con historia familiar positiva en progenitores) y EAP (probabilidad 80% más elevada en individuos con historia familiar positiva). El aumento en la probabilidad de desarrollar un padecimiento cardiovascular refleja el efecto combinado de factores genéticos, epigenéticos y ambientales.[12]

La coexistencia familiar de ECV pudiera estar relacionada a la agregación de comportamientos específicos (tabaquismo, uso de alcohol) o FR cardiovasculares (hipertensión, diabetes mellitus, obesidad), los cuales a su vez tienen contribución ambiental y genética. A diferencia de los FR de la genética mendeliana, en la cual usualmente una mutación directamente causa una enfermedad, los factores que contribuyen a una característica compleja, pueden incrementar el riesgo sin que necesariamente provoquen la condición. El tamaño del efecto de cualquier factor que contribuye al riesgo puede ser pequeño, pero ampliamente diseminado en una población, puede tener un efecto grande pero afecta a un sector pequeño de la población, o puede incrementar el riesgo cuando se encuentran presentes factores de riesgo ambientales.

La historia paterna positiva de ataque prematuro al corazón incrementa el riesgo en aproximadamente el doble de presentar ataque al corazón en los varones, mientras que en las mujeres el riesgo aumenta aproximadamente un 70%.[13,14] La historia de ataque al

corazón en ambos padres incrementa el riesgo del padecimiento, especialmente cuando uno de los progenitores tuvo ataque prematuro al corazón (Tabla 2).[15]

Tabla 2. Razón de momios para las combinaciones parentales de ataque al corazón.

	RM [IC 95%]
Sin historia familiar	1.00
Un progenitor con ataque al corazón ≥50 años de edad	1.67 [1.55-1.81]
Un progenitor con ataque al corazón <50 años de edad	2.36 [1.89-2.95]
Ambos progenitores con ataque al corazón ≥50 años de edad	2.90 [2.30-3.66]
Ambos progenitores con ataque al corazón, uno <50 años de edad	3.26 [1.72-6.18]
Ambos progenitores con ataque al corazón, ambos <50 años de edad	6.56 [1.39-30.95]

RA: razón de momios. IC: intervalo de confianza
Fuente: [15]

La historia de ECV en hermanos ha mostrado incrementar la probabilidad de la enfermedad en 45% tanto en hombres como en mujeres (razón de momios = 1.45; intervalo de confianza al 95% 1.19-1.91] después de ajustar por FR cardiovascular.[16]

La historia familiar de angina, infarto al miocardio, angioplastia o cirugía de revascularización prematuras incrementa el riesgo de mortalidad en aproximadamente 40 % tanto por enfermedad coronaria como por ECV.[17] En un estudio internacional en el que se incluyeron individuos con síndrome coronario agudo (edad ≤55 años), más mujeres (28%) que hombres (20%) tuvieron historia familiar positiva de EAC (p=0.008). Sin embargo, comparados con los pacientes sin historia familiar, aquellos con historia familiar de EAC tuvieron una prevalencia más alta de FR cardiovascular tradicionales, incluyendo dislipidemia y obesidad. Las mujeres con historia familiar tuvieron mayor

prevalencia de cada uno de los factores de riesgo tradicional (obesidad, diabetes mellitus, dislipidemia e hipertensión) excepto para tabaquismo.[18]

La heredabilidad es la relación de la variación debida a las causas genéticas con respecto a la variación total de una característica determinada. En la tabla 3 se muestra la heredabilidad estimada para factores de riesgo de ECV usando los datos generados del estudio del corazón de Framingham.

Tabla 3. Heredabilidad de factores de riesgo cardiovascular a partir del estudio del corazón de Framingham

Característica	Heredabilidad	Referencia
Tensión arterial sistólica	0.42	19
Tensión arterial diastólica	0.39	19
Masa ventricular izquierda	0.24-0.32	20
Índice de masa corporal	0.37(40 años promedio)-0.52 (60 años promedio)	21
Circunferencia de cintura	0.41	22
Grasa abdominal visceral	0.36	23
Grasa abdominal subcutánea	0.57	23
Glucosa de ayuno	0.34	24
Proteína C reactiva	0.30	25
Hemoglobina glucosilada	0.27	24
Triglicéridos	0.48	26
Colesterol HDL	0.52	26
Colesterol total	0.57	26
Colesterol LDL	0.59	26
Tasa de filtración glomerular estimada	0.33	27

Estos datos sugieren que la mayoría de los factores de riesgo cardiovascular tiene al menos una heredabilidad moderada. Estos datos en su conjunto muestran que la complejidad genética de la susceptibilidad a padecer EAC parece ser considerable y, además, que cada uno de los factores de riesgo clínicos para la EAC tiene su propia base genética.[28]

2.1.3 Estrategias genéticas y genómicas en la búsqueda de genes involucrados en el desarrollo de las enfermedades cardiovasculares.

Existen dos estrategias principales para identificar genes de susceptibilidad en humanos: a) los análisis de ligamiento y, b) los análisis de asociación, ambas estrategias han sido ampliamente usadas para identificar la participación de los genes en padecimientos cardiovasculares, no son mutualmente excluyentes y cada una presenta ventajas y desventajas.

2.1.4 Análisis de ligamiento

En los análisis de ligamiento se requiere de un número grande de individuos relacionados que van entre 200-300 familias con varios individuos que presenten la enfermedad en estudio. El término ligamiento se refiere a la co-segregación de marcadores genéticos (tales como marcadores de DNA) con el fenotipo de la enfermedad en familias con múltiples miembros afectados).[29] De esta manera, se puede analizar la co-segregación de marcadores genéticos ampliamente distribuidos sobre todo el genoma, tales como los microsatélites o los *Short tandem repetitive (STRs*) y *single nucleotide polymorphisms (SNPs*) y la enfermedad; es posible identificar una o varias regiones genómicas de ligamiento que contribuyen al desarrollo del padecimiento. Esta

región de ligamiento puede contener el gen responsable de la enfermedad. Hasta hace apenas unos pocos años, en los análisis clásicos de escaneo del ligamiento del genoma completo (*Genome-wide linkage scan: GWLS*) se utilizaban marcadores genéticos tipo STRs, los cuales son muy informativos, sin embargo, no se encuentran tan ampliamente distribuidos como otros polimorfismos dentro del genoma. De hecho, mediante algunos GWLS se han identificado varias regiones genómicas involucradas con el desarrollo de enfermedades cardiovasculares. Una vez confirmado el ligamiento, la búsqueda del o de los genes candidato debe iniciarse. En general las regiones de ligamiento asociadas a enfermedades comunes son grandes. En ocasiones se tiene que buscar un solo gen en 20-30 millones de pares de bases, y comúnmente puede haber más de 500 genes, entonces, cómo se pueden buscar genes en una región tan grande?. Lo que inicialmente se hace es buscar en bases de datos los genes ubicados en la región de ligamiento y definir cuales de ellos tienen un papel funcional. Actualmente, para este mismo tipo de estudio se pueden emplear los SNPs que tienen una distribución mayor dentro del genoma (se ha calculado que existen entre 10 y 20 millones). Los SNPs además son genéticamente estables y generalmente son bialélicos, esta última característica hace que técnicamente sean fáciles de analizar a gran escala, a un costo realmente bajo. Una de las principales ventajas que presenta este tipo de estudio es el análisis del genoma completo, de esta manera, este es el primer paso para hacer un mapeo más detallado con polimorfismos comunes. La principal desventaja que presenta esta estrategia es su bajo poder estadístico debido a la dificultad que implica obtener un gran número de familias con múltiples casos afectados.[29] Otra desventaja es que en las enfermedades multifactoriales, participan varios genes de baja penetrancia, así como factores ambientales de riesgo que contribuyen al desarrollo de la enfermedad. Por lo anterior, en

las enfermedades cardiovasculares los estudios de ligamiento han tenido poco éxito para definir cuáles son los genes involucrados en la etiología de la enfermedad. Sin embargo, se han logrado definir diferentes locus que contienen marcadores vinculados con el desarrollo de la aterogénesis empleando estudios de ligamiento genético. Entre éstos se tienen cuatro estudios en los que se encontraron regiones ligadas con el desarrollo de enfermedad arterial coronaria (EAC); seis estudios con regiones vinculadas al infarto agudo del miocardio (IAM), dos trabajos con información sobre regiones ligadas a EAC, IAM y síndrome isquémico coronario agudo (SICA), dos investigaciones que refieren el hallazgo de regiones ligadas con el incremento en el espesor de la íntima media de la carótida; dos estudios que informan acerca de regiones ligadas con la calcificación de la arteria coronaria; otros dos que detectaron regiones ligadas con evento vascular cerebral y una investigación sobre regiones ligadas a oclusión de la arteria periférica.[30] Por otro lado, los resultados de dos meta-análisis[31,32] demostraron la presencia de dos regiones de ligamiento en el brazo largo de los cromosomas 2 y 3, y cuatro regiones de ligamiento en los cromosomas 1p, 5p, 12 q y 13q. En dicho estudio se identificaron además cuatro regiones de ligamiento en los cromosomas 6p, 14p, y dos regiones en el cromosoma 8q, las cuales no se habían referido en la literatura. Otro meta-análisis reportó regiones de ligamiento en los cromosomas 2, 5 y 17 asocia- das con EAC, IAM e isquemia cerebral, de forma respectiva.[33] El grupo DeCode Genetics, de Islandia, mapeó un locus en el cromosoma 13q12-13 asociado con IAM y evento vascular cerebral. Este grupo identificó al gen ALOX5 como responsable de codificar para la proteína activadora 5-lipoxigenasa (ALOX5AP).[34]

2.1.5 Análisis de asociación

Los estudios de asociación dependen de la población más que de familias con múltiples miembros afectados. Su base es la siguiente, una vez que aparece una mutación esta tiende a extenderse en la población junto con los alelos que están presentes en los cromosomas ancestrales en los loci cercanos. Los alelos o haplotipos (combinación de dos o más alelos ubicados en un mismo cromosoma) de esos loci son analizados en estudios de asociación como marcadores de la enfermedad. Los estudios de asociación se pueden realizar en casos (individuos afectados) y controles (individuos sanos no relacionados) o los basados en familias (tríos: padres y probando). El objetivo es comparar frecuencias alélicas y genotípicas de la variante estudiada, y entonces observar si existe una diferencia estadística. Si la variante es más común en casos que en controles, entonces decimos que esta variante está asociada con susceptibilidad, si es más común en controles que en los individuos afectados entonces confiere protección. En el caso de los estudios de asociación basados en familias se emplea un método que se denomina análisis de desequilibrio de transmisión (*transmission disequilibrium test*: TDT). En este método se compara la frecuencia con la cual los padres heterocigotos transmiten un alelo específico de un SNP al hijo afectado; si la frecuencia del alelo transmitido muestra una significancia estadística entonces se dice que está asociado con la enfermedad. Algunas ventajas que representan los estudios de asociación de casos y controles son las siguientes: tienen mayor poder estadístico debido a que es mucho más sencillo conseguir un número mayor de individuos no relacionados que familias completas con varios individuos afectados, o incluso tríos, los SNPs se pueden elegir de acuerdo a su funcionalidad dentro del gen y evaluar su efecto directo en la enfermedad.

Cuando existe evidencia de una región genómica ligada al desarrollo de una enfermedad, lo que sigue es investigar cuáles genes son candidatos (genes que codifican a proteínas que por su función biológica pueden estar involucrados en su etiopatología) a estudiar en esta región, la cual puede comprender hasta cientos de ellos.[29] A diferencia de los estudios de ligamiento donde se escanea el genoma completo, en los análisis de asociación se pueden seguir varias estrategias, lo cual representa una gran ventaja; por ejemplo, se puede analizar uno o varios SNPs en un gen candidato sin evidencia previa de ligamiento o una región genómica que previamente haya mostrado evidencia de ligamiento. Actualmente se ha desarrollado un nuevo tipo de análisis de asociación denominado estudio de asociación amplio del genoma o estudio de asociación del genoma completo (GWAS). Con este tipo de estudio se puede hacer un mapeo genético más detallado de SNPs ubicados dentro de una región previa de ligamiento o escanear completamente al genoma de un individuo con un cierto fenotipo mediante microarreglos de SNPs de casas comerciales, estos chips pueden analizar dependiendo del modelo hasta más de un millón de marcadores, su parámetro principal está basado en el desequilibrio de ligamiento (*linkage disequilibrium*: LD), el cual se refiere a una asociación no azarosa de alelos en dos o más loci con la enfermedad. Además, estos mismos microarreglos pueden analizar a otro tipo de polimorfismos en el genoma denominados variantes en el número de copias (*copy number variants*: CNVs), los cuales son segmentos de DNA que tienen un tamaño igual o mayor a 1 kb, estas CNVs incluyen inserciones, deleciones, duplicaciones, y no causan enfermedades sino susceptibilidad. Se han examinado diversos genes candidatos en pacientes con ateroesclerosis. Ozaki y colaboradores analizaron 92 788 SNPs en población japonesa, e identificaron cinco polimorfismos de los genes LTA (linfotoxina α), NFKBIL1 y BAT1 ubicados en el

cromosoma 6, asociados con el desarrollo de IAM.[35] El estudio antes mencionado se replicó en pacientes con EAC e IAM en diferentes poblaciones y obtuvo datos similares a los reportados por Ozaki y colaboradores.[36] Por otro lado, Shiffman y colaboradores en un estudio GWAS de 11 053 SNP, probaron la asociación de polimorfismos ubicados en los genes PALLDIN, ROS1, TAS2R50, OR13G1 con el desarrollo de IAM.[37] Un estudio analizó 11747 SNP en pacientes con IAM y comprobó la relación de este padecimiento con polimorfismos ubicados en los genes VAMP8 y HNRPUL1.[38] En fechas recientes, Palmer y colaboradores analizaron 1536 SNPs en una población hispanoamericana e identificaron varios genes vinculados con el desarrollo de enfermedades subyacentes a la ateroesclerosis, entre los que destacan MAG11, DMRT3, gen hipotético AK097474, KLHL25, EFCAB7, ALG6 y MYH13 ubicados en los cromosomas 3, 8, 10, 15 y 17, respectivamente.[39] En otro estudio GWAS, O'Donenell y colaboradores analizaron más de 100 000 SNPs presentes en 20 de los 23 cromosomas, en una población caucásica americana.[40] Este grupo informó sobre más de 20 sitios polimórficos de diferentes genes (AB12, PCSK2, NOS3, ESRI, entre otros) distribuidos a lo largo de 20 cromosomas, relacionados con la susceptibilidad al desarrollo de ateroesclerosis subclínica.

Los GWAS permiten identificar asociaciones entre genotipos y fenotipos de forma precisa. El consorcio CARDIoGRAMplusC4D,[41] que representa el estudio genético más grande para EAC a la fecha, ha mostrado asociaciones modestas (RM 1.06 a 1.51 por copia del alelo de riesgo), sin embargo, estas asociaciones incluyen alelos comunes, por lo que se sugiere que el riesgo atribuible puede ser sustancial. Análisis adicionales sugieren que los *loci* asociados a EAC incluyen genes que codifican para moléculas que están involucradas tanto en las vías de metabolismo de lípidos como en inflamación.[41]

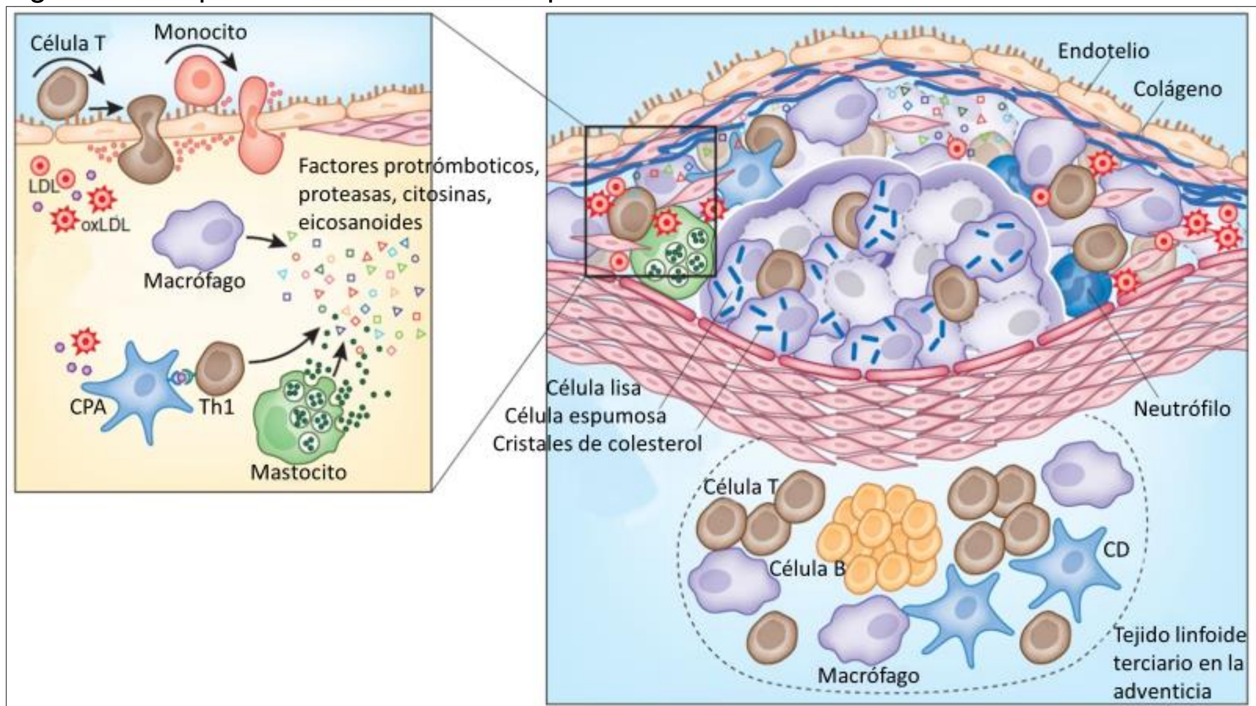
2.1.6 Sistema inmune en la aterosclerosis

Como se mencionó previamente, la ECV es la principal causa de mortalidad en varios países. La EAC y la EVC constituyen las dos formas más comunes de la ECV, ambas tienen severas consecuencias tanto para el individuo, como para la sociedad. El proceso subyacente a estas manifestaciones clínicas es la aterosclerosis, una enfermedad crónica de progresión lenta que afecta a las arterias de tamaño medio y grande y que se manifiesta clínicamente cuando provoca trombosis.[42] La concepción de la aterosclerosis ha cambiado de ser un proceso pasivo de acumulación de colesterol en la pared del vaso, a ser definida y considerada como una enfermedad inflamatoria crónica.

La placa aterosclerosa se caracteriza por la acumulación de lípidos en la pared de la arteria, junto con inmunocitos tales como macrófagos, células T y mastocitos, y la formación de una capa fibrosa por acción de células vasculares del músculo liso, compuesta fundamentalmente de colágeno. La lesión temprana, conocida como estría grasa consiste en depósito subendotelial de lípidos, células espumosas cargadas con colesterol y células T (Figura 4).

Con el paso del tiempo se desarrolla una lesión más compleja, con células apoptóticas, necróticas, desechos celulares y cristales de colesterol conformando un centro necrótico en la lesión. Esta estructura está cubierta por una capa fibrosa de grosor variable; la región superior, conocida como hombro de la lesión, se encuentra infiltrada con células T activadas, macrófagos y mastocitos, lo cuales producen mediadores proinflamatorios y enzimas.[43] El crecimiento de la placa puede provocar estenosis y estrechamiento de la luz del vaso, contribuyendo así a la isquemia del tejido aledaño.

Figura 4. componentes inmunes de la placa aterosclerosa



El ateroma tiene un centro lipídico que incluye cristales de colesterol, células vivas y apoptóticas y una capa fibrosa con células de músculo liso y colágeno. Las lipoproteínas del plasma se acumulan en la región subendotelial. Diferentes tipos de células de la respuesta inmune se encuentran presentes en el ateroma, incluyendo macrófagos, células T, mastocitos y células dendríticas. El ateroma crece en la íntima. Fuera de la íntima, la media contiene células del músculo liso que regulan la tensión sanguínea y la perfusión de la región. La adventicia continua en el tejido conectivo de alrededor. Ahí, se acumulan células de la respuesta inmune fuera del ateroma que pudieran desarrollar una estructura linfoide terciaria con centros germinales.
Tomada y adaptada de: Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol. 2011;12:204-212. **Licence number: 4216611092994**

La trombosis se presenta en la superficie tras la ruptura de la placa. Esto expone material trombogénico presente en el centro de la lesión, se produce agregación plaquetaria, coagulación humoral y la formación de un trombo que puede obstruir la luz del vaso o bien desprenderse un coagulo que es capaz de bloquear el flujo sanguíneo de un vaso en un punto lejano al lugar de origen. La aterotrombosis provoca isquemia, con el consecuente infarto al miocardio o infarto cerebral.

2.1.7 Lipoproteína de baja densidad e inflamación

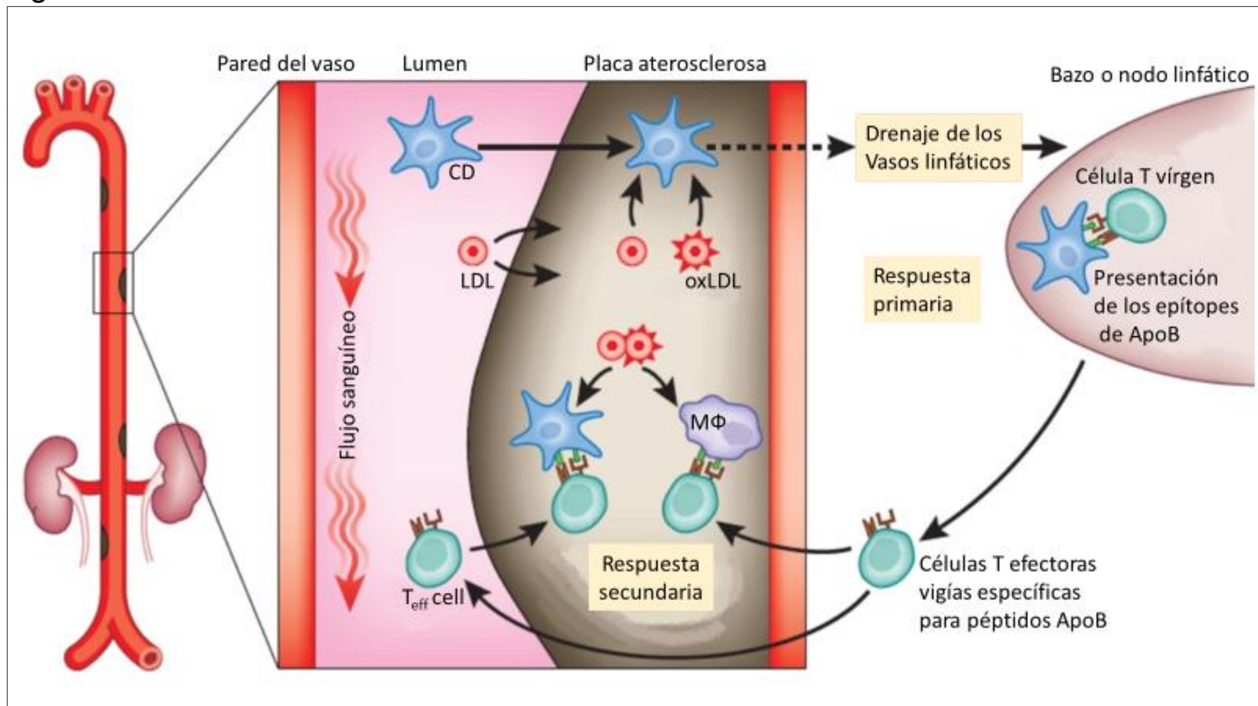
En la sangre, el colesterol es transportado desde el hígado hacia los tejidos por la lipoproteína de baja densidad o LDL (por sus siglas en inglés). Los estudios en modelos animales, clínicos y epidemiológicos han mostrado que el aumento en el número de partículas de LDL circulantes promueve la ECV aterosclerosa. La LDL está formada por un centro de ésteres de colesterol y triglicéridos, rodeados por fosfolípidos y colesterol libre y contiene una molécula de apolipoproteína B100 (apoB100). Mediante interacciones iónicas, esta proteína se une a proteoglicanos de la matriz extracelular,[44] un factor importante en la iniciación del proceso ateroscleroso temprano.[45] La consecuencia de la retención subendotelial de las partículas de LDL es que éstas quedan atrapadas en la íntima donde son susceptibles de ser modificadas químicamente por ataques enzimáticos o por especies reactivas de oxígeno. La peroxidación de los residuos de ácidos grasos presentes en los fosfolípidos, ésteres de colesterol y triglicéridos generan aldehídos reactivos capaces de iniciar la respuesta inflamatoria innata.

Inicialmente, los antioxidantes lipofílicos previenen la modificación de la lipoproteínas, sin embargo, cuando los antioxidantes se consumen, inicia la oxidación de los ácidos grasos polinsaturados y la partícula LDL se considera “mínimamente modificada”.[46] Una vez iniciada, la lipoperoxidación se autocataliza generando una reacción en cadena promoviendo la acumulación de gran cantidad de productos de lipoperoxidación que provocan la modificación química de la apoB100. Esto origina la disminución de la carga positiva de la partícula y la formación de nuevos epítopes que ya no son reconocidos por el receptor natural de la LDL, pero son reconocidos ávidamente por el receptor tipo basurero (“scavenger”) de los macrófagos.[47,48]

La LDL oxidada no es un tipo de molécula definida, sino un espectro de partículas que han sufrido una variedad de cambios fisicoquímicos que dependen del agente oxidante. Los lípidos oxidados presentes en la LDL activan a las células endoteliales y macrófagos, lo que resulta en producción de moléculas de adhesión como E-selectina y VCAM-1 (molécula de adhesión vascular celular 1) y quimiocinas, tales como CCL2, CCL5, CXCL10 y CX3CL1 que atraen monocitos, células dendríticas y células T a la íntima.[49] En la íntima, mediante el factor estimulador de colonias, los macrófagos estimulan la diferenciación de monocitos a macrófagos, proceso necesario para el desarrollo de la aterosclerosis.[50] Los macrófagos regulan a la alta los receptores tipo basurero para fagocitar a las LDL oxidadas. Estos receptores carecen de regulación a la baja, lo que conduce a captación continua y en grandes cantidades de LDL oxidada, favoreciendo la acumulación de lípidos en su interior y su conversión a células espumosas. Estas, al acumularse, forman la llamada estría grasa, considerada la lesión inicial de la aterosclerosis.

Las células dendríticas monitorean las arterias y pueden captar componentes de partículas oxidadas de LDL para su subsecuente presentación en los nódulos linfáticos (Figura 5). En la pared de una arteria normal, las células dendríticas residentes promueven la tolerancia a los antígenos silenciando a las células T, sin embargo, señales de peligro generadas durante la aterogénesis pueden activar a las células dendríticas, provocando el cambio de tolerante a reactiva de la inmunidad adaptativa.[51,52] Las células T reclutadas en la lesión aterosclerosa producen mediadores proaterogénicos y contribuyen tanto al crecimiento como a la mayor gravedad de la lesión.[43,53]

Figura 5. Activación de células T en el vaso



La aorta que se muestra a la izquierda tiene varias placas (óvalos obscuros). las células dendríticas emigran de la sangre a la arteria, donde captan antígenos tales como fragmentos de apoB100 y migran a los nódulos linfáticos donde presentan los antígenos a células T naïve. Después de la activación, estas células se diferencian a células efectoras y entran al torrente sanguíneo. Cuando las células efectoras son reclutadas en las placas aterosclerosas, son reactivadas mediante la presentación de antígeno por macrófagos locales y células dendríticas.
Tomada y adaptada de: Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol. 2011;12:204-212. **Licence number: 4216611092994**

Las células B y los mastocitos se encuentran presentes solo ocasionalmente en las lesiones, pero son abundantes en el lado extra luminal y de la adventicia de la arteria aterosclerosa.[54,55] Es claro que los mecanismos adaptativos, así como los innatos, tienen una participación importante en la aterosclerosis.

2.1.8 Familia de la interleucina 12

La familia de la interleucina 12 (IL-12), evolutivamente ligada a la superfamilia de la interleucina 6 (IL-6), consiste en un grupo único de citocinas heterodiméricas compuestas de una de tres subunidades alfa (p19, p28 o p35) y una de dos subunidades beta (p40 o gen 3 inducido por virus de Epstein-Barr (EBI3))[56] (Figura 6). La α hélice es un grupo de

4 hélices estructuralmente similar al de las citocinas de IL-6 tipo I, mientras que la cadena β está compuesta por dos dominios tipo II de fibronectina que forman una región homologa de unión a citocinas y un dominio N-terminal de inmunoglobulina homologo al del receptor soluble de IL-6.[57] La unión de las diferentes subunidades α y β, da como resultado 6 citocinas heterodiméricas (Figura 6). Los miembros de la familia de la IL-12 tiene una participación clave tanto en la promoción como en la supresión de múltiples respuestas inmunológicas bajo condiciones fisiológicas, así como patológicas. Las citocinas de la familia de la IL-12 señalizan mediante receptores heteródiméricos expresados en la superficie de las células.[56] Cada cadena se une individualmente a su subunidad correspondiente del receptor (Figura 6).

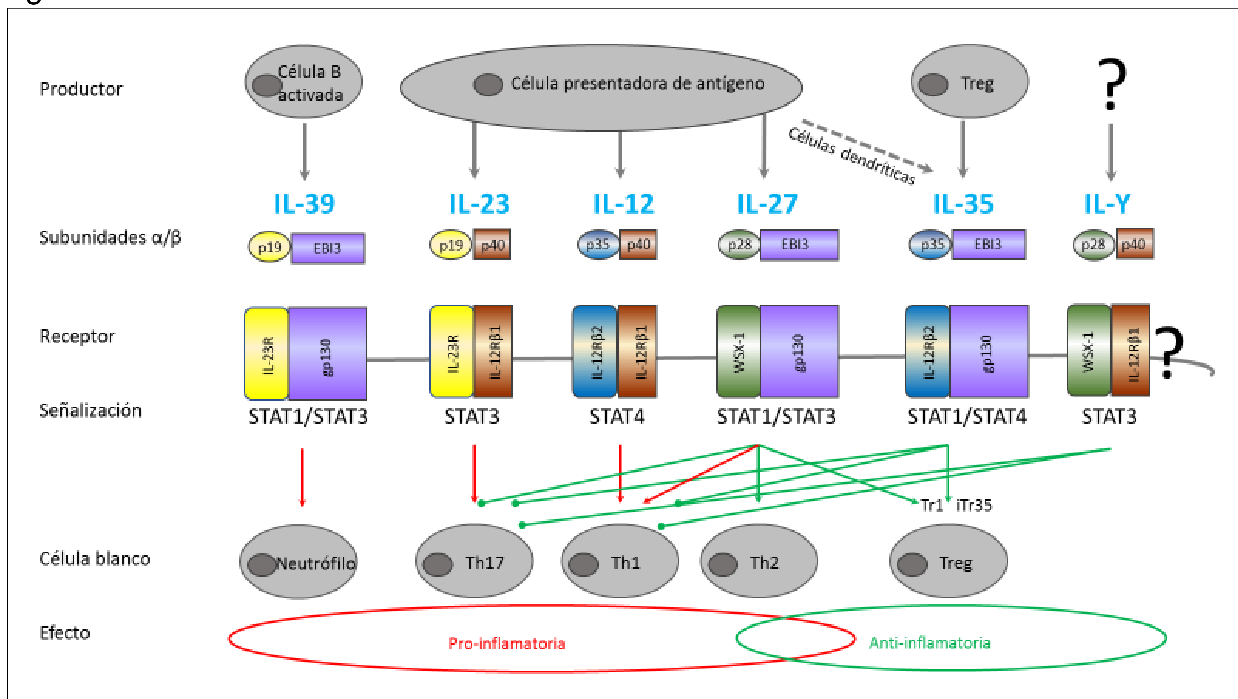
La interleucina 12 es el patriarca de la familia, fue descubierta independientemente por Kobayashi y colaboradores[58] y Stern y colaboradores[59] como "factor estimulador de las células asesinas naturales (natural killer-NK)" y como "factor de maduración de linfocitos citotóxicos, respectivamente. La interleucina 12 es una proteína heterodimérica de 75 kDa, formada por las subunidades p40 y p35 unidas por un puente disulfuro[60] y se une al complejo heterodimérico formado por las cadenas IL-12Rβ1/IL-12Rβ2. La subunidad β p40 también se puede unir covalentemente a la p19 mediante un puente disulfuro para formar a la interleucina 23 (IL-23),[61] que señaliza mediante su unión al receptor IL-12Rβ1/IL-23R. La interleucina 27 (IL-27) está constituida por la unión no covalente de las cadenas p28 y EBI3 (Figura 6) y su señalización es vía el complejo del receptor WSX-1/glicoproteína 130 (gp130) [62]. La unión no covalente de las subunidades p35 y EBI3 forma la interleucina 35 (IL-35), esta citocina tiene una característica única de señalización, no compartida por los otros miembros de la familia, señaliza vía cuatro complejos de receptores: IL-12Rβ2/gp130, IL-12Rβ2/IL12Rβ2, gp130/gp130 y IL-

12Rβ2/WSX-1.[63,64] La recientemente descubierta IL-39, está formada por la cadena p19 y la cadena EBI3 y su vía de señalización es a través de IL-23R/gp130.[65] El heterodímero p28/p40, tentativamente nombrado IL-Y, se ha demostrado que antagoniza la señalización de IL-12 e IL-27.[66]

La señalización de los receptores mencionados anteriormente, es mediada por la familia de los miembros de traducción de señal de la cinasa Janus y los activadores de la transcripción (JAK-STAT).[67] La vía de señalización de la IL-12 es STAT4,[68] de la IL-23 es STAT3/-STAT4,[69] de IL-27 e IL-39 es STAT1/STAT3,[57,70] y la IL-Y lo hace vía STAT3[57] (Figura 6).

La coexpresión de las cadenas α y β es un pre-requisito para la secreción de la citocina activa.[71] La expresión de la cadena α limita la producción de cada una de las citocinas heterodiméricas. En comparación con EBI3 y p40, la p35 se expresa a niveles más bajos, limitando por tanto el ensamblaje de la IL-12 e IL-35. De forma similar, la restricción a nivel celular y de tejido de la p19, limita la secreción de la IL-23. Las citocinas de la familia de la IL-12 pueden directamente inducir el desarrollo de subpoblaciones de células T, así como alterar la función y el destino de varias poblaciones celulares, dictaminando el desenlace de la enfermedad; de esta manera, actúan como reguladores inmunológicos, dando forma a la respuesta inmune.[56] Las células presentadoras de antígeno, como las células dendríticas y macrófagos, expresan y secretan IL-12, IL-23 e IL-27. La activación de receptores de inmunidad innata induce su expresión. La IL-35 se expresa principalmente por células T reguladoras FoxP3+, aunque también la pueden expresar las células Tγδ, CD8+ y trofoblastos placentarios.[72] Wang y colaboradores[73] demostraron que las células B activadas pueden secretar IL-39.

Figura 6. Miembros de la familia de la interleucina 12.



Esta interesante y fascinante familia está compuesta por citocinas heterodiméricas que comparten tres cadenas α (p19, p28, p35) y dos cadenas β (p40 y EBI3). Sus receptores son heterodiméricas y comparten sus subunidades también. La señalización de estas citocinas se realiza vía la familia Jak-STAT y tiene diferentes efectos sobre subpoblaciones de células efectoras. Las flechas rojas indican efectos proinflamatorias, las verdes propiedades antinflamatorias.
Tomada y modificada de: Ringkowski S, Thomas PS, Herbert C. Interleukin-12 family cytokines and sarcoidosis. *Frontiers in Pharmacology*. 2014;5:233.

Los miembros de la familia de la IL-12 exhiben propiedades inmunoreguladoras debido a sus efectos sobre la diferenciación y función de células T. La IL-12 e IL-23 son citocinas proinflamatorias, la primera es capaz de inducir células Th1 mientras que la segunda participa de manera importante en la inducción de células Th17. La IL-27 tiene un fenotipo de efectos pleiotrópicos capaz de aumentar la respuesta tanto pro como anti-inflamatoria.[62] La IL-35 es una citocina supresora que inhibe el desarrollo de células Th17, la proliferación y la respuesta de células T efectoras [56,72] de manera dependiente de interleucina 10 (IL-10).[74] La IL-39 induce la diferenciación y/o expansión de neutrófilos [73] (Figura 6).

Como se mencionó previamente, en las etapas tempranas de la aterosclerosis, la respuesta inflamatoria se inicia por la acumulación de lípidos en la pared del vaso. La

respuesta inflamatoria es dirigida por las células Th1 y Th17, con el incremento en la producción de IL-6, interleucina 17 (IL-17), interferón gama y anticuerpos IgG2a dirigidos contra las LDL modificadas.[75,76] Las citocinas de la familia de la IL-12 tiene una participación crítica en la diferenciación de células T CD4+ vírgenes mediada por células dendríticas activadas.

2.2 ANTECEDENTES.

La EAC es una patología poligénica y de origen multifactorial que resulta de una respuesta inflamatoria exacerbada. Recientemente se han descrito dos nuevas citocinas que participan en este proceso: la IL-27 y la IL-35, miembros de la familia heterodimérica de la IL-12.

La IL-27 se produce principalmente por células dendríticas,[77] está compuesta por la subunidad β EBI-3[78] y la subunidad α p28,[79] y participa como iniciador y atenuador de la respuesta inmune/inflamatoria;[80] promueve la diferenciación temprana de Th1[81] y suprime la diferenciación de Th2[82] y Th17,[83] e induce la producción de citocinas antiinflamatorias tales como IL-10.[84] Estudios en cultivos celulares,[85,86] modelos animales[85] y en pacientes coronarios[86] así como la detección de la IL-27 en placas aterosclerosas[87] sugieren la participación de esta citocina en la EAC.

La IL-35 es una citocina antiinflamatoria, está formada por la subunidad α, p35 y la subunidad β, EBI3,[88] es producida por células T CD4+Foxp4+ (T reguladoras-Tregs) y se requiere para que estas células realicen su función supresora,[88] suprime la actividad de las células T efectoras (Th1, Th2 y Th17) y disminuye la progresión de enfermedades tanto inflamatorias como autoinmunes.[89–91] La IL-35 puede transformar células T naïve en células supresoras Treg llamadas iTr35.[89] Estudios en modelos animales[92] y en

pacientes coronarios[93] apoyan la participación de la IL-35 en el desarrollo de la aterosclerosis.

2.2.1 Interleucina-27

La IL-27 (Figura 7) es producida principalmente por células dendríticas, monocitos y macrófagos activados después de reconocer patrones específicos de patógenos (PAMS por sus siglas en inglés).[69,70] Mas aún, macrófagos activados con lipopolisacáridos expresan niveles elevados de p28 y EBI3 en cualquier tejido o tipo celular descrito;[69,70,94] la interacción entre el receptor de superficie CD40 y el ligando de CD40 entre las células dendríticas y las células T incrementa la producción de IL-27, IL-23 e IL-12.[95]

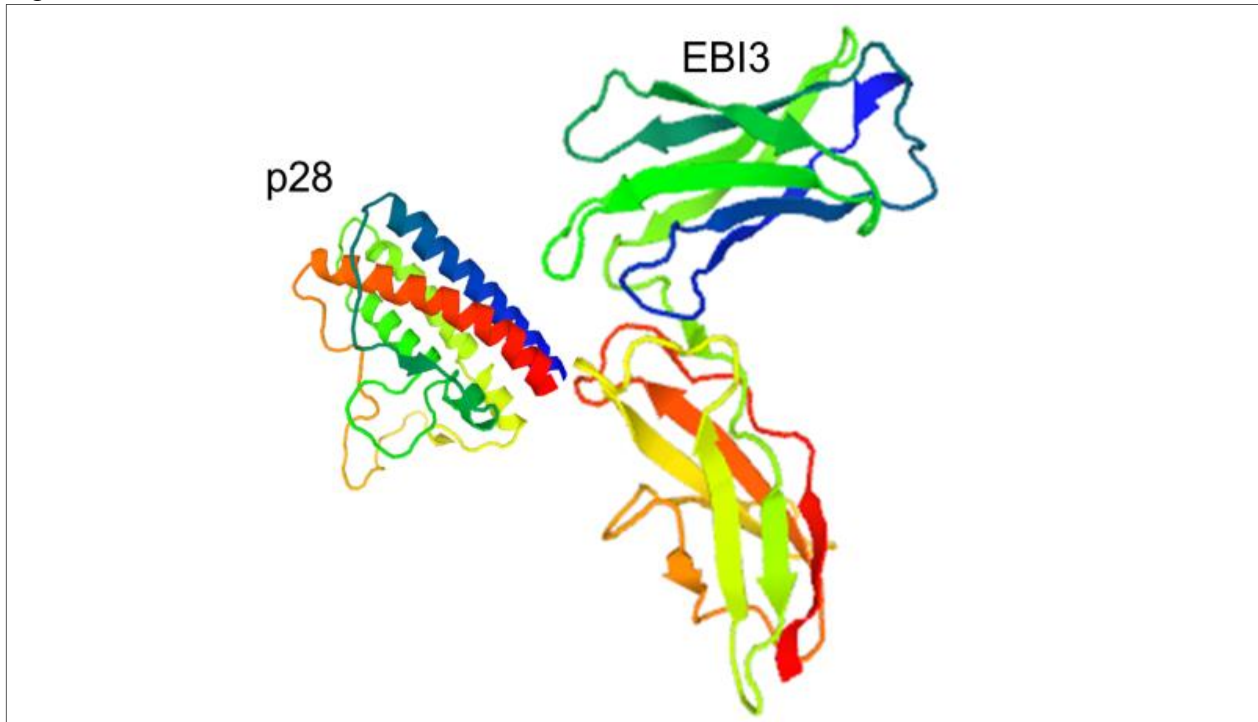
La IL-27 es considerada una citocina pleiotrópica debido a que modula la respuesta anti-inflamatoria así como la pro-inflamatoria dependiendo de la respuesta inmune predominante, tipo y severidad de la enfermedad. Los efectos pleiotrópicos de esta citocina sugieren que su amplia participación en diversas enfermedades inflamatorias puede deberse a su efecto en mecanismos comunes involucrados en este tipo de patologías.[96] Inicialmente se reportó que la IL-27, junto con la IL-12, inician la expansión clonal de las células T naïve e incrementan la producción de IFNγ en estas células, así como en células NK,[70,79] lo que sugiere que la IL-27 sensibiliza a las células T a los efectos de IL-12 y, por tanto, es crítica en los eventos tempranos que dan lugar a la proliferación y diferenciación de células Th1.[97] Estudios posteriores han demostrado que la IL-27 también tiene actividad antiinflamatoria. La IL-27 puede inhibir la actividad de células Th1 durante las infecciones,[98] el desarrollo de células Th17 en diferentes condiciones inflamatorias,[99] la formación de células Treg inducida por el factor transformante de crecimiento β (transforming growth factor-β),[100] y la secreción de

interleucina 4 (IL-4); también antagoniza la producción de interleucina 2 (IL-2), limitando, por tanto, la diferenciación de células Th2 y la función celular efectora.[101] La IL-27 convierte a células inflamatorias T CD4+ activadas en células Th1 o Tr1 productoras de IL-10.[99,102] Al parecer, la IL-27 tiene bidireccionalidad en sus funciones y, así como parece inducir la respuesta inflamatoria en células naïve, hace lo opuesto en células activadas. Adicionalmente, se ha sugerido un incremento en la expresión de la IL-27 durante el desarrollo fetal, consecuentemente la IL-27, similar a la IL-35, pudiera tener un papel en la tolerancia materno-fetal.[103] En este sentido, la IL-27 es el único miembro de la familia de la IL-12 que posee ambas actividades, tanto inflamatoria como inmunomoduladora.

La subunidad p28 de la IL-27, conocida como IL-30, sola actúa como agonista de la señalización mediada por gp130.[104] La IL-30 bloquea la señal mediada por IL-6, IL-12 e IL-27, incluyendo la respuesta Th17 dependiente de IL-6.

El receptor de la IL-27 es un complejo formado por las cadenas WSX-1 y gp130.[105] Las subunidades WSX-1 y gp130 se co-expresan en diferentes tipos celulares como células dendríticas, mastocitos, monocitos, macrófagos, células NK, células endoteliales, linfocitos T y B.[79,106,107] La complejidad de su receptor podría explicar el amplio margen de función inmunomoduladora de esta citocina. La activación o supresión de la inflamación por la IL-27 puede variar dentro de diferentes patologías. La evidencia disponible muestra que la IL-27 suprime la inflamación en la infección por *leishmania donovani*,[108] en la inflamación intraocular,[109] inflamación crónica del sistema nervioso central,[110] artritis autoinmune,[111] encefalitis autoinmune experimental,[83] asma alérgico.[112] En contraste, promueve la inflamación en esclerosis sistémica,[113] glomerulonefritis experimental,[114] colitis experimental[115] y en hepatitis.[116]

Figura 7. Modelo molecular de la interleucina 27



Se observan las moléculas de las subunidades p28 y EBI3 que conforman a la interleucina 27.

La participación de la IL-27 en la regulación de la inmunidad innata y adaptativa apoya su posible contribución en la aterosclerosis. La IL-27 se expresa en placas ateroscleróticas,[117] y su papel en la aterosclerosis ha sido reportado en cultivos celulares, modelos animales y pacientes coronarios, con hallazgos inconsistentes. En ratones susceptibles al desarrollo de la aterosclerosis (Ldlr$^{-/-}$) y deficientes de IL-27 (Ldlr$^{-/-}$Ebi3$^{-/-}$) o del receptor para esta citocina (Ldlr$^{-/-}$WSX-1$^{-/-}$), el proceso de desarrollo de la enfermedad se acelera y, el tratamiento con IL-27 recombinante, inhibe la aterosclerosis *in vivo* y la activación de macrófagos *in vitro*.[85] Adicionalmente, en un modelo de daño por isquemia/reperfusión en rata, la administración de IL-27 cinco minutos antes de la reperfusión redujo el daño tisular y mejoró de forma importante la recuperación post-isquemia en los corazones aislados perfundidos, sugiriendo que esta citocina protege al miocardio contra el daño de la isquemia, y facilita la recuperación de los cardiomiocitos

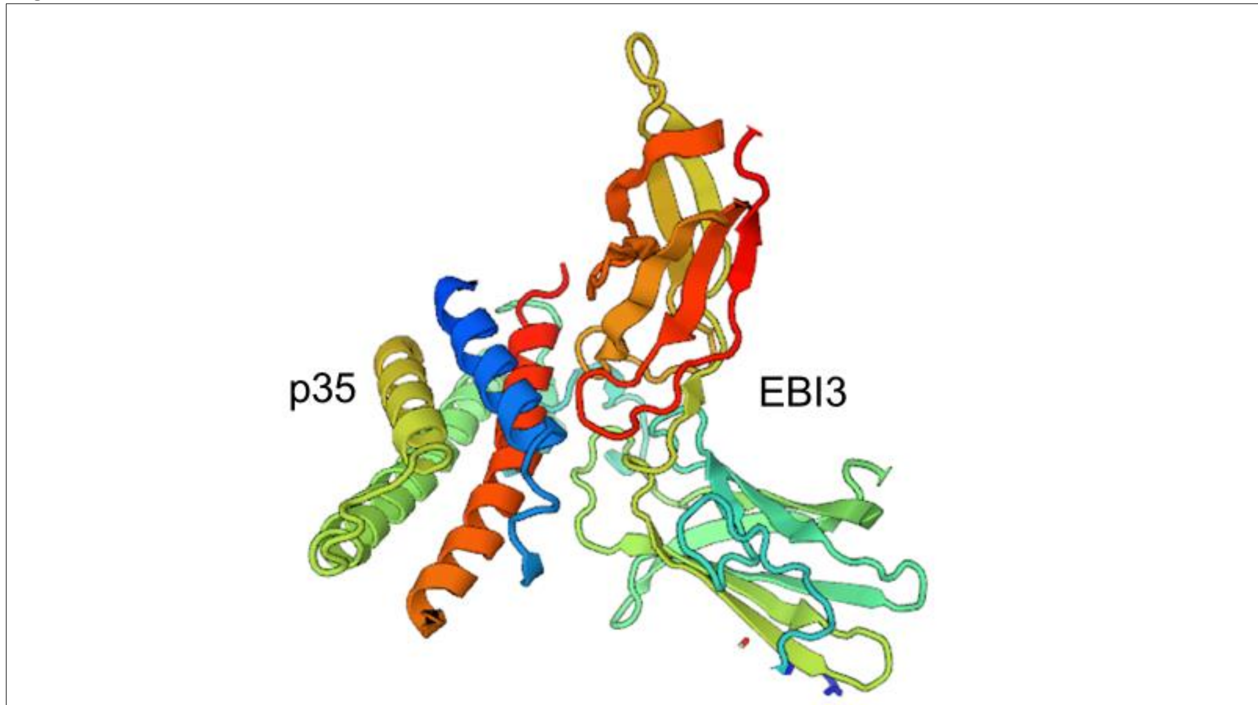
dañados.[118] Un estudió *in vitro* mostró que, la IL-27 reduce la acumulación de lípidos en macrófagos THP-1 e incrementa de manera importante el eflujo de colesterol a través del aumento en la expresión tanto del RNA mensajero como de la proteína del transportador de membrana ABCA1.[119] Estos hallazgos sugieren que la IL-27 reduce la acumulación de lípidos en las células espumosas gracias a la regulación de la expresión del transportador transmembranal ABCA1. En contraste, en un estudio clínico que incluyo 136 pacientes con EAC y 29 sujetos control, Jin y colaboradores observaron que las concentraciones en plasma de la IL-27 eran mayores y correlacionaban con el grado de severidad de la lesión aterosclerótica coronaria en pacientes con EAC al compararlos con los sujetos control.[86] Más aún, células dendríticas incubadas con LDL oxidadas producen IL-27, sugiriendo que estas partículas modificadas pudieran tener una participación importante en la activación de las células dendríticas y la producción de IL-27.[86] Estos datos en conjunto sugieren que, la IL-27 pudiera tener un papel crucial en la inmunidad y en la red reguladora del proceso inflamatorio en la aterosclerosis (Tabla 4).

2.2.2 Interleucina-35

La IL-35 (Figura 8) es considerada una citocina anti-inflamatoria e inmunosupresora.[120] Se secreta constitutivamente por las células T CD4+ reguladoras.[64] En ratones y en humanos, la IL-35 es capaz de inducir la conversión de células Treg convencionales en células supresoras Treg productoras de IL-35, conocidas como iTr35.[63] La IL-35 no se expresa constitutivamente en tejidos.[88] Los genes que codifican para las subunidades de la IL-35 se transcriben en células B activadas y en menor grado en monocitos, células del músculo liso y células endoteliales tras la activación con lipopolisacáridos y citocinas proinflamatorias.[88] No obstante la IL-35 comparte características estructurales con los

miembros de la familia de la IL-12, su función parece ser estrictamente reguladora. Contrario a los efectos inflamatorios de la IL-12 y 23, la IL-35 puede inhibir de manera efectiva células Th1 y Th17 mediante la expansión de células Treg y producción de IL-10, es también importante para que las células Treg ejerzan su actividad de forma óptima.[64] Devergne y colaboradores, reportaron una elevada expresión de las subunidades de la IL-35, EBI3 y p35, en trofoblastos placentarios, lo que sugiere que, de forma similar a la IL-27, la IL-35 pudiera ser un inmunomodulador de las barrera materno-fetal.[121] Así, la IL-35 tiene participación crítica en la prevención de autoinmunidad, en mantener autotolerancia y suprimir la respuesta inmune antitumoral. En modelos experimentales, la IL-35 ha mostrado suprimir el desarrollo de la artritis inducida por colágeno,[122] diabetes autoinmune,[90] encefalomielitis autoinmune,[123] enfermedad inflamatoria intestinal[124] y enfermedad alérgica de las vías aéreas dependiente de IL-17.[125]

Figura 8. Modelo molecular de la IL-35



Se observan las moléculas de las subunidades p35 y EBI3 que conforman a la interleucina 35.

Tabla 4 Estudios y sus hallazgos en la participación de las interleucinas 27 y 35 en aterosclerosis

Estudio	Hallazgo	Referencia
IL-27		
Cultivo celular	IL-27 reduce la acumulación de lípidos en macrófagos THP-1 y aumenta el eflujo de colesterol	119
Modelo animal	La deficiencia de IL-27 y su receptor acelera la aterosclerosis. El tratamiento con IL-27 recombinante inhibe la aterosclerosis *in vivo* y la activación de macrófagos *in vitro*	85
	En un modelo de rata de daño por isquemia/reperfusión, la administración de IL-27 reduce el daño del tejido y mejora la recuperación post-isquemia.	118
Humano	La IL-27 se expresa en placas aterosclerosas.	117
	En comparación con controles, los pacientes coronarios tienen concentraciones en plasma de IL-27 más altas, que correlaci0onan con la severidad de la lesión aterosclerosa.	86
IL-35		
Modelo animal	El tratamiento con IL-35 reduce la lesión aterosclerosa en ratones *ApoE*$^{-/-}$	126
	Ratones *Apo E-/-* con lesión aterosclerosa establecida tiene concentraciones más bajas en comparación con ratones control	92
Humano	Se ha demostrado la coexpresión de las subunidades α y β de la IL-35 en placas ateroscleróticas avanzadas	117
	En comparación con sujetos con síndrome de dolor de pecho, los pacientes con síndrome coronario agudo tienen niveles más bajos de IL-35. Los niveles de IL-35 correlacionas con la fracción de eyección ventricular izquierda	93

Como se mencionó con anterioridad, los genes que codifican para las subunidades de la IL-35 son transcritos en las células endoteliales, del músculo liso y monocitos después de ser activadas,[88] por tanto la IL-35 pudiera participar en el desarrollo de la aterosclerosis. Se ha reportado que el tratamiento con IL-35 reduce el área de lesión aterosclerótica en ratones *ApoE*$^{-/-}$ alimentados con dieta alta en grasa, al mejorar la supresión mediada por células Treg.[126] Más aún, en ratones con deficiencia en apo E (*ApoE*$^{-/-}$) con lesiones ateroscleróticas establecidas, Wang y colaboradores[92] recientemente han mostrado niveles más bajos de IL-35 comparados con ratones silvestres C57BL/6 de la misma edad sin presencia de placa aterosclerosa. Por otro lado, la expresión de la IL-35 aumenta significativamente en ratones deficientes de apo E (*ApoE*$^{-/-}$) con atenuación de la placa aterosclerosa. En humanos, se ha demostrado la co-

expresión de las subunidades de la IL-35 en placas ateroscleróticas avanzadas.[117] Mas aún, se han reportado concentraciones bajas de IL-35 en pacientes con síndrome coronario agudo (angina inestable e infarto al miocardio) comparadas con sujetos con síndrome de dolor de pecho. En este caso, los niveles de IL-35 correlacionaron positivamente con la fracción de eyección del ventrículo izquierdo cuya reducción se asocia a falla cardiaca.[93] En su conjunto, estos datos apoyan la participación de la IL-35 en el proceso ateroscleroso (Tabla 4).

2.2.3 Genes que codifican para las subunidades α y β de las interleucinas 27 y 35 y su asociación con enfermedad cardiovascular

Los genes que codifican para las subunidades que forman a las interleucinas 27 y 35 son polimórficos y algunos de estos polimorfismos pudieran tener efectos funcionales que cambien o modulen la expresión de estas subunidades y en consecuencia, la expresión y producción de estas citocinas. Polimorfismos genéticos funcionales que alteran la expresión de las citocinas pudieran modular el desarrollo y progresión de la aterosclerosis y ECV. Los genotipos de los polimorfismos en genes que codifican para las cadenas α y β de estas citocinas miembros de la familia de la IL-12, podrían influenciar la producción y actividad de la citocina y, quizá definir, el balance de la respuesta células Th en aterosclerosis.

2.2.3.1 Gen *IL-27p28*

En el humano el gen *IL-27p28* que codifica para la subunidad α de la IL-27 se encuentra ubicado en el locus 16p11, está formado por 5 exones y es altamente polimórfico.[127] Algunos genotipos de polimorfismos localizados en este gen se han asociado con mayor

riesgo de padecer enfermedad inflamatoria intestinal,[128] artritis reumatoide,[129] asma,[127] rinitis alérgica[130] y enfermedad pulmonar crónica obstructiva[131] y con protección para colitis ulcerativa.[132]

Un estudio de casos y controles realizado en población china, mostró la asociación de defectos del septo atrial y ventricular con el alelo *G* del polimorfismo rs153109 del gen *IL-27p28*.[133] En un número importante de pacientes con EAC que pertenecen a la población Han de China se determinaron 4 polimorfismos blanco o Tag SNPs, los cuales son SNPs con alto desequilibrio de ligamiento y que representan a un grupo de polimorfismos conocido como haplotipo (rs181206, rs17855750, rs37833 y rs153109). Después de ajustar por variables potencialmente confusoras, no se observó asociación independiente y significativa entre la presencia de los polimorfismos y la EAC, la edad de inicio de la enfermedad o la severidad de la misma.[134]

2.2.3.2 Gen *IL-12A*

La subunidad α p35 presente en las interleucinas 12 y 35, esta codificada por el gen *IL-12A* formado por 7 exones y localizado en el cromosoma 3q25.33. Estudios previos han mostrado la asociación de los polimorfismos del gen *IL-12A* con enfermedades inflamatorias. En población china, el polimorfismo *IL-12A* rs2243115 se asoció con riesgo de padecer enfermedad pulmonar crónico obstructiva.[135] mientras que el polimorfismo *IL-12A* rs568404 se asoció con riesgo de presentar asma.[136] En un estudio de asociación de casos y controles, se determinaron cinco Tag SNPs del gen *IL-12A* (rs2243115, rs2243123, rs583911, rs568408 y rs2243143) en dos cohortes chinas independientes. Una cohorte piloto en Shanghai y la cohorte de réplica de la isla Xiamen. El análisis de haplotipos mostró que el haplotipo *TTAAG* se asoció con riesgo de presentar enfermedad

de Graves en ambas cohortes.[137] Recientemente, Shen y colaboradores reportaron que el genotipo *IL-12A* rs2243115*GG* pudiera incrementar el riesgo de padecer artritis reumatoide en individuos chinos con factor reumatoide negativo.[138] Un estudio coreano de casos y controles mostró que el haplotipo *TA* (rs582054 y rs2243151) del gen *IL-12A* se asoció significativamente con el fenotipo de dermatitis atópica.[139]

Hasta el momento de la realización del presente trabajo, no existían estudios que hubiesen analizado la asociación de polimorfismos presentes en el gen I*L-12A* con el desarrollo de la enfermedad arterial coronaria.

2.2.3.3 Gen *EBI3*

La subunidad β (EBI3) de las interleucinas 27 y 35 está codificada por el gen *EBI3* localizado en el cromosoma 19q13.3 y contiene 5 exones. En un estudio de asociación de casos y controles en población china, se evaluó la asociación entre polimorfismos presentes en los genes *FOXP3* y *EBI3*. Los análisis de regresión logística, ajustados por edad y sexo, mostraron una asociación significativa entre el polimorfismo *EBI3* rs428253 y menor riesgo para el desarrollo de rinitis alérgica.[140] En otra cohorte china, el mismo polimorfismo se asoció con la presencia de rinosinusitis crónica.[141] En población mexicana, los polimorfismos *EBI3* rs428253, rs4740 y rs4905 se asociaron con riesgo para la presencia de colitis ulcerativa.[132] Zheng y colaboradores encontraron que el polimorfismo *EBI3* rs4740 se asoció con susceptibilidad para tuberculosis pulmonar en sujetos chinos.[142]

De manera similar como sucede con el gen *IL-12A*, al momento de la realización del presente trabajo, no existían estudios que hubiesen analizado la asociación de

polimorfismos presentes en el gen *EBI3* con el desarrollo de la enfermedad arterial coronaria.

3. PLANTEAMIENTO DEL PROBLEMA.

Existe evidencia de que tanto el desarrollo como la progresión de la aterosclerosis están relacionadas con el desequilibrio de citocinas anti y pro-inflamatorias,[53,143] por ello, actualmente se considera a la aterosclerosis como una enfermedad inflamatoria crónica.[144] Células inflamatorias y citocinas pro-inflamatorias se encuentran en lesiones tempranas y avanzadas, así como tras la ruptura de la placa y durante la trombosis. Sin embargo, las citocinas antinflamatorias no se incrementan en forma paralela con las citocinas pro-inflamatorias, por lo tanto, el desequilibrio de estas citocinas da lugar a la progresión de la aterosclerosis, inestabilidad de la placa y la subsecuente presencia de síndrome coronario agudo.[53,143,145] Varios estudios han mostrado que una baja o elevada expresión, así como, las deficiencias funcionales de algunas moléculas inflamatorias conducen al fenómeno inflamatorio lo mismo en individuos asintomáticos, que en pacientes con EAC, hipertensión y aterosclerosis.[49,146–149] Además de las citocinas clásicas que se sabe participan en el proceso inflamatorio, como la IL-1, el TNF-α, la IL-6 y el TGF-beta, recientemente se han descrito nuevas citocinas que participan de manera importante en este proceso. Entre estas citocinas descritas recientemente, destacan las interleucinas 27 y 35, dos miembros de la familia de la IL-12 que se han encontrado asociadas con el desarrollo de AE en modelos animales, cultivos celulares y en humanos. Polimorfismos ubicados en los genes que codifican para estas citocinas podrían estar influenciando su producción y por tanto el desarrollo de la aterosclerosis.

4. JUSTIFICACIÓN

La aterosclerosis es una de las principales causas de muerte a nivel mundial. Según datos de la Secretaria de Salud, en México se registran 50 mil muertes al año por algún evento cardiovascular a causa de la aterosclerosis. Este padecimiento es de origen multifactorial, es decir, en su inicio y progresión participan tanto factores genéticos como ambientales. Evidencia reciente señala que la inflamación e inmunidad, interaccionan con factores de riesgo metabólico, clínicos, ambientales y conductuales, para mediar todas las etapas de la aterosclerosis, desde la acumulación de las LDL dentro del espacio subendotelial y la progresión de la placa aterosclerosa, hasta la ruptura y la trombosis.[42] El estudio de los genes que codifican para moléculas relevantes en la patogénesis de la aterosclerosis, tales como las citocinas, es importante para definir marcadores de susceptibilidad y/o resistencia a este padecimiento. Estas consideraciones sustentan el interés y la importancia de investigar e identificar marcadores genéticos de susceptibilidad para el desarrollo de la EAC aterosclerosa prematura en nuestra población. Los resultados del estudio tienen el potencial de permitir en un futuro no lejano, el establecimiento de tratamientos preventivos y mejor dirigidos al tener en consideración las características individuales. En la literatura internacional no existen estudios que hayan evaluado la asociación de la EAC prematura con polimorfismos de los genes que codifican para las subunidades que constituyen a la IL-27 y la IL-35.

5. PREGUNTA DE INVESTIGACIÓN

¿Los polimorfismos de los genes que codifican para las subunidades que forman la IL-27 e IL-35 confieren susceptibilidad y/o resistencia para el desarrollo de la EAC prematura?

6. HIPÓTESIS

Si las interleucinas 27 y 35 modulan el proceso inflamatorio que participa en el desarrollo de la aterosclerosis y los polimorfismos de los genes que codifican para las subunidades que las forman pueden regular su función y producción, entonces dichos polimorfismos podrían ser considerados como marcadores de susceptibilidad y/o protección para el desarrollo de EAC prematura.

7. OBJETIVOS

7.1 OBJETIVO GENERAL

Establecer si los polimorfismos de los genes que codifican para las subunidades de las interleucinas 27 y35 se asocian con la presencia de la EAC prematura.

7.2 OBJETIVOS ESPECIFICOS:

1. Determinar la frecuencia de los alelos y genotipos de los polimorfismos de los genes que codifican para las subunidades de las interleucinas 27 y 35 en individuos con y sin EAC prematura y, evaluar si algún polimorfismo de los genes estudiados pudiera conferir susceptibilidad para el desarrollo de la EAC prematura.

2. Analizar el desequilibrio de ligamiento entre los polimorfismos estudiados, construir los haplotipos y definir si alguno de éstos pudiera conferir susceptibilidad al desarrollo del padecimiento.

3.- Definir si alguno de los polimorfismos se asocia con algún factor de riesgo cardiovascular y/o parámetro metabólico en individuos con y sin EAC prematura.

4.- Establecer si existen diferencias en las concentraciones de las interleucinas 27 y 35 entre individuos con y sin EAC prematura y, si existe relación entre los genotipos de cada polimorfismo con los niveles séricos de las citocinas en los grupos de estudio.

8. METODOLOGIA

8.1 Población

El estudio Genética de la Enfermedad Aterosclerosa (GEA) fue diseñado en el Instituto Nacional de Cardiología "Ignacio Chávez", para examinar las bases genéticas de la EAC prematura y la relación entre los factores de riesgo tradicionales y los emergentes con la aterosclerosis subclínica en población adulta mexicana. El estudio incluyó 1240 pacientes con EAC prematura y un grupo control de 1500 individuos, de 30 a 75 años de edad. Todos los participantes son mestizos mexicanos no relacionados, con por lo menos 3 generaciones nacidas en México. La EAC prematura se definió como historia de infarto del miocardio, angioplastia, cirugía de revascularización o estenosis coronaria >50% en la angiografía. Se consideró EAC prematura cuando el diagnóstico se realizó antes de los 55 años de edad en los hombres y 65 años en las mujeres. Los pacientes con un evento cardiovascular agudo en los 3 meses previos no fueron incluidos en el estudio. Los participantes del grupo control, sin historia familiar ni manifestaciones clínicas de EAC prematura, se seleccionaron de la población que acude al banco de sangre del

instituto o fueron reclutados mediante invitación por medios escritos. Los individuos con historia o evidencia de enfermedad hepática, renal, oncológica o tiroidea, y aquellos en terapia corticoesteroidea no fueron incluidos. El estudio GEA fue aprobado por el Comité de Investigación y Bioética del Instituto Nacional de Cardiología “Ignacio Chávez” y realizado con base a los lineamientos de la Declaración de Helsinki. Todos los participantes firmaron el consentimiento informado.

Para el presente trabajo, de los 1500 sujetos del grupo control se seleccionaron 1107 participantes, sin evidencia de aterosclerosis subclínica (calcificación arterial coronaria = 0) y 1162 pacientes con EAC prematura, en quienes fue posible obtener los datos completos de la genotipificación de los polimorfismos estudiados. A todos los participantes se les aplicaron cuestionarios estandarizados para obtener información demográfica, antecedentes familiares y personales de factores de riesgo cardiovascular, hábitos de alimentación, actividad física, consumo de alcohol y uso de medicamentos.

8.2 Mediciones antropométricas y definición de factores de riesgo cardiovascular

El peso se midió en kilogramos (kg) y la talla en centímetros (cm), utilizando una báscula calibrada y un estadímetro de pared. El índice de masa corporal (IMC) fue calculado con la fórmula peso (kg) / talla (m^2). La circunferencia de cintura se midió con una cinta métrica de fibra de vidrio, en el punto medio de la distancia entre la parte inferior de la última costilla y la cresta iliaca. La tensión arterial se midió en posición sedente después de por lo menos 10 minutos de reposo empleando un esfigmomanómetro digital Welch Allyn, series 5200 (Shaneateies Fails, N.Y., E.U.A.), y el promedio de las dos últimas de tres mediciones consecutivas se utilizó para el análisis. Se consideró sobrepeso con un IMC de 25 a 29.9 Kg/m^2 y obesidad con un IMC $\geq$ 30 kg/m^2. La obesidad abdominal se definió

con los valores de circunferencia de cintura de ≥ 80 cm. en mujeres y ≥ 90 cm. en hombres.[150] La presencia de diabetes mellitus tipo 2 (DMT2) se definió de acuerdo a los criterios de la Asociación Americana de Diabetes.[151] El síndrome metabólico (SM) se definió cuando se presentaron tres o más de los siguientes componentes: obesidad abdominal,[150] tensión arterial ≥130/85 mmHg, glucosa de ayuno ≥100 mg/dl, triglicéridos ≥150 mg/dl y colesterol de la lipoproteína de alta densidad (C-HDL) <40 en hombres y <50 en mujeres.[152] Los puntos de corte para definir hiperuricemia fueron valores de ácido úrico sérico > 6.0 mg/dl en mujeres y > 7.0 mg/dl en hombres.[153] Las dislipidemias se definieron de acuerdo a los siguientes puntos de corte: hipercolesterolemia: colesterol total > 200 mg/dL o C-LDL ≥ 130 mg/dL; hipertrigliceridemia: triglicéridos ≥ 150 mg/dL; hipoalfalipoproteinemia: C-HDL < 40 mg/dL en hombres y < 50 mg/dL en mujeres; colesterol no HDL elevado (CnoHDL) >160mg/dL, o cuando el individuo se encontraba en tratamiento regulador de lípidos. Se consideró proteína C reactiva de alta sensibilidad (PCRas) elevada, o inflamación, cuando el valor fue ≥3.0 mg/L. La hipertensión arterial se definió con cifras de tensión arterial sistólica y diastólica ≥ 140/90 mmHg y/o uso de medicamentos antihipertensivos. Para definir los puntos de corte de otros factores de riesgo cardiometabólico en la muestra estudiada, de la base de datos del grupo control GEA se seleccionó una submuestra de 131 hombres y 185 mujeres, sin obesidad y con valores normales de lípidos, glucosa y tensión arterial. Se consideró actividad elevada de aspartato aminotransferasa (AST) con: ≥ 27 UI/L en mujeres, y ≥ 29 UI/L en hombres; actividad elevada de alanina aminotransferasa (ALT) con: ≥ 21 UI/L en mujeres, y ≥ 24.5 UI/L en hombres; gamma glutamiltranspeptidasa (GGT) con: ≥ 21 UI/L en mujeres, y ≥ 27.5 UI/L en hombres; hipoadiponectinemia con concentraciones de adiponectina: ≤ 8.67 μg/mL en mujeres y ≤ 5.50 μg/mL en hombres; hiperinsulinemia con valor de insulina: ≥

16.97 µU/mL en mujeres y ≥ 15.20 µU/mL en hombres; resistencia a la insulina con un valor de HOMA-RI: ≥ 3.66 en mujeres, y ≥ 3.38 en hombres. Se consideró grasa abdominal visceral (GAV) aumentada cuando el valor fue ≥p75 (≥122 cm^2 en mujeres, ≥151.5 cm^2 en hombres).

8.3 Análisis de laboratorio

Después de 10-12 horas de ayuno, en posición sedente se colectaron muestras de sangre venosa. En el plasma se determinaron las concentraciones de glucosa, colesterol total, triglicéridos y C-HDL, y en suero apolipoproteína AI (apoA), apolipoproteína B (apoB), ALT, AST, GGT, ácido úrico y creatinina con métodos enzimático-colorimétricos (Roche/Hitachi, Alemania), en un autoanalizador Hitachi 902 (Hitachi LTD, Tokio Japón). Se estimaron las concentraciones de C-LDL. El colesterol no HDL (CnoHDL) se estimó restando el valor de C-HDL del colesterol total. La reproducibilidad y precisión de las determinaciones de lípidos en nuestro laboratorio son evaluadas periódicamente por el Centro de Control y Prevención de Enfermedades de Atlanta (CDC, por sus siglas en inglés, Atlanta, GA, E.U.A). Los coeficientes de variación intra e interensayo fueron menores al 3%. La concentración de insulina en suero se determinó por radioinmunoanálisis (Millipore RIA Kit ST, Charles, Missouri, E.U.A.). Los coeficientes de variación intra e interensayo fueron de 2.1% y 6.8%, respectivamente. La resistencia a la insulina fue estimada con el modelo homeostático de resistencia a la insulina (HOMA-RI).[154] La concentración de PCRas se determinó mediante inmunonefelometría (BN ProSpec Nefelometer, Dade Behring, Marburg, Alemania); los coeficientes de variación intra e interensayo fueron menores de 6%. La concentración de adiponectina en suero se

determinó por inmunoensayo enzimático (R&D Systems, Minneapolis, Minnesota, E.U.A.). Los coeficientes de variación intra e interensayo fueron menores al 10%.

8.4 Estudio de tomografía axial computada

La tomografía axial computada es un método validado para cuantificar la calcificación arterial coronaria (CAC), la grasa abdominal total (GAT), la grasa abdominal subcutánea (GAS) y la GAV e identificar la presencia de esteatosis hepática. En este estudio, las imágenes del tórax y abdomen se obtuvieron con un tomógrafo de 64 canales (Somaton Sensation, Forcheim, Alemania). Las imágenes tomográficas fueron interpretadas por radiólogos experimentados, para determinar y cuantificar: 1) la GAT, GAS y GAV;[155] 2) el índice de atenuación hígado:bazo;[156] y 3) el puntaje de la CAC utilizando el método Agatston.[157] Para medir la grasa abdominal se realizó un solo corte tomográfico a nivel del espacio intervertebral L4-L5. Las áreas de GAV y GAS, fueron separadas mediante un trazo manual siguiendo la pared muscular abdominal. Se cuantificó la GAT y la GAV en cm^2 y la GAS se calculó restando el área de GAV del área de GAT.[155]

8.5 Estudio genético

8.5.1 Selección de los polimorfismos

La predicción del efecto funcional de los polimorfismos de los genes *IL-27p28*, *IL-12A* y *EBI3* se realizó usando herramientas bioinformáticas, incluyendo los programas FastSNP, SplicePort: An Interactive Splice Site Analysis Tool (http://www.spliceport.cbcb.umd.edu/), SNPs3D (http://www.snps3d.org/), PESX: Putative Exonic Splicing enhancers/Silencers (http://cubweb.biology.columbia.edu/pesx/), y ESEfinder release 3.0 (http://rulai.cshl.edu/cgi-

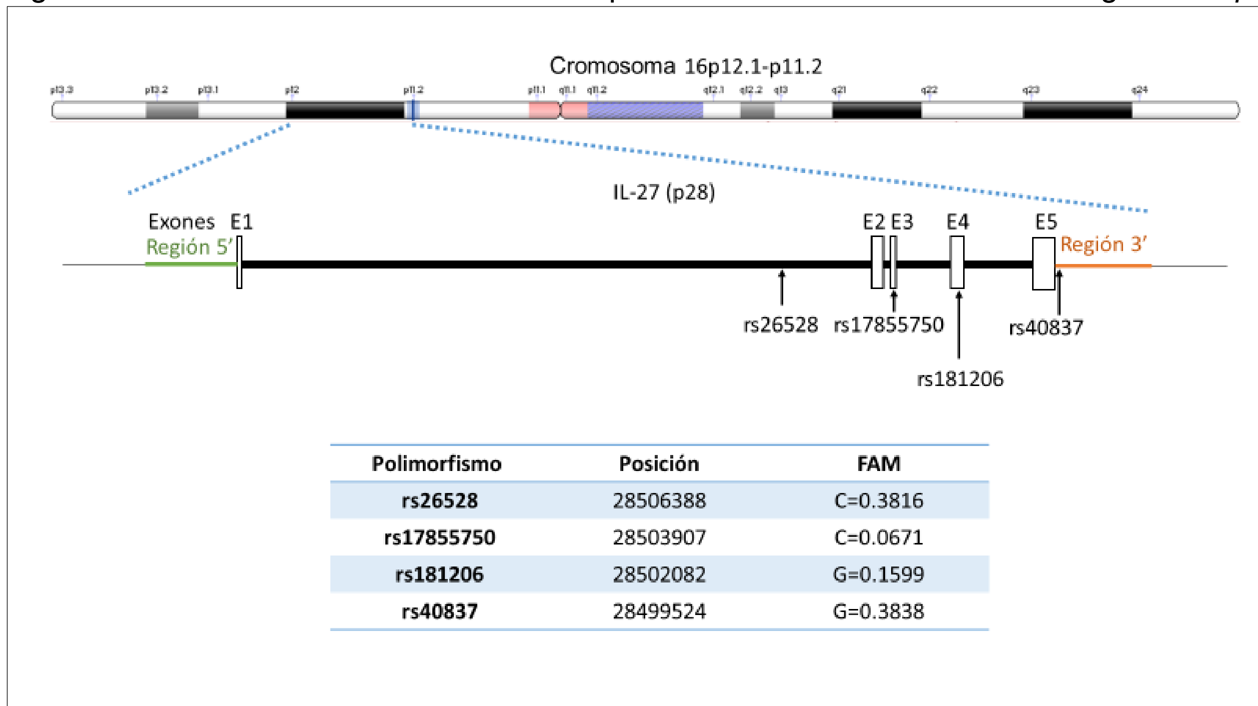
bin/tools/ESE3/esefinder.cgi?process=home).

Con el fin de predecir potenciales interacciones del polimorfismo rs40837, situado en el 3'-UTR del gen *IL-27p28* con miRNAs, se utilizaron los algoritmos TargetScan (http://www.targetscan.org), Diana-MicroT3.0 (http://diana.cslab.ece.ntua.gr), y Miranda (http://www.microrna.org). El análisis mostró que el polimorfismo se encuentra en una región que puede ser reconocido por el miR-379-5p y miR-1225-5p.

8.5.2 Detección de los polimorfismos

El presente proyecto incluyó el estudio de 11 polimorfismos, 4 presentes en el gen *IL-27p28* que codifica para la subunidad p28 de IL-27 (rs26528, rs17855750, rs181206 y rs40837) (Figura 9), 4 en el gen *IL-12A* que codifica para la subunidad p35 de IL-35 (rs2243115, rs2243123, rs583911, y rs568408) (Figura 10) y 3 en el gen *EBI3* que codifica para la subunidad EBI3 presente en IL-27 e IL-35 (rs428253, rs4740 y rs4905) (Figura 11). Todos ellos fueron seleccionados por su posible efecto funcional definido con herramientas informáticas (Tabla 5) y/o frecuencia del alelo menor mayor al 5%. Los polimorfismos se determinaron utilizando sondas TaqMan (Applied Biosystem) en un equipo de PCR en tiempo real.

Figura 9. Posición en el cromosoma de los polimorfismos seleccionados en el gen *IL-27p28*



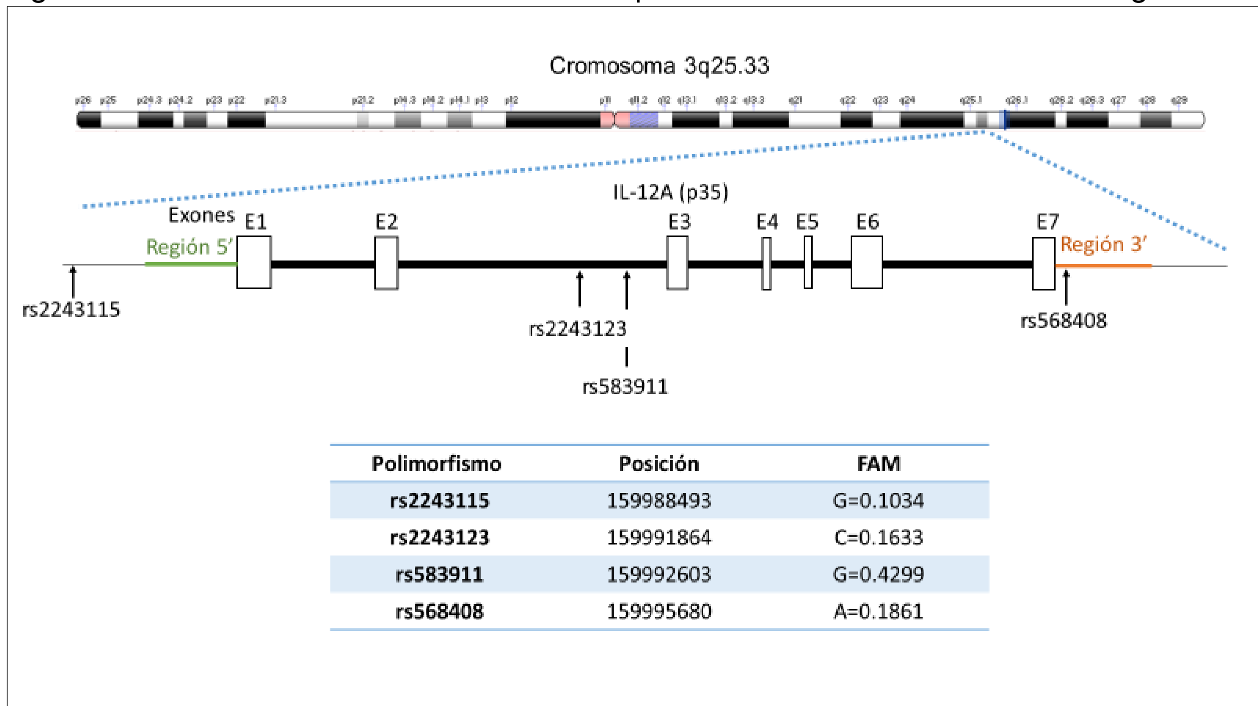
Polimorfismo	Posición	FAM
rs26528	28506388	C=0.3816
rs17855750	28503907	C=0.0671
rs181206	28502082	G=0.1599
rs40837	28499524	G=0.3838

FAM : frecuencia del alelo menor

8.5.3 Determinación de los genes de ancestría

En los estudios de casos y controles pueden existir sesgos debido a las diferencias étnicas de los individuos que componen los grupos de comparación. Considerando esto, en los individuos incluidos en nuestro estudio fueron analizados diferentes marcadores de ancestría. Para este análisis, se determinaron 265 marcadores de ancestría definidos de acuerdo al HapMap y se determinaron en una plataforma GoldenGate de Illumina. Se determinó el porcentaje de ancestría caucásico, indígena y africano utilizando el programa ADMIXTURE. Los resultados no mostraron diferencias significativas entre los pacientes con EAC prematura y los sujetos control en la frecuencia de genes indígenas (55.8% en pacientes y 54.0% en controles), caucásicos (34.3% en pacientes y 35.8% en controles) y africanos (9.8% en pacientes y 10.1% en controles), P > 0.05 para todas las comparaciones. Por tanto, en nuestro estudio no existe sesgo étnico.

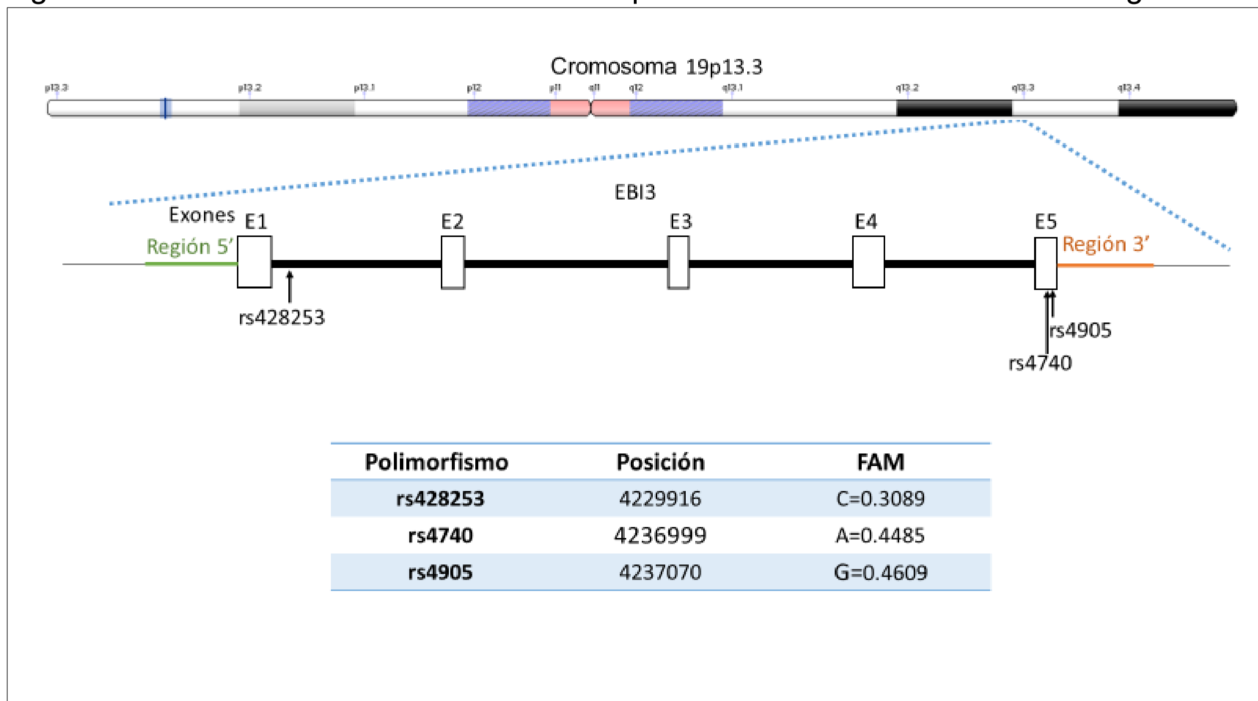
Figura 10. Posición en el cromosoma de los polimorfismos seleccionados en el gen *IL-12A*



Polimorfismo	Posición	FAM
rs2243115	159988493	G=0.1034
rs2243123	159991864	C=0.1633
rs583911	159992603	G=0.4299
rs568408	159995680	A=0.1861

FAM : frecuencia del alelo menor

Figura 11. Posición en el cromosoma de los polimorfismos seleccionados en el gen *EBI3*



Polimorfismo	Posición	FAM
rs428253	4229916	C=0.3089
rs4740	4236999	A=0.4485
rs4905	4237070	G=0.4609

FAM : frecuencia del alelo menor

Tabla 5. Polimorfismos seleccionados para el estudio

Gen	Identificación	Localización	Cambio alelo	Efecto funcional
IL-27p28				
	rs26528	Intrón 1	C>T	No funcional
	rs17855750	Exón 2	A>C	Cambio de codón **T**CC por GCC, serina por alanina en posición 59, modifica unión a SF2ASF2 y SF2ASF1
	rs181206	Exón 4	A>G	Cambio de codón CTG por C**C**G, leucina por prolina en posición 119, modifica unión a SF2ASF2 y SF2ASF1
	rs40837	3'UTR	G>A	Alelo A puede crear sitios de unión a miRNAs
IL-12A				
	rs2243115	Promotor	T>G	G crea sitio de unión para AP2
	rs2243123	Intrón	T>C	No funcional
	rs583911	Intrón	A>G	No funcional
	rs568408	3'UTR	G>A	Alelo A crea sitio de unión a miRNAs
EBI3				
	rs428253	Intrón 1	G>C	Unión a LEF1
	rs4740	Exón 5	G>A	Cambio de codón de GTC por **A**TC (valina a isoleucina posición 201)
	rs4905	Exón 3	A>G	No, cambio de codón ACA por ACG (treonina a treonina posición 224)

Análisis in silico
FastSNP, SplicePort: An Interactive Splice Site Analysis Tool (http://spliceport.cbcb.umd. edu/SplicingAnalyser.html SNPs3D (http://www.snps3d.org/), PESX: Putative Exonic Splicing enharcers/Silencers (http://cubweb.biology.columbia.edu/pesx/)
ESEfinder release 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi)

8.6 Aproximación funcional

8.6.1 Determinación de las concentraciones de IL-27 e IL-35

Las concentraciones en plasma de IL-27 e IL-35 se determinaron en un subgrupo de pacientes con EAC e individuos control sin obesidad y con valores de proteína C reactiva <3mg/L, utilizando el equipo Luminex.

8.7 Ensayo de luciferasa

Fragmentos de 266 pb de la región 3'-UTR del gen humano *IL-27p28* que contenía los alelos *A* o *G* del polimorfismo rs40837, se amplificaron por PCR con los oligonucleótidos F 5'-CGCACGCGTCCCCCACCCTTTAGAACTTT-3' y R 5'-GCGCAAGCTTTGGATGAGAGTGCTTTATTGG-3' a partir de muestras de ADN

genómico humano homocigotos para cada alelo. Los productos de PCR se separaron en un gel de agarosa, se purificaron y se clonaron en plásmidos pMIR-REPORT (Applied Biosystems, Foster City, CA, E.U.A) previamente digeridos con las enzimas MluI y HindIII (Figura 1B). Células HEK293 se cultivaron en DMEM (InvitrogenTM) suplementado con 10% de suero fetal bovino y 1% de antibióticos antimicóticos (InvitrogenTM) a 37 °C con 5% de CO_2. Un total de 100,000 células se sembraron en placas de 12 pozos con medio Opti-MEM® libre de suero (Invitrogen Life Technologies, Inc., Carlsbad, CA, E.U.A). Cuarenta y ocho horas después las células fueron co-transfectadas con Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, E.U.A) según las instrucciones del fabricante. Cada co-transfección contenía 500 ng de los vectores pPMIR-REPORT (rs40837) con los alelos *A* o *G* y 100 ng del plásmido pRL CMV *Renilla reniformis* luciferasa utilizado como control interno de transfección. Veinticuatro horas después las células fueron nuevamente transfectadas con 75 nmoles del control negativo de RNA (secuencia no específica) o con el RNA miR-379-5p (Dharmacon GE Life Sciences) utilizando el agente de transfección amina SIPORT (Applied Biosystems, Foster City, CA, E.U.A). Cuarenta y ocho horas después de la transfección con los miRNA's, se cuantificaron actividades de luciferasa producida por los vectores pMIR-REPORTE y pRL CMV Renilla reniformis con el sistema de detección doble de luciferasa (Promega, Madison, WI, E.U.A), y la actividad relativa de luciferasa se calculó de acuerdo con las instrucciones del fabricante en un TD- 20/20 luminómetro (Turner Biosystems, Sunnyvale, CA, E.U.A).

8.8 Análisis Estadístico

Las variables categóricas se expresan como proporción, las variables continuas como media ± desviación estándar (DE) o mediana (rango intercuartil). La comparación entre grupos se realizó utilizando la prueba de Chi cuadrada para variables categóricas, t de Student para variables continuas paramétricas y U de Mann Whitney para variables no paramétricas. Las características generales se muestran en la población de estudio estratificada de acuerdo a la presencia o ausencia de EAC prematura. Las frecuencias alélicas y genotípicas de los polimorfismos analizados se obtuvieron por conteo directo. Se evaluó el equilibrio de Hardy-Weinberg con la prueba de Chi cuadrada. El análisis de regresión logística multivariado (razón momios (RM) e intervalo de confianza al 95% (IC 95%)) se usó para determinar la independencia de la asociación de los polimorfismos con la presencia de EAC prematura, de acuerdo a diferentes modelos de herencia (aditivo, dominante, heterocigoto, recesivo, y codominante) ajustados por edad, sexo, IMC, tabaquismo, GAT, HOMA-RI, AST, adiponectina y ácido úrico. Para evaluar la independencia de la asociación entre los polimorfismos estudiados con los factores de riesgo cardiovascular y parámetros metabólicos en función a diferentes modelos de herencia, se realizaron análisis de regresión logística ajustados por edad, sexo e IMC. El análisis de desequilibrio de ligamiento (D'), la construcción y análisis de haplotipos se realizaron mediante el programa Haploview versión 4:1 (Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA). Los valores de $p<0.05$ se consideraron estadísticamente significativos. Todos los análisis se realizaron con el paquete estadístico SPSS v15.0 (SPSS Chicago, IL).

9. RESULTADOS

9.1 Resultados interleucina 27

9.1.1Características clínicas y parámetros metabólicos

Las características clínicas de los 1162 pacientes con EAC prematura y 1107 sujetos control incluidos en el estudio se muestran en la Tabla 6.

Tabla 6. Características clínicas y factores de riesgo cardiovascular en los grupos estudiados.

	Control (n=1107)	EAC (n=1162)	*p
Edad (años)	51 ± 9	54 ± 8	<0.001
Sexo (% hombres)	41.2	81.1	<0.001
Índice de masa corporal (kg/m^2)	27.8 [25.4-30.8]	28.3 [25.9-31.1]	0.003
Circunferencia de cintura (cm)	94 ± 11	98 ± 10	<0.001
Tensión arterial sistólica (mmHg)	102 [104-122]	116 [106-127]	<0.001
Tensión arterial diastólica (mmHg)	70 [65-76]	71 [66-78]	0.013
Grasa abdominal visceral (cm^2)	139 [10.4-180]	168 [129-215]	<0.001
Alanina aminotransferasa (IU/L)	24 [18-34]	26 [19-36]	0.031
Aspartato aminotransferasa (UI/L)	25 [21-30]	26 [22-31]	0.001
Colesterol total > 200mg/dl (%)	36.5	20.3	<0.001
Colesterol LDL ≥ 130 mg/dL (%)	29.6	16.1	<0.001
Hipoalfalipoproteinemia (%)	52.0	67.2	<0.001
Hipertrigliceridemia (%)	47.3	56.2	<0.001
Colesterol no HDL>160 mg/dL (%)	27.9	19.5	<0.001
Obesidad (%)	30.1	35.0	0.003
Obesidad abdominal (%)	81.0	83.6	0.060
Diabetes mellitus tipo 2 (%)	10.1	35.4	<0.001
Resistencia a la insulina (%)	54.2	77.0	<0.001
Síndrome metabólico (%)	40.7	71.9	<0.001
Hipertensión (%)	6.7	68.1	<0.001
Grasa abdominal visceral elevada (%)	54.5	64.6	<0.001
Tabaquismo actual (%)	22.8	11.6	<0.001
Hipoadiponectinemia (%)	41.5	56.5	<0.001
PCR de alta sensibilidad ≥3 mg/L (%)	26.6	21.3	0.002
Hiperuricemia (%)	19.9	35.9	<0.001
Pacientes tratados con estatinas (%)	5.2	91.4	<0.001

Los datos se muestran como media ± desviación estándar, mediana [rango intercuartil] o porcentaje. *U de Mann Whitney, t de Student o Chi cuadrada. EAC = enfermedad arterial coronaria, LDL = lipoproteína de baja densidad (por sus siglas en inglés), HDL = lipoproteína de alta densidad (por sus siglas en inglés), PCR = proteína C reactiva.

En los pacientes con EAC prematura, los valores de edad, porcentaje de varones, IMC, circunferencia de cintura, tensión arterial sistólica y diastólica y la GAV fueron significativamente más altos que en los sujetos del grupo control ($p < 0.05$ para todas las

comparaciones). En comparación con los participantes del grupo control, los pacientes tuvieron prevalencias menores de hipercolesterolemia (definida como colesterol total >200 mg/dL o C-LDL ≥130 mg/dL), niveles elevados de colesterol no HDL, inflamación (definida como niveles de proteína C reactiva de alta sensibilidad ≥3mg/L) y tabaquismo actual ($p < 0.05$) (Tabla 6). Por el contrario, las prevalencias de hipoalfalipoproteinemia, hipertrigliceridemia, obesidad general, obesidad abdominal, DMT2, hiperinsulinemia, resistencia a la insulina, síndrome metabólico, hipertensión, GAV incrementada, hiperuricemia e hipoadectinemia, fueron significativamente más altas en los pacientes con EAC prematura ($p < 0.05$) (Tabla 6). Debido al efecto del tratamiento con estatinas, en los pacientes coronarios las concentraciones de colesterol total, C-LDL, colesterol no HDL y PCRas fueron significativamente menores con respecto a los sujetos control (Tabla 6, $p < 0.001$ para todas las variables).

De forma similar a lo observado para la prevalencia de los factores de riesgo cardiovascular, las concentraciones de triglicéridos, ALT, AST, glucosa, insulina, HOMA-RI y ácido úrico fueron más altas y las de C-HDL y adiponectina más bajas en los pacientes con EAC prematura que en los individuos del grupo control, todas las diferencias fueron estadísticamente significativas ($p < 0.05$) (Tabla 6).

9.1.2 Asociación de los polimorfismos con la EAC prematura

Las frecuencias observadas de los genotipos en los sitios polimórficos se encontraron en equilibrio de Hardy-Weinberg ($p>0.05$). La distribución de las frecuencias de los polimorfismos rs17855750 y rs181206 fueron similares en los individuos control y pacientes con EAC prematura. Por el contrario, los alelos rs26528 *T* y rs40837 *A*, tuvieron una prevalencia significativamente más baja en el grupo de pacientes con EAC prematura

(Tabla 7). En comparación con el grupo control y bajo modelos dominante, heterocigoto y codominante 1, el alelo rs26528 *T* se asoció significativamente con reducción en el riesgo de padecer EAC prematura ($p_{dominante}$, $p_{heterocigoto}$, $p_{codominante\ 1} < 0.05$). Para el caso del alelo rs40837 *A*, esta asociación fue significativa bajo los modelos heterocigoto y codominate 1 ($p_{heterocigoto}$ y $p_{codominante\ 1} < 0.05$). Todos los modelos se ajustaron por edad, sexo, IMC, tabaquismo, grasa abdominal total, HOMA-RI, AST, adiponectina y ácido úrico (Tabla 7).

Tabla 7. Asociación de los polimorfismos del gen *IL27p28* con enfermedad arterial coronaria prematura

Polimorfismo	Frecuencia del genotipo			FAM	Modelo	RM [IC 95%]	p
rs26528	***CC***	***CT***	***TT***				
					Aditivo	0.958 [0.817-1.122]	0.594
Control (n=1107)	0.378	0.481	0.142	0.382	**Dominante**	**0.794 [0.634-0.996]**	**0.046**
Coronarios (n=1162)	0.410	0.441	0.149	0.369	Recesivo	1.302 [0.958-1.771]	0.092
					Heterocigoto	**0.701 [0.562-0.875]**	**0.002**
					Codominante 1	**0.718 [0.564-0.913]**	**0.007**
					Codominante 2	1.086 [0.778-1.517]	0.627
rs17855750	***AA***	***AC***	***CC***				
					Aditivo	0.967 [0.777-1.205]	0.767
Control (n=1107)	0.729	0.248	0.023	0.147	Dominante	0.932 [0.724-1.199]	0.582
Coronarios (n=1162)	0.731	0.242	0.024	0.146	Recesivo	1.240 [0.610-2.522]	0.553
					Heterocigoto	0.900 [0.694-1.169]	0.431
					Codominante 1	0.906 [0.697-1.177]	0.460
					Codominante 2	1.211 [0.594-2.470]	0.599
rs181206	***AA***	***AG***	***GG***				
					Aditivo	1.003 [0.855-1.178]	0.967
Control (n=1107)	0.451	0.426	0.123	0.336	Dominante	0.955 [0.768-1.187]	0.677
Coronarios (n=1162)	0.442	0.441	0.117	0.337	Recesivo	1.135 [0.810-1.590]	0.463
					Heterocigoto	0.923 [0.740-1.151]	0.476
					Codominante 1	0.936 [0.742-1.182]	0.581
					Codominante 2	1.073 [0.749-1.536]	0.701
rs40837	***GG***	***GA***	***AA***				
					Aditivo	0.992 [0.847-1.162]	0.923
Control (n=1107)	0.378	0.480	0.143	0.382	Dominante	0.847 [0.676-1.062]	0.150
Coronarios (n=1162)	0.405	0.445	0.150	0.372	Recesivo	1.316 [0.970-1.786]	0.078
					Heterocigoto	**0.740 [0.593-0.923]**	**0.008**
					Codominante 1	**0.768 [0.604-0.977]**	**0.031**
					Codominante 2	1.140 [0.818-1.589]	0.440

FAM = frecuencia del alelo menor, RM = razón de momios, IC95%= intervalo intercuartil al 95%. Todos los modelos se ajustaron por edad, sexo, IMC, tabaquismo, grasa abdominal total, HOMA-RI, ALT, adiponectina y ácido úrico.

9.1.3 Análisis de los haplotipos del gen *IL27p28*

En la Figura 12 se muestra el mapa de DL para los cuatro polimorfismos del gen *IL-27p28* analizados. Los marcadores rs40837 y rs26528 se encontraron en desequilibrio de ligamiento (D' = 0.98, r^2 = 0.956). Ninguno de los haplotipos formados se asoció con la susceptibilidad al desarrollo de la EAC prematura (Tabla 8).

Figura 12. Mapa de DL del gen *IL27p28*

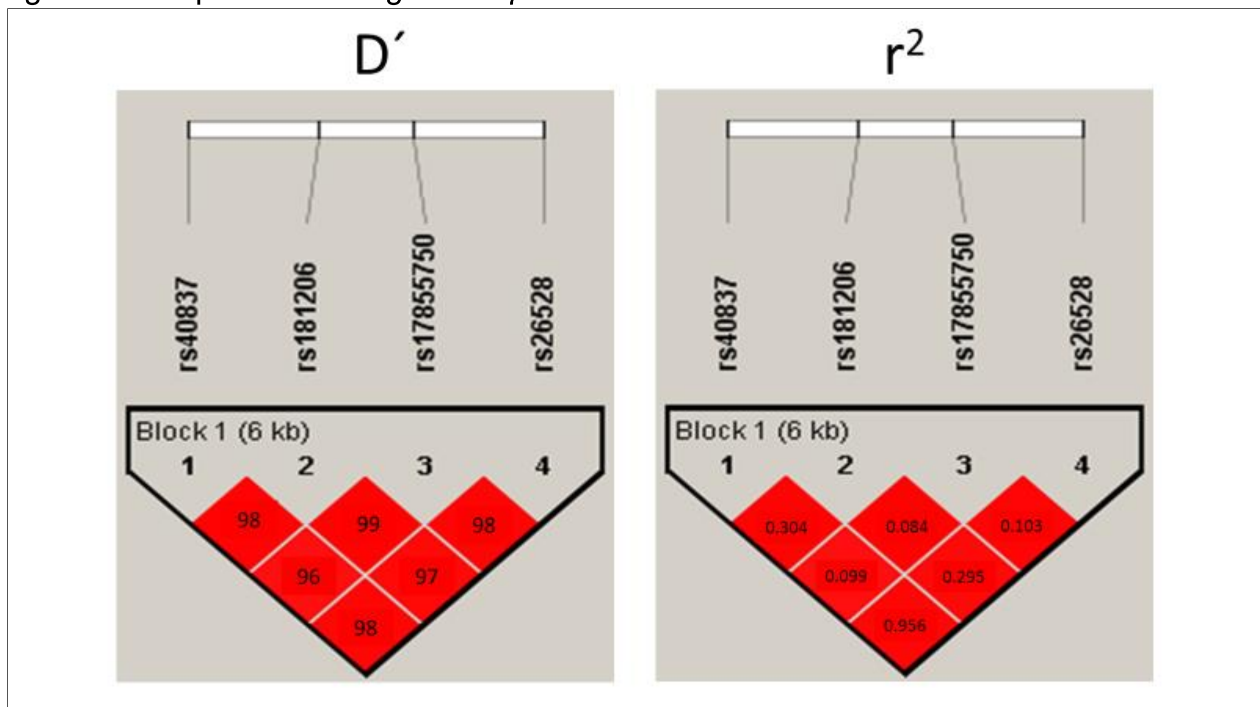


Tabla 8. Frecuencias de haplotipos del gen *IL-27p28*

Haplotipos	Secuencia	Coronarios	Controles	Chi²	p
H1	AAAT	0.366	0.382	1.409	0.2353
H2	GGAC	0.332	0.328	0.086	0.7693
H3	GACC	0.148	0.138	1.202	0.2732
H4	GAAC	0.147	0.136	1.290	0.2561

El orden de los polimorfismos corresponde a la posición que ocupan en el cromosoma (rs40837, rs181206, rs17855750, rs26528)

9.1.4 Asociación de los polimorfismos del gen *IL27p28* con parámetros clínicos y metabólicos

Las asociaciones de los polimorfismos del gen *IL-27p28* con los parámetros metabólicos y factores de riesgo cardiovascular se analizaron por separado en cada uno de los grupos. Los polimorfismos rs26528, rs181206 y rs40837 se asociaron con parámetros cardiometabólicos bajo diferentes modelos de herencia ajustados por edad, sexo e IMC, tanto en el grupo de controles (Tabla 9) como de pacientes (Tabla 10).

Tabla 9. Asociación de los polimorfismos del gen *IL27p28* con parámetros metabólicos en los sujetos control

Polimorfismo	Frecuencia del genotipo			MAF	Modelo	RM [IC 95%]	p
rs26528	***CC***	***CT***	***TT***				
Resistencia a la insulina							
No (n=507)	0.358	0.466	0.176	0.408	Recesivo	0.623 [0.423-0.916]	0.016
Si (n=600)	0.395	0.492	0.113	0.359	Codominante 2	0.610 [0.401-0.930]	0.021
AST >p75							
No (n=705)	0.368	0.471	0.161	0.396	Aditivo	0.777 [0.633-0.954]	0.016
Si (n=402)	0.391	0.500	0.109	0.359	Recesivo	0.654 [0.431-0.992]	0.046
					Codominante 2	0.583 [0.373-0.912]	0.018
rs181206	***AA***	***AG***	***GG***				
Hiperuricemia							
No (n=887)	0.438	0.434	0.128	0.345	Aditivo	0.788 [0.624-0.994]	0.044
Si (n=220)	0.495	0.405	0.100	0.302			
rs40837	***GG***	***GA***	***AA***				
Resistencia a la insulina							
No (n=506)	0.354	0.470	0.176	0.411	Aditivo	0.646 [0.468-0.892]	0.008
Si (n=601)	0.399	0.486	0.115	0.358	Dominante	0.616 [0.381-0.994]	0.047
					Recesivo	0.488 [0.274-0.867]	0.014
					Codominante 2	0.404 [0.210-0.775]	0.006
AST >p75							
No (n=705)	0.366	0.475	0.159	0.396	Aditivo	0.771 [0.628-0.947]	0.013
Si (n=402)	0.339	0.489	0.112	0.357	Codominante 2	0.603 [0.388-0.938]	0.025

FAM = frecuencia del alelo menor, RM = razón de momios, IC95%= intervalo intercuartil al 95%, p75 = percentila 75, AST = aspartato aminotrasferasa. Todos los modelos se ajustaron por edad, sexo e índice de masa corporal. Únicamente se muestran los polimorfismos y modelos con diferencias significativas.

Tabla 10. Asociación de los polimorfismos del gen *IL27p28* con parámetros metabólicos en pacientes con EAC prematura

Polimorfismo	Frecuencia del genotipo			FAM	Modelo	RM [IC 95%]	p
rs26528	***CC***	***CT***	***TT***				
AST>p75							
No (n=710)	0.382	0.459	0.159	0.389	Aditivo	0.802 [0.673-0.957]	0.014
Si (n=452)	0.454	0.407	0.139	0.343	Dominante	0.689 [0.539-0.882]	0.003
					Heterocigoto	0.737 [0.576-0.944]	0.016
					Codominante 1	0.678 [0.521-0.883]	0.004
rs181206	***AA***	***AG***	***GG***				
Hiperuricemia							
No (n=745)	0.439	0.426	0.135	0.348	Recesivo	0.546 [0.359-0.830]	0.005
Si (n=417)	0.455	0.460	0.085	0.314	Codominante 2	0.566 [0.365-0.878]	0.011
rs40837	***GG***	***GA***	***AA***				
Resistencia a la insulina							
No (n=316)	0.393	0.470	0.137	0.372	Heterocigoto	0.702 [0.504-0.978]	0.037
Si (n=846)	0.414	0.433	0.153	0.369			
AST>p75							
No (n=710)	0.375	0.469	0.156	0.391	Aditivo	0.799 [0.670-0.953]	0.013
Si (n=452)	0.456	0.407	0.137	0.341	Heterocigoto	0.720 [0.563-0.922]	0.009
					Codominante 1	0.662 [0.509-0.863]	0.002

FAM = frecuencia del alelo menor, RM = razón de momios, IC95%= intervalo intercuartil al 95%, p75 = percentila 75, AST = aspartato aminotransferasa. Todos los modelos se ajustaron por edad, sexo e índice de masa corporal
Únicamente se muestran los polimorfismos y modelos con diferencias significativas.

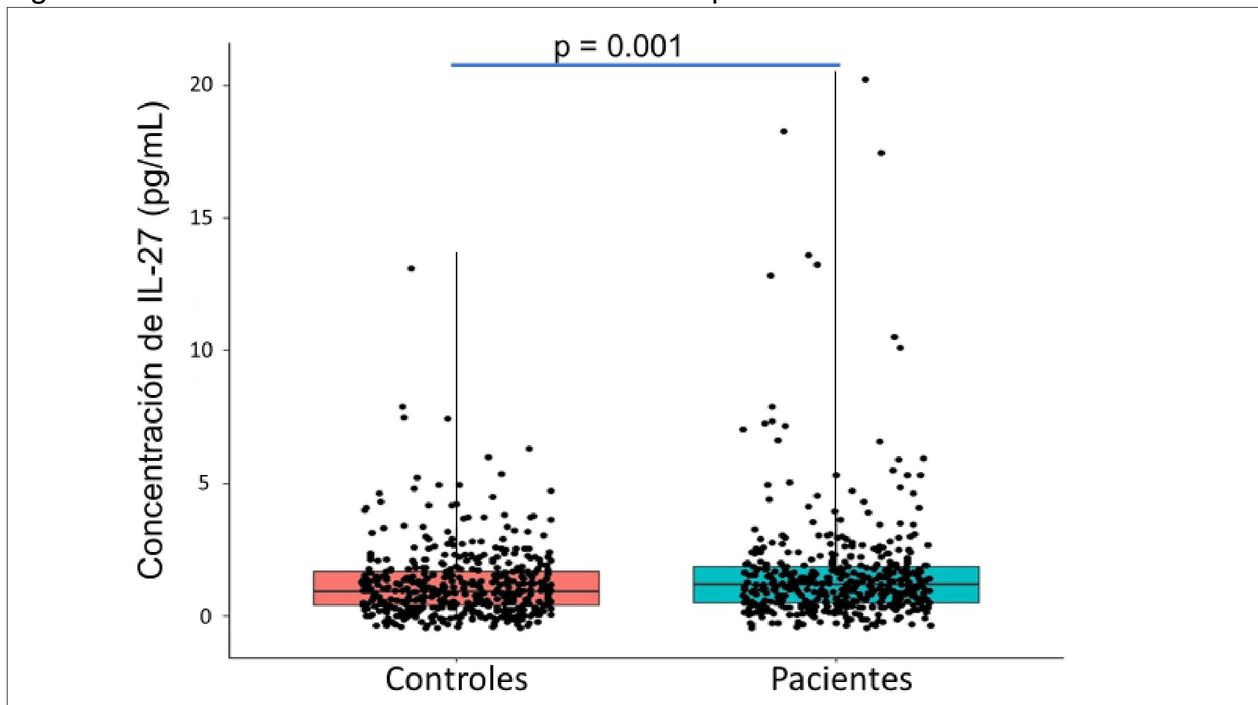
En los sujetos control, los polimorfismos rs26528 y rs40837 se asociaron con bajo riesgo de presentar resistencia a la insulina ($p_{recesivo}$ y $p_{codominante\ 2} < 0.05$ para rs26528 y $p_{aditivo}$, $p_{dominante}$, $p_{recesivo}$, $p_{codominante\ 2} < 0.05$ para rs40837) y bajo riesgo de presentar actividad elevada de AST ($p_{aditivo}$, $p_{recesivo}$, $p_{codominante\ 2} < 0.05$ para rs26528 y $p_{aditivo}$, $p_{codominante\ 2} < 0.05$ para rs40837); el polimorfismo rs181206 se asoció con bajo riesgo de presentar hiperuricemia ($p_{aditivo} = 0.044$) (Tabla 9). En los pacientes con EAC prematura (Tabla 10), los polimorfismos rs26528 ($p_{aditivo}$, $p_{dominante}$, $p_{heterocigoto}$, $p_{codominante\ 1} < 0.02$) y rs40837 ($p_{aditivo}$, $p_{heterocigoto}$, $p_{codominante\ 1} < 0.02$) se asociaron con bajo riesgo de presentar actividad elevada de AST (>p75); el polimorfismo rs181206 se asoció con bajo riesgo de presentar

hiperuricemia ($p_{recesivo}$, $p_{codomianate\ 2}$ < 0.02) y el polimorfismo rs40837 con bajo riesgo de presentar resistencia a la insulina ($p_{heterocigoto}$ = 0.037).

9.1.5 Concentraciones de IL-27 en pacientes con EAC prematura y controles.

Los pacientes con EAC prematura mostraron concentraciones significativamente más altas de IL-27 que los individuos control (1.2 [0.5–1.9] pg/mL vs 0.9 [0.4–1.7] pg/mL, respectivamente, p = 0.001, Figura 13). No se observaron diferencias significativas en las concentraciones de IL-27 cuando se analizaron en función de cada uno de los genotipos de los cuatro polimorfismos estudiados (Tabla 11).

Figura 13. Concentraciones de interleucina 27 en plasma



Concentraciones en plasma de la interleucina 27 en 447 sujetos control y 447 pacientes con EAC prematura sin obesidad y con valores de proteína C reactiva de alta sensibilidad <3mg/L. Las comparaciones se hicieron con la prueba de U de Mann Whitney.

Tabla 11. Concentraciones de IL-27 en plasma en los grupos estudiados en función de los genotipos de *EBI3* e *IL-27p28*.

			Controles (n=447)			EAC prematura (n=447)		
	Polimorfismo	Genotipo	n	Concentración (pg/mL)	p	n	Concentración (pg/mL)	p
EBI3								
	rs428253	*GG*	275	0.94 [0.29-1.66]		286	1.15 [0.47-1.87]	
		GC	151	0.78 [0.31-1.66]	0.933	140	1.15 [0.73-1.93]	0.082
		CC	21	1.00 [0.43-1.34]		21	0.78 [0.40-1.15]	
	rs4740	*GG*	250	0.78 [0.40-1.66]		233	1.23 [0.73-1.90]	
		GA	160	0.78 [0.08-1.55]	0.259	185	1.00 [0.43-1.71]	0.104
		AA	37	1.15 [0.43-1.87]		29	0.78 [0.47-1.49]	
	rs4905	*AA*	248	0.78 [0.40-1.60]		233	1.15 [0.73-1.90]	
		AG	164	0.78 [0.09-1.62]	0.405	186	1.00 [0.43-1.75]	0.139
		GG	35	1.15 [0.42-1.90]		28	0.91 [0.45-1.60]	
IL27p28								
	rs26528	CC	164	0.94 [0.09-1.62]		175	1.15 [0.47-1.87]	
		CT	202	0.78 [0.40-1.66]	0.915	199	1.15 [0.47-1.90]	0.675
		TT	81	0.94 [0.40-1.71]		73	0.88 [0.42-1.71]	
	rs17855750	AA	321	0.94 [0.29-1.66]		334	1.15 [0.47-1.90]	
		AC	117	0.78 [0.40-1.49]	0.056	106	1.03 [0.42-1.49]	0.352
		CC	9	0.01 [0.01-078]		7	1.43 [1.24-2.99]	
	rs181206	AA	223	0.78 [0.31-1.54]		202	1.02 [0.42-1.66]	
		AG	173	0.88 [0.40-1.51]	0.546	190	1.15 [0.47-1.87]	0.080
		GG	51	1.03 [0.09-2.08]		55	1.32 [0.73-2.22]	
	rs40837	GG	163	0.94 [0.09-1.58]		173	1.15 [0.47-1.87]	
		GA	202	0.78 [0.40-1.66]	0.916	201	1.14 [0.47-1.87]	0.817
		AA	82	0.94 [0.09-1.71]		73	1.00 [0.42-1.71]	
	rs153109	GG	115	0.94 [0.08-1.66]		140	1.15 [0.47-1.87]	
		GA	192	0.88 [0.40-1.49]	0.780	197	1.13 [0.47-1.87]	0.964
		AA	97	0.94 [0.40-1.90]		79	1.14 [0.42-1.90]	

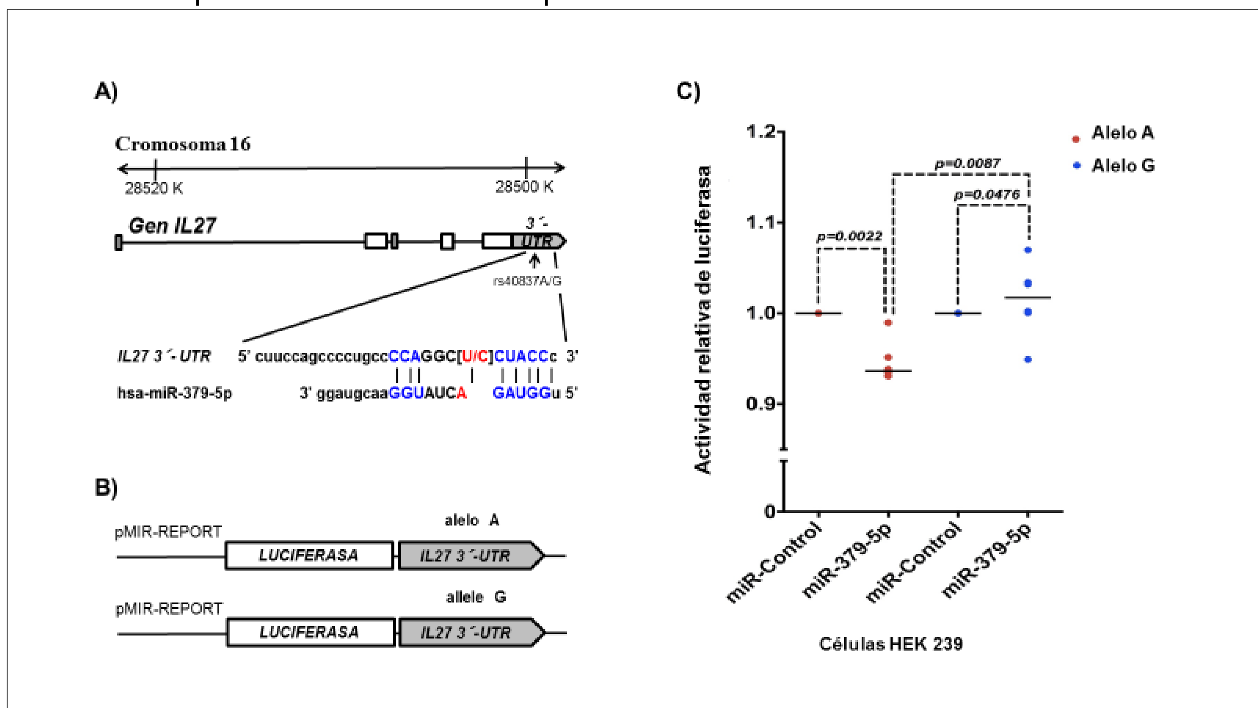
Los datos se muestran como mediana [intervalo intercuartil]. Las comparaciones se realizaron empleando la prueba de Kruskal Wallis. EAC = Enfermedad arterial coronaria.

9.1.6 Ensayos de luciferasa

Dado que el polimorfismo rs40837, localizado en la región 3'-UTR del gen IL-27, puede ser blanco de los miR-379-5p y miR-1225-5p, se decidió examinar el efecto de los alelos *G* o *A* sobre la expresión del gen de luciferasa en presencia del miR-379-5p y del miR-1225-5p utilizando un sistema dual de luciferasa. Con este fin, se transfectaron células HEK 293 con construcciones del gen reportero luciferasa que tenían parte de la secuencia 3'-UTR con el alelo *G* o *A* del polimorfismo rs40837 (Figura 14A). Las células cotransfectadas con las clonas portadoras del alelo *A* (Figura 14B) y el hsa-miR-379-5p

mostraron una reducción de aproximadamente del 5% en la actividad de luciferasa ($p = 0.0022$), en comparación con las células cotransfectadas con el alelo *A* y el miR-Control (Figura 14C). Por el contrario, cuando las células se cotransfectaron con la clona que alberga el alelo *G* en presencia del miR-379-5p se observó un aumento en la expresión del gen de la luciferasa, en comparación con las células cotransfectadas con el alelo *G* y el miR-Control ($p = 0.0476$). En comparación con las clonas cotransfectadas con el alelo *G* y el miR-379-5p, aquellas cotransfectadas con el alelo *A* y el miR-379-5p, mostraron una disminución estadísticamente significativa en la expresión del gen de la luciferasa ($p = 0.0087$). La cotransfección con el miR-1225-5p no afectó el nivel de expresión de la luciferasa en presencia ya sea del alelo *A* o del alelo *G*.

Figura 14. El polimorfismo rs40837 del gen *IL-27p28* afecta el nivel de expresión de luciferasa en presencia del miR-379-5p.



A) Representación esquemática del gen IL27 que indica la ubicación de las variantes alélicas rs40837 (A / G) en la región 3'-UTR y su complementariedad con la secuencia de miR-379-5p. B) La secuencia con cada alelo se clonó en el vector pMIR-Report para evaluar la expresión del gen indicador de luciferasa como se describe en Materiales y Métodos. C) Resultados de los análisis de expresión de luciferasa cuando las células se cotransfectaron con el miR-379-5p en comparación con las células cotransfectadas con el miR-control en presencia de los alelos *A* o *G*. n = 5 experimentos con duplicados independientes; las medianas se compararon utilizando U de Mann Whitney.

9.2 RESULTADOS INTERLEUCINA 35

9.2.1 Características clínicas y parámetros metabólicos

Análisis preliminares de los datos mostraron que la prevalencia de DMT2 varía de forma significativa en función de los genotipos de polimorfismos de *EBI3* e *IL-12A*. Por esta razón, para evaluar la asociación de los polimorfismos estudiados con la presencia de EAC prematura, en esta parte del estudio se seleccionaron sujetos del grupo control con valores de calcificación coronaria igual a cero, que fueran normoglucémicos y sin diagnótico de DMT2 (n=873). Las tablas 12 y 13 muestran las características clínicas y demográficas de los individuos estudiados. Como era de esperarse, todas las variables analizadas fueron diferentes y estadísticamente significativas entre los dos grupos, excepto por la grasa abdominal total (Tabla 12) y proteína C reactiva de alta sensibilidad (Tabla 13).

Tabla 12. Características clínicas y metabólicas de los grupos estudiados.

	Control (n=873)	EAC prematura (n=1162)	p
Edad (años)	51 ± 9	54 ± 8	<0.001
Sexo (% hombres)	40.7	81.1	<0.001
Índice de masa corporal (kg/m^2)	27.3 [24.9-30.2]	28.3 [25.9-31.1]	<0.001
Circunferencia de cintura (cm)	92 ± 11	98 ± 10	<0.001
Tensión arterial sistólica (mmHg)	111 [103-121]	116 [106-127]	<0.001
Tensión arterial diastólica (mmHg)	70 [65-76]	71 [66-78]	0.001
Grasa abdominal total (cm^2)	416 [330-514]	425 [340-523]	0.147
Grasa abdominal visceral (cm^2)	130 [98-172]	168 [129-215]	<0.001
Grasa abdominal subcutánea (cm^2)	280 [209-356]	245 [193-313]	<0.001
Colesterol total (mg/dL)	190 [167-210]	160 [132-193]	<0.001
Colesterol lipoproteína de alta densidad (mg/dL)	46 [37 -56]	37 [32-44]	<0.001
Colesterol lipoproteína de baja densidad (mg/dL)	116 [95-133]	91 [68-116]	<0.001
Triglicéridos (mg/dL)	138 [102-190]	162 [119-219]	<0.001
Colesterol no HDL (mg/dL)	141 [119-162]	120 [93-151]	<0.001
Alanina aminotransferasa (IU/L)	23 [17-32]	26 [19-36]	<0.001
Aspartato aminotransferasa (UI/L)	24 [20-30]	26 [22-31]	<0.001
Glucosa (mg/dL)	87 [82-92]	95 [87-117]	<0.001
Insulina (µUI/mL)	16 [12-21]	20 [15-28]	<0.001
Modelo homeostático de resistencia a la insulina	3.3 [2.4-4.7]	5.1 [3.5-7.7]	<0.001
Proteína C reactiva de alta sensibilidad (mg/L)	1.4 [0.7-2.9]	1.2 [0.6-2.6]	0.005
Adiponectina (µg/mL)	8.5 [5.3-13.6]	5.2 [3.2-8,1]	<0.001
Ácido úrico (mg/dL)	5.3 [4.3-6.3]	6.5 [5.4-7.4]	<0.001

Los datos se muestran como media ± desviación estándar, mediana [intervalo intercuartil] o porcentaje. Las comparaciones se realizaron usando las pruebas de t de Student o U de Mann Whitney, para variables continuas, y con la prueba de Chi cuadrada para variables categóricas. EAC = Enfermedad arterial coronaria.

Tabla 13. Prevalencia de factores de riesgo cardiovascular en la población estudiada.

	Control (n=873)	EAC prematura (n=1162)	p
Colesterol total > 200mg/dl (%)	36.3	20.3	<0.001
Colesterol LDL ≥ 130 mg/dL (%)	29.2	16.1	<0.001
Hipoalfalipoproteinemia (%)	49.3	67.2	<0.001
Hipertrigliceridemia (%)	42.8	56.2	<0.001
Colesterol non HDL >160 mg/dL (%)	26.0	19.5	<0.001
Obesidad (%)	26.1	35.0	<0.001
Obesidad abdominal (%)	77.6	83.6	<0.001
Diabetes mellitus tipo 2 (%)	0	35.4	<0.001
Hiperinsulinemia (%)	45.8	71.4	<0.001
Resistencia a la insulina (%)	44.2	77.0	<0.001
Síndrome metabólico (%)	29.7	71.9	<0.001
Hipertensión (%)	5.7	68.1	<0.001
Grasa abdominal visceral aumentada (%)	49.8	64.6	<0.001
Tabaquismo actual (%)	23.5	11.6	<0.001
Hipoadiponectinemia (%)	40.0	56.5	<0.001
Proteína C reactiva de alta sensibilidad ≥ 3 mg/L (%)	23.6	21.3	0.114
Hiperuricemia (%)	16.8	35.9	<0.001

Los datos se muestran como porcentaje. Las comparaciones se realizaron usando la prueba de Chi cuadrada. EAC = enfermedad arterial coronaria, LDL= lipoproteína de baja densidad (por sus siglas en inglés), HDL = lipoproteína de alta densidad (por sus siglas en inglés).

Las concentraciones de colesterol total, colesterol LDL y colesterol no HDL, de PCRas y las prevalencias de hipercolesterolemia (colesterol total > 200mg/dl, C-LDL ≥ 130 mg/dL), y tabaquismo actual fueron significativamente menores en los pacientes en comparación con los controles ($p < 0.01$ para todas las variables). Lo anterior probablemente debido al efecto del tratamiento hipolipemiante con estatinas, así como a los cambios en el estilo de vida que se indican a los pacientes después de presentar el evento cardiovascular.

9.2.2 Asociación de los polimorfismos de *EBI3* e *IL-12A* con EAC prematura

La distribución de los polimorfismos de *EBI3* (rs4740 y rs4905) e *IL-12A* (rs2243123, rs568408 y rs583911) fue similar en ambos grupos. Sin embargo, bajo los modelos aditivo, recesivo y codominante 2, ajustados por edad, sexo, índice de masa corporal, tabaquismo actual, ALT, AST y ácido úrico, el polimorfismo de *EBI3* rs428253 se asoció con menor riesgo de presentar EAC prematura ($p_{aditivo}$, $p_{recesivo,\ codominante\ 2} < 0.030$).

Tabla 14. Asociación entre los polimorfismos de *EBI3* e *IL-12A* y enfermedad arterial coronaria prematura.

Polimorfismo	**Frecuencia del genotipo**			**FAM**	**Modelo**	**RM [IC 95%]**	**p**
EBI3*							
rs428253	***GG***	***GC***	***CC***				
Control (n=873)	0.614	0.317	0.069	0.227	**Aditivo**	**0.831 [0.699-0.988]**	**0.036**
					Dominante	0.842 [0.681-1.042]	0.115
					Recesivo	**0.614 [0.392-0.963]**	**0.033**
Coronarios (n=1162)	0.637	0.319	0.044	0.204	Heterocigoto	0.935 [0.750-1.167]	0.553
					Codominante 1	0.895 [0.715-1.120]	0.334
					Codominante 2	**0.591 [0.375-0.933]**	**0.027**
rs4740	***GG***	***GA***	***AA***				
Control (n=873)	0.547	0.376	0.078		Aditivo	0.922 [0.797-1.066]	0.274
					Dominante	0.884 [0.734-1.064]	0.191
					Recesivo	0.973 [0.687-1.377]	0.876
Coronarios (n=1162)	0.532	0.395	0.073	0.271	Heterocigoto	0.886 [0.733-1.071]	0.211
					Codominante 1	0.877 [0.722-1.065]	0.186
					Codominante 2	0.920 [0.644-1.316]	0.649
rs4905	AA	AG	GG				
Control (n=873)	0.544	0.377	0.079	0.268	Aditivo	0.926 [0.800-1.071]	0.300
					Dominante	0.892 [0.741-1.073]	0.224
					Recesivo	0.968 [0.684-1.370]	0.854
Coronarios (n=1162)	0.528	0.399	0.073	0.273	Heterocigoto	0.896 [0.742-1.082]	0.254
					Codominante 1	0.886 [0.730-1.076]	0.224
					Codominante 2	0.919 [0.643-1.314]	0.642
IL-12A*							
rs2243115	***TT***	***TG***	***GG***				
Control (n=873)	0.855	0.137	0.008	0.077	**Aditivo**	**0.674 [0.499-0.909]**	**0.010**
					Dominante	**0.676 [0.494-0.925]**	**0.014**
					Recesivo	0.294 [0.048-1.785]	0.183
Coronarios (n=1162)	0.902	0.096	0.002	0.050	**Heterocigoto**	**0.698 [0.508-0.956]**	**0.027**
					Codominante 1	**0.694 [0.505-0.954]**	**0.024**
					Codominante 2	0.282 [0.046-1.712]	0.169
rs568408	***GG***	***GA***	***AA***				
Control (n=873)	0.893	0.103	0.003	0.055	Aditivo	0.989 [0.720-1.359]	0.947
					Dominante	1.023 [0.738-1.418]	0.893
					Recesivo	0.212 [0.025-1.797]	0.155
CAD (n=1162)	0.884	0.114	0.002	0.059	Heterocigoto	1.059 [0.761-1.473]	0.734
					Codominante 1	1.055 [0.758-1.468]	0.751
					Codominante 2	0.213 [0.025-1.808]	0.156
rs2243123	***TT***	***TC***	***CC***				
Control (n=873)	0.416	0.452	0.132	0.055	Aditivo	0.853 [0.735-1.091]	0.197
					Dominante	0.827 [0.671-1.020]	0.076
					Recesivo	0.783 [0.582-1.053]	0.105
CAD (n=1162)	0.379	0.464	0.157	0.059	Heterocigoto	0.939 [0.764-1.154]	0.549
					Codominante 1	0.838 [0.612-1.147]	0.270
					Codominante 2	0.723 [0.525-1.095]	0.106
rs583911	***AA***	***AG***	***GG***				
Control (n=873)	0.255	0.502	0.243	0.492	Aditivo	0.953 [0.825-1.101]	0.513
					Dominante	0.915 [0.725-1.156]	0.458
					Recesivo	0.961 [0.756-1.222]	0.747
CAD (n=1162)	0.275	0.483	0.242	0.480	Heterocigoto	0.961 [0.783-1.180}	0.705
					Codominante 1	0.918 [0.717-1.177]	0.501
					Codominante 2	0.909 [0.681-1.215}	0.520

*Los modelos de ajustaron por edad, sexo, IMC, tabaquismo actual, ALT, AST y ácido úrico. ** Los modelos de ajustaron por edad, sexo, IMC y tabaquismo actual. Los individuos del grupo control eran normoglucémicos no diabéticos. FAM = frecuencia del alelo menor, RM = razón de momios, IC95%= intervalo intercuartil al 95%.

De manera similar, el polimorfismo *IL-12A* rs2243115 se asoció con menor riesgo de presentar EAC prematura (Tabla 14) bajo diferentes modelos ($p_{aditivo}$, $p_{dominante}$, $p_{heterocigoto}$, $p_{codominante\ 1} < 0.030$) ajustados por edad, sexo, IMC y tabaquismo actual.

9.2.3 Análisis de los haplotipos del gen *EBI 3*

De los tres polimorfismos de *EBI3* estudiados el rs4740 y rs4905 se encontraron en desequilibrio de ligamiento (D' = 0.99, r^2 = 0.97, Figura 15). El análisis de desequilibrio de ligamiento entre los polimorfismos del gen *EBI3* generó cuatro haplotipos H1: ***GGA***, H2: ***CAG***, H3: ***GAG*** y H4: ***CGA*** (el orden de los polimorfismos en las secuencias de los haplotipos es rs42853, rs4740, rs4905) (Tabla 15). El haplotipo H4: ***CGA*** fue menos frecuente en los pacientes coronarios en comparación con el grupo control (p = 0.0328).

Figura 15. Mapa de DL del gen *EBI3*

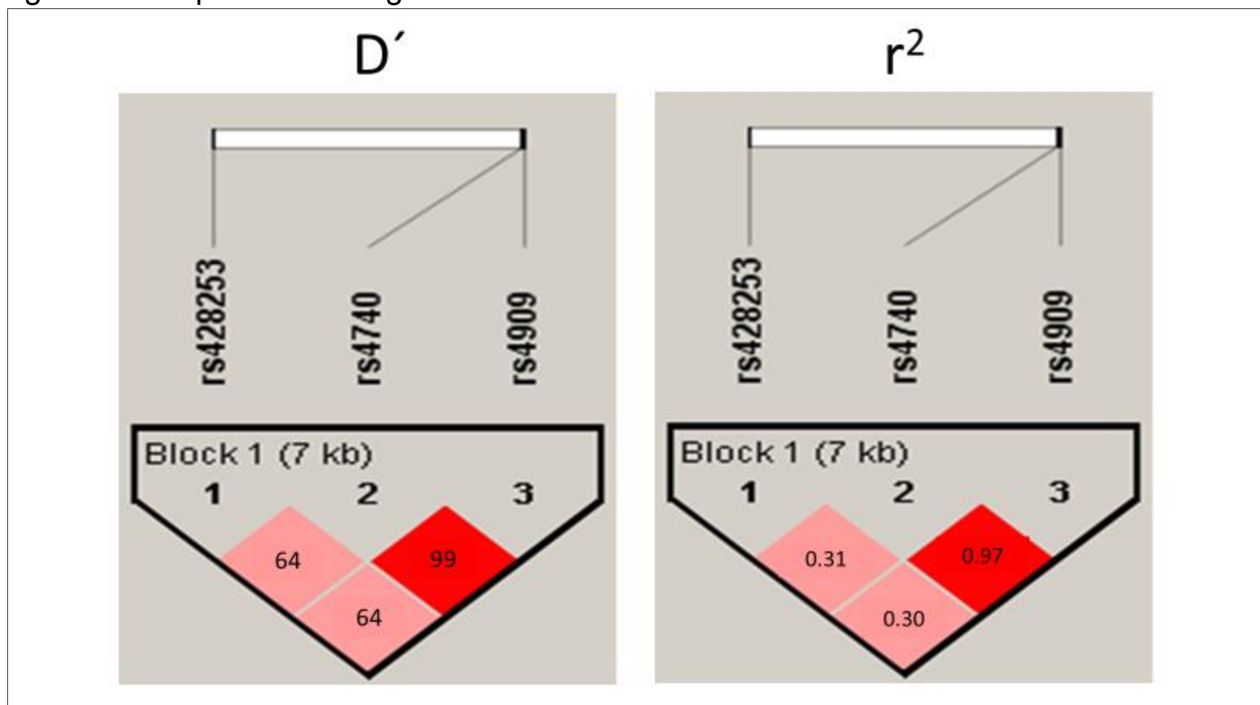


Tabla 15. Frecuencias de haplotipos del gen *EBI3*

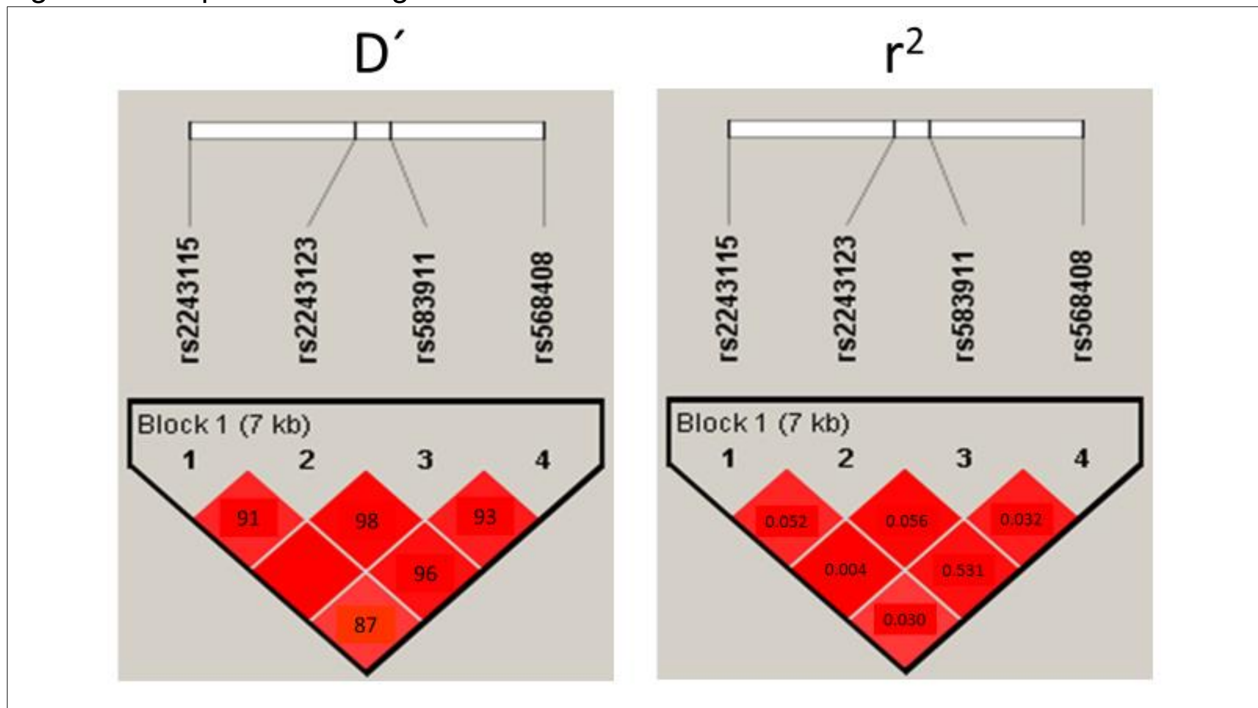
Haplotipos	Secuencia	Coronarios	Controles	Chi2	p
H1	GGA	0.678	0.667	0.479	0.4889
H2	CAG	0.156	0.165	0.578	0.4473
H3	GAG	0.112	0.102	1.207	0.2718
H4	**CGA**	**0.047**	**0.063**	**4.556**	**0.0328**

El orden de los polimorfismos corresponde a la posición que ocupan en el cromosoma (rs42853, rs4740, rs4905)

9.2.4 Análisis de haplotipos del gen *IL-12A*

De los cuatros polimorfismos analizados, los marcadores rs2242123 y rs 568408 del gen *IL-12A* se encontraron en desequilibrio de ligamiento (D'>0.96, r^2 = 0.531, Figura 16).

Figura 16. Mapa de DL del gen *IL-12A*



El análisis de desequilibrio de ligamiento entre los polimorfismos del gen *IL-12A* generó cinco haplotipos H1: ***TTGG***, H2 ***TCAG***, H3: ***TTAA***, H4: ***GTAG*** y H5: ***TTAG*** (el orden de los polimorfismos en las secuencias de los haplotipos es rs2243115, rs2243123,

rs583911, rs568408) (Tabla 16). El haplotipo H4: ***GTAG*** fue menos frecuente en los pacientes coronarios en comparación con el grupo control ($p = 0.0031$).

Tabla 16. Frecuencias de haplotipos del gen *IL-12A*

Haplotipos	Secuencia	Coronarios	Controles	Chi2	p
H1	TTGG	0.474	0.481	0.219	0.6396
H2	TCAG	0.379	0.344	5.073	0.0243
H3	TTAA	0.058	0.055	0.170	0.6797
H4	**GTAG**	**0.046**	**0.068**	**8.735**	**0.0031**
H5	TTAG	0.031	0.034	0.205	0.6507

El orden de los polimorfismos corresponde a la posición que ocupan en el cromosoma (rs2243115, rs2243123, rs583911, rs568408)

9.2.5 Asociación de los polimorfismos de los genes *EBI3* e *IL-12A* con parámetros metabólicos

En los pacientes con EAC prematura, bajo diferentes modelos, el polimorfismo *EBI3* rs428253 se asoció con niveles elevados de ALT (>p75) ($p_{aditivo}$, $p_{dominante}$, $p_{hetetocigoto}$, $p_{codominante\ 1} < 0.02$) y de AST (>p75) ($p_{codominante\ 2} = 0.042$) y con menor riesgo de presentar DMT2 ($p_{dominante}$, $p_{heterocigoto}$, $p_{codominante\ 1} < 0.03$). El polimorfismo rs4905 de *EBI3* se asoció con niveles elevados de ALT ($p_{aditivo}$, $p_{dominante}$, $p_{codominante\ 1} < 0.05$). Por otro lado, el polimorfismo rs2243123 de la *IL-12A* se asoció con mayor riesgo de presentar DMT2 ($p_{recesivo}$, $p_{codominante\ 2} < 0.03$), mientras que el polimorfismo rs2243115 se asoció a reducción en el riesgo para síndrome metabólico ($p_{aditivo}$, $p_{dominante}$, $p_{heterocigoto}$, $p_{codominante\ 1}$ < 0.025). El polimorfismo rs583911 se asoció con menor riesgo de inflamación, definida como concentraciones de PCR de alta sensibilidad ≥ 3 mg/L ($p_{recesivo} = 0.017$), actividad incrementada de AST ($p_{aditivo}$, $p_{dominante}$, $p_{recesivo}$, $p_{codominante\ 2} < 0.05$) y de GGT (>p75, $p_{recesivo} = 0.042$) (Tabla 17).

Tabla 17. Asociación entre los polimorfismos de *EBI3* e *IL-12A* y anormalidades metabólicas en pacientes con enfermedad arterial coronaria prematura.

Polimorfismo	**Frecuencia del genotipo**			**FAM**	**Modelo**	**RM [IC 95%]**	**p**
EBI3							
rs428253	***GG***	***GC***	***CC***				
ALT >p75					Aditivo	1.330 [1.083-1.632]	0.006
No (n=590)	0.679	0.283	0.038	0.179	Dominante	1.429 [1.121-1.821]	0.004
Si (n=572)	0.594	0.357	0.049	0.227	Heterocigoto	1.392 [1.084-1.787]	0.010
					Codominante 1	1.425 [1.107-1.835]	0.006
AST >p75							
No (n=752)	0.650	0.316	0.034	0.191	Codominante 2	1.823 [1.022-3.250]	0.042
Si (n=410)	0.613	0.328	0.059	0.223			
Diabetes mellitus tipo 2					Dominante	0.753 [0.580-0.978]	0.033
No (n=750)	0.611	0.345	0.044	0.217	Heterocigoto	0.727 [0.554-0.954]	0.022
Si (n=412)	0.684	0.272	0.044	0.180	Codominante 1	0.726 [0.522-0.955]	0.022
rs4905	*AA*	*AG*	*GG*				
ALT >p75					Aditivo	1.241 [1.031-1.495]	0.023
No (n=752)	0.532	0.402	0.066	0.267	Dominante	1.309 [1.037-1.653]	0.024
Si (n=410)	0.517	0.394	0.089	0.285	Codominante 1	1.284 [1.006-1.640]	0.045
IL-12A							
rs2243123	***TT***	***TC***	***CC***				
Diabetes mellitus tipo 2							
No (n=750)	0.387	0.477	0.137	0.375	Recesivo	1.148 [1.061-2.063]	0.021
Si (n=412)	0.365	0.441	0.194	0.415	Codominante 2	1.511 [1.048-2.178]	0.028
rs2243115	***TT***	***TG***	***GG***				
Síndrome metabólico					Aditivo	0.591 [0.386-0.905]	0.015
No (n=327)	0.875	0.122	0.003	0.064	Dominante	0.590 [0.381-0.912]	0.017
Si (n=835)	0.913	0.086	0.001	0.044	Heterocigoto	0.599 [0.386-0.929]	0.022
					Codominante 1	0.592 [0.385-0.927]	0.021
rs583911	***AA***	***AG***	***GG***				
Inflamación							
No (n=930)	0.274	0.466	0.260	0.493	Recesivo	0.633 [0.435-0.921]	0.017
Si (n=232)	0.287	0.532	0.181	0.446			
AST>p75					Aditivo	1.236 [1.046-1.460]	0.013
No (n=712)	0.296	0.482	0.222	0.463	Dominante	1.318 [1.004-1.730]	0.046
Si (n= 450)	0.242	0.483	0.275	0.517	Recesivo	1.344 [1.021-1.769]	0.035
					Codominante 2	1.529 [1.096-2.133]	0.013
GGT >p75							
No (n=625)	0.285	0.494	0.221	0.468	Recesivo	1.329 [1.011-1.748]	0.042
Si (n=537)	0.263	0.468	0.269	0.504			

En la tabla se muestran los modelos con asociación significativa. Todos los modelos se ajustaron por edad, sexo e índice de masa corporal. FAM = frecuencia del alelo menor, RM = razón de momios, IC95%= intervalo intercuartil al 95%, ALT = alanina aminotransferasa, AST = aspartato aminotransferasa, GGT = Gamma-glutamil transferasa. Solo se muestran los polimorfismos y los modelos con diferencias significativas.

Tabla 18. Asociación entre los polimorfismos de *EBI3* e *IL-12A* y anormalidades metabólicas en el grupo control.

Polimorfismo	Frecuencia del genotipo			FAM	Modelo	RM [IC 95%]	p
EBI3							
rs428253	***GG***	***GC***	***CC***				
Hiperuricemia							
No (n=726)	0.625	0.303	0.072	0.223	Heterocigoto	1.595 [1.064-2.389]	0.024
Si (n=147)	0.555	0.390	0.055	0.248	Codominante 1	1.567 [1.038-2.365]	0.032
rs4740	***GG***	***GA***	***AA***				
Obesidad central							
No (n=196)	0.503	0.400	0.097	0.296	Heterocigoto	0.391 [0.163-0.937]	0.035
Si (n=677)	0.557	0.371	0.072	0.258	Codominante 2	0.386 [0.157-0.949]	0.038
Aspartato aminotransferasa >p75					Aditivo	1.250 [1.004-1.557]	0.046
No (n=567)	0.576	0.348	0.076	0.250	Dominante	1.430 [1.076-1.899]	0.014
Si (n=306)	0.485	0.433	0.082	0.299	Heterocigoto	1.433 [1.073-1.913]	0.015
					Codominante 1	1.473 [1.093-1.985]	0.011
rs4905	***AA***	***AG***	***GG***				
Obesidad central							
No (n=196)	0.503	0.400	0.097	0.296	Recesivo	0.404 [0.170-0.960]	0.040
Si (n=677)	0.552	0.371	0.074	0.261	Codominante 2	0.403 [0.165-0.983]	0.046
Aspartato aminotransferasa >p75					Dominante	1.399 [1.054-1.858]	0.020
No (n=567)	0.571	0.352	0.078	0.253	Heterocigoto	1.410 [1.056-1.882]	0.020
Si (n=306)	0.485	0.433	0.082	0.299	Codominante 1	1.445 [1.072-1.946]	0.016
IL-12A							
rs568408	***GG***	***GA***	***AA***				
Síndrome metabólico							
No (n=614)	0.883	0.112	0.005	0.061	Aditivo	0.583 [0.347-0.981]	0.042
Si (n=259)	0.915	0.081	0.004	0.044			
rs583911	***AA***	***AG***	***GG***				
Grasa abdominal subcutánea >p75							
No (n=483)	0.268	0.459	0.273	0.503	Heterocigoto	1.776 [1.203-2.622]	0.004
Si (n=390)	0.226	0.563	0.211	0.492	Codominante 1	1.776 [1.107-2.849]	0.017

En la tabla se muestran los modelos con asociación significativa. Todos los modelos se ajustaron por edad, sexo e índice de masa corporal. FAM = frecuencia del alelo menor, RM = razón de momios, IC95%= intervalo intercuartil al 95%. Solo se muestran los polimorfismos y los modelos con diferencias significativas.

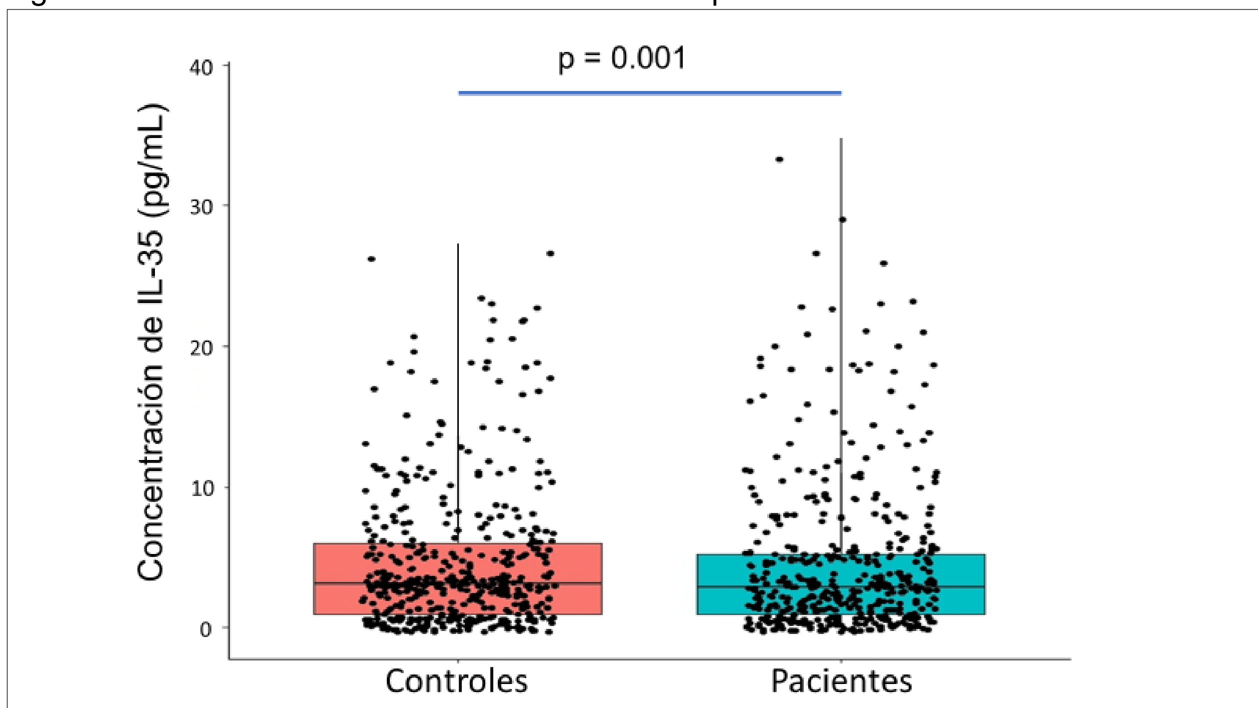
En los participantes del grupo control, el polimorfismo *EBI3* rs428253 se asoció con la presencia de hiperuricemia ($p_{heterocigoto}$, $p_{codominante\ 1} < 0.05$), el *EBI3* rs4740 se asoció con reducción en el riesgo de presentar obesidad de tipo central ($p_{heterocigoto}$, $p_{codominante\ 2} < 0.05$) y con riesgo aumentado de actividad de AST elevada (>p75, $p_{aditivo}$, $p_{dominante}$,

$p_{heterocigoto}$, $p_{codominente\ 1} < 0.05$) y el *EBI3* rs4905 se asoció con menor riesgo para obesidad de tipo central ($p_{recesivo}$, $p_{codominante\ 2} < 0.05$) y riesgo incrementado de actividad AST elevada (>p75, $p_{dominante}$, $p_{heterocigoto}$, $p_{codominante1} < 0.025$). Además, encontramos que el polimorfismo *IL-12A* rs568408 correlacionó con menor riesgo de síndrome metabólico ($p_{aditivo} = 0.042$) y el *IL-12A* rs583911 se asoció con grasa abdominal subcutánea incrementada SAT >p75 ($p_{heterocigoto}$, $p_{codominante\ 1} < 0.02$) (Tabla 18).

9.2.6 Asociación de los polimorfismos de los genes *EBI3* e *IL-12A* con las concentraciones de IL-35

Los pacientes con EAC prematura tuvieron concentraciones significativamente más altas de IL-35 en comparación con los controles (3.2 [1.6–6.7] pg/mL vs 2.7 [0.8–5.2] pg/mL, respectivamente, p = 0.001, Figura 17).

Figura 17. Concentraciones de interleucina 35 en plasma



Concentraciones en plasma de interleucina 35 en 447 sujetos control y 447 pacientes con EAC prematura sin obesidad y con valores de proteína C reactiva <3mg/L. Las comparaciones se hicieron con la prueba de U de Mann Whitney.

Tabla 19. Concentraciones de IL-35 en plasma en los grupos estudiados en función de los genotipos de *EBI3* e *IL-12A*.

			Controles (n=447)			EAC prematura (n=447)		
	Polimorfismo	Genotipo	n	Concentración (pg/mL)	p	n	Concentración (pg/mL)	p
EBI3								
	rs428253	*GG*	275	2.72 [0.88-4.97]		286	3.16 [1.03-6.51]	
		GC	151	3.00 [1.62-5.23]	0.273	140	3.40 [1.19-7.76]	0.433
		CC	21	1.98 [0.19-5.10]		21	2.20 [1.63-3.78]	
	rs4740	*GG*	**250**	**2.52 [0.88-4.50]**		233	3.23 [1.62-7.61]	
		GA	**160**	**3.00 [1.63-5.23]**	**0.020**	185	3.16 [0.95-6.15]	0.311
		AA	**37**	**3.40 [0.88-7.90]**		29	3.16 [1.62-5.23]	
	rs4905	*AA*	**248**	**2.52 [0.88-4.40]**		233	3.23 [1.62-7.61]	
		AG	**164**	**3.00 [1.63-5.23]**	**0.017**	186	3.16 [0.95-6.15]	0.338
		GG	**35**	**3.78 [0.88-7.90]**		28	3.08 [1.62-5.23]	
IL12A								
	rs2243115	*TT*	389	2.72 [0.88-4.97]		407	3.16 [1.62-6.44]	
		TG+GG	58	3.08 [0.83-5.23]	0.376	40	3.56 [0.38-7.95]	0.985
	rs568408	*GG*	398	2.81 [0.88-4.97]		396	3.23 [1.62-6.74]	
		GA+AA	49	2.32 [0.31-7.17]	0.763	51	2.72 [0.95-6.44]	0.254
	rs2243123	*TT*	188	2.72 [0.59-4.97]		183	3.40 [1.03-7.09]	
		TC	198	2.90 [0.88-5.23]	0.702	190	3.00 [1.62-6.31]	0.714
		CC	61	3.00 [0.88-5.23]		74	3.32 [1.47-8.38]	
	rs583911	*AA*	113	3.00 [0.88-5.23]		130	3.23 [1.30-8.56]	
		AG	224	2.90 [0.88-5.23]	0.570	194	3.00 [0.95-6.19]	0.215
		GG	110	2.46 [0.88-4.82]		123	3.40 [1.62-7.09]	

Los datos se muestran como mediana [intervalo intercuartil]. Las comparaciones se realizaron empleando la prueba de U de Mann Whitney o Kruskal Wallis. EAC = Enfermedad arterial coronaria.

En el grupo control se observó que las concentraciones de IL-35 fueron significativamente diferentes dependiendo del genotipo *EBI3* rs4740 (*AA* = 3.40 [0.88–7.90] pg/mL, *GA* = 3.00 [1.63–5.23] pg/mL, *GG* = 2.52 [0.88–4.50] pg/mL; p = 0.020) y rs4905 (*GG* = 3.78 [0.88–7.90] pg/mL, *AG* = 3.00 [1.63–5.23] pg/mL, *AA* = 2.52 [0.88–4.40] pg/mL; p = 0.017) (Table 19).

10. DISCUSIÓN

Polimorfismos *IL27p28*

Este estudio muestra por primera vez que los polimorfismos del gen *IL-27p28* se asocian con la presencia de EAC prematura, actividad de AST aumentada, riesgo de hiperuricemia y resistencia a la insulina en población mexicana. La distribución de las variantes rs26528, rs17855750, rs181206, y rs40837 se analizó en pacientes con EAC prematura y en sujetos control. Los polimorfismos fueron seleccionados por tener posibles consecuencias funcionales y/o frecuencia del alelo menor >5%. Después de ajustar los modelos estadísticos por variables con potencial confusor, los alelos rs26528 *T* y rs40837 *A* se asociaron significativamente con menor riesgo de presentar EAC prematura. En forma separada para casos y controles, también se analizó la asociación de los polimorfismos del gen *IL-27p28* con factores cardiometabólicos. Los alelos rs26528 *T* y rs40837 *A* se asociaron con menor actividad elevada de AST (>p75), el alelo rs181260 *G* se asoció con menor riesgo para hiperuricemia y el alelo rs40837 *A* con reducción en el riesgo para presentar resistencia a la insulina. Las asociaciones de los polimorfismos con las anormalidades metabólicas fueron similares en pacientes y controles, sugiriendo que estas asociaciones son independientes de la presencia del problema cardiovascular. Los análisis informáticos mostraron que el polimorfismo rs40837 crea sitios de unión para los miR-379-5p y miR-1225-5p. La evaluación del efecto funcional de este polimorfismo, se realizó utilizando ensayos de luciferasa, lo cual permitió establecer el efecto de cada uno de los alelos en la expresión del gen en presencia de ambos miRNAs. En presencia del miR-379-5p, el alelo rs40837*A* mostró reducción significativa de la expresión del gen de la luciferasa. Existen evidencias del papel que desempeñan varios miRNAs en la aterosclerosis. Entre otros efectos, se ha mostrado su participación en la regulación del

metabolismo de lípidos, biosíntesis de insulina, adipogénesis, disfunción endotelial, neoangiogénesis, desarrollo y ruptura de la placa, así como en la homeostasis de la glucosa.[158,159] Es interesante señalar que el polimorfismo rs181206, asociado con menor riesgo para hiperuricemia, genera sitios de unión para las proteínas SF/ASF, las cuales regulan el *splicing* alternativo.[160] Es posible que este sitio polimórfico pueda regular la generación de isoformas de IL-27 relevantes en el desarrollo de la elevación del ácido úrico.

La IL-27 participa de manera importante en la inflamación y la patogenia de la aterosclerosis, con efecto dual pro- y antiinflamatorio.[84,110] Promueve la diferenciación temprana de Th1[81] y suprime la diferenciación de Th2[82] y Th17.[83] Los efectos de la IL-27 en la inflamación, se han descrito en varias enfermedades. En hepatitis[116] y esclerosis sistémica[113] promueve la inflamación, mientras que en la artritis autoinmune, asma alérgica[111] y encefalomielitis autoinmune, la suprime.[83] Además, los estudios sobre la participación de la IL-27 en aterosclerosis de modelos animales y de humanos, han también mostrado datos inconsistentes. En ratones, la administración de IL-27 suprime la activación de macrófagos y el desarrollo de la aterosclerosis.[85] Los ratones deficientes de IL-27 o de su receptor tienen mayor susceptibilidad para el desarrollo de aterosclerosis.[85] Estos hallazgos sugieren que en el modelo murino la citocina tiene un papel protector. Por el contrario, en pacientes coronarios se encontraron concentraciones elevadas de IL-27, que correlacionaron con la severidad de la estenosis,[86] hallazgos que sugieren un papel proaterogénico de esta interleucina en humanos. En consistencia con lo reportado por Jin y colaboradores,[86] nuestros resultados mostraron que, en comparación con el grupo control, las concentraciones de IL-27 en plasma son más altas en pacientes con EAC prematura.

A pesar de la amplia evidencia sobre la participación de la IL-27 en la ateroclerosis, la extensa búsqueda en la literatura nos permitió identificar únicamente un trabajo publicado durante el curso de nuestro estudio sobre la asociación de las variantes del gen *IL-27p28* con el desarrollo de la enfermedad cardiovascular.[134] En ese estudio, 4 marcadores polimórficos (tag SNPs) del gen *IL-27p28* (rs181206, rs17855750, rs37833 y rs153190) se determinaron en un número grande de casos con EAC pertenecientes al estudio chino GeneID de la población Han. Después de ajustar por variables confusoras, los polimorfismos no se asociaron con la presencia de la EAC, edad de presentación de la patología o severidad de la misma.[134] Los resultados de esa investigación en población china son consistentes con los hallazgos del presente estudio para las variantes genéticas rs181206 y rs17855750 del gen *IL-27p28*. Sin embargo, las asociaciones observadas en el presente estudio (rs26528, rs40837), consideramos que deben ser investigadas en otras cohortes para confirmar los resultados. Nuestros hallazgos sugieren que los alelos rs26528*T* y rs40837*A* pudieran ser considerados como marcadores potenciales de susceptibilidad para la presencia de EAC prematura y resistencia a la insulina. Aunque estos polimorfismos no se asociaron significativamente con los niveles en plasma de IL-27, los pacientes mostraron concentraciones significativamente más altas de IL-27 en comparación con los sujetos control, hallazgo similar a los resultados de un estudio previo.[86] La ausencia de asociación entre las concentraciones de IL-27 y los polimorfismos del gen *IL-27p28* se puede explicar considerando que, en forma semejante a lo que sucede con otras moléculas, la producción de IL-27 se realiza por un mecanismo complejo que involucra no solo cambios a nivel de DNA sino también modificaciones epigenéticas. Mas aún, es importante considerar que en nuestro estudio los niveles de IL-27 se cuantificaron únicamente en

submuestras de pacientes con EAC y del grupo control que reunían características específicas. Tomando en consideración que la población mexicana tiene características genéticas particulares por tratarse de una población mestiza, conformada por la mezcla de genes amerindios, caucásicos y africanos, las asociaciones observadas con los polimorfismos *IL-27p28* deben ser investigados en otras poblaciones para establecer si son específicas de nuestra población o se comparten con otras etnias.

Polimorfismos *IL-12A* y *EBI3*

Hasta donde sabemos, este es el primer estudio que evalúa el papel de los polimorfismos de los genes *IL-12A* y *EBI3* en EAC prematura. En nuestro estudio dos polimorfismos (*EBI3* rs428253 e *IL-12A* rs2243115), se asociaron con riesgo de presentar esta patología. Detectamos además dos haplotipos (*CGA* de *EBI3* y *GTAG* de *IL-12A*) con menor frecuencia en el grupo de pacientes al comparar con el grupo control. Los dos polimorfismos asociados con EAC prematura, también se asociaron con menor riesgo de presentar DMT2 (*EBI3* rs428253) y síndrome metabólico (*IL-12A* rs2243115) en los pacientes coronarios. Sin embargo, en los sujetos control solo el *EBI3* rs428253 correlacionó con riesgo de hiperuricemia. Los polimorfismos que no se asociaron con EAC prematura se asociaron con otros parámetros clínicos y metabólicos. En los pacientes, el *EBI3* rs4905 se asoció con aumento en la actividad de ALT, el *IL-12A* rs2243123 se asoció con riesgo para la presencia de DMT2, e *IL-12A* rs583911 correlacionó con inflamación, y actividad de AST y GGT. En el grupo control, los polimorfismos *EBI3* rs4905 y rs4740 se asociaron con menor riesgo de obesidad central y mayor riesgo de aumento en la actividad de AST, mientras que el *IL-12A* rs583911

correlacionó con mayor riesgo de tener grasa abdominal subcutánea aumentada y el *IL-12A* rs568404 con menor riesgo de presentar síndrome metabólico.

De acuerdo con las herramientas informáticas, los dos polimorfismos asociados con menor riesgo de presentar EAC prematura, tienen posibles efectos funcionales. Específicamente, el *EBI3* rs428253 modifica el sitio de unión para LEF1 (lymphoid enhancer-binding factor 1, por sus siglas en ingles) que es un factor de trascripción decisivo en el control de la proliferación y diferenciación de granulocitos.[161] Por otro lado, el polimorfismo *IL-12A* rs2243115 localizado en la región promotora, produce sitios de unión para los factores de transcripción AP2, LRH1 y SF1. Así, después de considerar que los polimorfismos estudiados pudieran tener efecto en la producción de la IL-35, analizamos la concentración de la citocina en plasma de submuestras de pacientes con EAC prematura y del grupo control. En comparación con el grupo control, los pacientes coronarios mostraron concentraciones significativamente más altas de IL-35. Con estos resultados, no podemos definir si estas diferencias pudieran tener efecto sobre el desarrollo de la aterosclerosis. La aterosclerosis es una enfermedad multifactorial y múltiples citocinas, tanto pro- como antiinflamatorias, participan en la génesis y progresión del proceso inflamatorio. En este análisis, ni *EBI3* rs42853 ni *IL12A* rs2243115 (los polimorfismos asociados con EAC prematura y con posible efecto funcional) se asociaron con las concentraciones en plasma de la IL-35. Esta falta de asociación se puede explicar considerando que la producción de la IL-35 y otras moléculas está regulada por un mecanismo complejo que no involucra solo cambios a nivel de DNA sino también modificaciones epigenéticas. Además, es importante considerar que, en nuestro estudio, los niveles de la citocina se cuantificaron en circulación y no en el sitio de la lesión. Por otro lado, los polimorfismos *EBI3* rs4740 y rs4905 se asociaron con los niveles

de IL-35 en el grupo control. De estos dos polimorfismos, el *EBI3* rs4740 es funcional, de acuerdo con las herramientas informáticas empleadas para la selección de los polimorfismos. El polimorfismo produce un sitio de unión para Srp40 y SRp55, que pertenecen a la familia de las proteínas SR que regulan el splicing alternativo.[160]

Los polimorfismos del gen *IL-12A* se han asociado con varias enfermedades, tales como artritis reumatoide,[138] enfermedad de Alzheimer,[162] enfermedad de Graves',[137] y asma.[136] Mientras que los polimorfismos de *EBI3* se han asociado con colitis ulcerativa,[132] tuberculosis pulmonar,[142] rinosinusitis crónica[141] y rinitis alérgica.[140] En estos estudios, los genes *IL-12A* y *EBI3* se analizaron independientemente. Hasta donde sabemos, a la fecha no existen estudios que hayan investigado ambos genes simultáneamente en una enfermedad determinada.

Debido a que este es el primer estudio que reporta la asociación de los polimorfismos de la IL-35 con EAC prematura y parámetros cardiovasculares, se requieren estudios adicionales en cohortes independientes que validen los resultados del presente trabajo.

Fortalezas y debilidades del estudio:

Las principales fortalezas de este estudio son las siguientes:

a) El estudio incluye una cohorte grande de casos y controles mexicanos que fueron genotipificados y que han sido bien caracterizados desde el punto de vista tomográfico, clínico y bioquímico lo que nos permitió ajustar los análisis por un número considerable de variables confusoras.
b) En el grupo control, se incluyeron solo sujetos sin evidencia tomográfica de aterosclerosis subclínica (puntaje de calcificación arterial coronaria = 0).

c) La población es étnicamente homogénea, Las proporciones de genes de ancestría caucásicos, africanos e indígenas fueron similares en ambos grupos ($p > 0.05$).

Sin embargo, es importante tener en consideración que el presente estudio tiene las siguientes limitaciones.

1) debido al carácter transversal del estudio, no podemos asegurar la causalidad de las asociaciones.

2) la selección de los participantes no fue aleatoria, por lo que los hallazgos pudieran no ser aplicables a la población mexicana en general.

3) la resistencia a la insulina no se evalúo empleando el estándar de oro que es la pinza euglicémica/hiperinsulinémica, sin embargo, el índice HOMA-RI ha mostrado ser una medición confiable para determinar sensibilidad a la insulina.[163]

4) la cuantificación de las citocinas en plasma se realizó únicamente en una subpoblación del estudio. Sin embargo, se emplearon los mismos criterios para la selección de los sujetos en ambos grupos, por lo que se esperaría que la selección no haya sesgado la submuestra seleccionada.

11. CONCLUSIONES

Este estudio muestra por primera vez, que los polimorfismos de los genes que codifican para las subunidades de las interleucinas 27 y 35 se asocian en forma independiente y significativa a menor riesgo de cardiopatía coronaria. Los resultados sugieren que los alelos rs26528*T* y rs40837*A* del gen *IL-27p28* y los polimorfismos *EBI3* rs42853 e *IL-12A* rs2243115, pudieran ser considerados como marcadores genéticos de protección para EAC prematura en nuestra población. Algunos de los polimorfismos estudiados se asociaron con factores de riesgo cardiovascular o parámetros metabólicos.

Los tres polimorfismos de *EBI3* y los cuatro de *IL-12A* e *IL-27p28* se encontraron en desequilibrio de ligamiento. El análisis de *IL-27p28* y *EBI3* generó cuatro haplotipos y el de *IL-12A* cinco haplotipos. El haplotipo H4 de *EBI3* (CGA) y el de *IL-12A* (GTAG) fueron menos frecuentes en los pacientes que en el grupo control. No se observaron diferencias en las frecuencias de los haplotipos de *IL-27p28* entre los grupos estudiados.

En comparación con el grupo control, los pacientes con EAC prematura mostraron concentraciones significativamente más altas de IL-27 e IL-35 en plasma. No se observó relación entre los valores de IL-27 y los genotipos de los polimorfismos de *IL-27p28* estudiados. Los genotipos de *EBI3* rs4740 y rs4905 se asociaron con la variación en las concentraciones de IL-35 en el grupo control.

El alelo rs40837*A* en presencia del miR-379-5p disminuye significativamente la expresión del gen de la luciferasa. Esta observación sugiere que los individuos con este alelo podrían presentar concentraciones más bajas de IL-27 y, como consecuencia, tener menor riesgo de desarrollar EAC prematura.

Dado que la población mexicana tiene características genéticas particulares y diferentes a otros grupos étnicos,[164–167] las asociaciones detectadas deben ser evaluadas en otras

cohortes y poblaciones independientes, para confirmar nuestros hallazgos y establecer si estas asociaciones son específicas de la población mexicana o se comparten con otras etnias.

12. PERSPECTIVAS

El proyecto GEA se encuentra actualmente en la última etapa de la fase de seguimiento. El seguimiento a 5 años de los participantes en la etapa basal, permitirá evaluar si los portadores de los polimorfismos asociados con EAC prematura o alguna variable metabólica, que no presentaban la patología, la han o no desarrollado. De esta manera, la asociación transversal que se describe en el presente trabajo, será evaluada y posiblemente confirmada en la fase prospectiva del estudio.

Por otro lado, deseamos investigar si existe efecto aditivo de los alelos y/o genotipos de los genes *IL-27p28*, *IL-12A* y *EBI3*, en la susceptibilidad para EAC prematura y/o algún parámetro metabólico.

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14. ANEXOS

14.I.- Publicaciones generadas del proyecto de tesis de doctorado

1.- **Posadas-Sánchez R**, Pérez-Hernández N, Rodríguez-Pérez JM, Coral-Vázquez RM, Roque-Ramírez B, Llorente L, Lima G, Flores-Domínguez C, Villarreal-Molina T, Posadas-Romero C, Vargas-Alarcón G. Interleukin-27 polymorphisms are associated with premature coronary artery disease and metabolic parameters in the Mexican population: the genetics of atherosclerotic disease (GEA) Mexican study. Oncotarget. 2017;38:64459-64470.

2.- **Posadas-Sánchez R**, Pérez-Hernández N, Angeles-Martínez J, López-Bautista F, Villarreal-Molina T, Rodríguez-Pérez JM, Fragoso JM, Posadas-Romero C, Vargas-Alarcón G. Interleukin 35 Polymorphisms Are Associated with Decreased Risk of Premature Coronary Artery Disease, Metabolic Parameters, and IL-35 Levels: The Genetics of Atherosclerotic Disease (GEA) Study. Mediators Inflamm. 2017;2017:6012795.

3.- **Posadas-Sánchez R**, Vargas-Alarcón G. Innate Immunity in coronary disease. The role of Interleukin-12 family cytokines in atherosclerosis. Rev Inves Clin 2017. In press

14.2.- Otras publicaciones derivadas del proyecto

14.2.1.- Como primer autor o autor correspondiente

1.- **Posadas-Sánchez R**, Ángeles-Martínez J, Pérez-Hernández N, Rodríguez-Pérez JM, López-Bautista F, Villarreal-Molina T, Fragoso JM, Posadas-Romero C, Vargas-Alarcón G. Receptor-interacting protein 2 (RIP2) gene polymorphisms are associated with increased risk of subclinical atherosclerosis and clinical and metabolic parameters. The Genetics of Atherosclerotic Disease (GEA) Mexican study. Exp Mol Pathol. 2017 Feb;102(1):1-6.

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Clinical Research Paper

Interleukin-27 polymorphisms are associated with premature coronary artery disease and metabolic parameters in the Mexican population: the genetics of atherosclerotic disease (GEA) Mexican study

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ABSTRACT

Several studies suggest an important role of Interleukin-27 in the development of atherosclerosis. The aim of this study was to establish whether the *IL-27p28* gene polymorphisms are associated with premature coronary artery disease and/or other cardiovascular risk factors. Four *IL-27p28* gene polymorphisms were selected and genotyped in 1162 premature coronary artery disease cases and 1107 controls. rs26528 *T* and rs40837 *A* alleles were significantly associated with a lower risk of premature coronary artery disease under different inheritance models ($P_{dominant}$ = 0.046; $P_{over-dominant}$ = 0.002; $P_{co-dominant1}$ = 0.007 for rs26528T; $P_{over-dominant}$ = 0.008 and $P_{co-dominant1}$ = 0.031 for rs40837). The rs40837 *A* allele was also associated with a lower risk of insulin resistance, in cases ($P_{over-dominant}$ = 0.037) and controls ($P_{additive}$ = 0.008; $P_{dominant}$ = 0.047; $P_{recessive}$ = 0.014; $P_{co-dominant2}$ = 0.006), while the rs26528 *T* allele was associated with a lower risk of insulin resistance only in the control group ($P_{recessive}$ = 0.016; $P_{co-dominant2}$ = 0.021). Interleukin-27 plasma levels were measured in 450 controls and 450 cases, and were significantly higher in cases compared to controls (P = 0.004). However, Interleukin-27 plasma levels were not associated with *IL-27p28* polymorphisms. Luciferase assays showed that co-transfection of the rs40837 *A* allele and miR-379-5p significantly decreased luciferase gene expression. Our study shows for the first time, that IL-*27p28* gene polymorphisms are associated with premature coronary artery disease and with some metabolic parameters. The rs40837 *A* allele in presence of miR-379-5p significantly decreased luciferase gene expression.

INTRODUCTION

Cardiovascular disease (CVD) is the main cause of morbidity in developed and emerging countries. Coronary arterial disease (CAD) is the most common CVDs mainly caused by atherosclerosis, a multifactorial

disease involving both genetic and environmental factors [1]. Several studies suggest atherosclerosis could be considered an inflammatory disease [2-4]. Macrophage and T cell infiltrates are known to play an important role in atherosclerotic lesions in humans and in animal models [5,6]. Macrophages [7,8] and Th1 cells [9-11] secreted cytokines and chemokines that amplify local immune responses, while Th2 cells are known to have a protective effect on the development of atherosclerosis [12-13].

Interleukin 27 (IL-27), a member of the interleukin 12 family, is a heterodimeric cytokine, conformed by an α p28 and a β EBI3 subunit [14]. IL-27 is an immune/inflammatory response regulator [15] by promoting early Th1 differentiation [16], suppressing Th2 and Th17 differentiation [17-20], and inducing the production of anti-inflammatory cytokines such as IL-10 by activated T cell [21]. IL-27 is expressed in atherosclerotic plaques [22], and its role in atherosclerosis has been studied in cultured cells, animal models and coronary patients, with inconsistent findings. IL-27-deficient (*Ldlr*$^{-/-}$ *Ebi3*$^{-/-}$) and IL-27 receptor-deficient (*Ldlr*$^{-/-}$*WSX-1*$^{-/-}$) *Ldlr*$^{-/-}$ knockout mice were more susceptible to develop atherosclerosis, and IL-27 administration suppressed macrophage activation and atherosclerosis development [23]. However, coronary patients showed higher IL-27 levels as compared to controls, and IL-27 levels showed a significant correlation with stenosis severity [24]. Moreover, dendritic cells incubated with oxidized LDL (low density lipoprotein) produced IL-27, suggesting these modified lipoproteins could play an important role in dendritic cell activation and IL-27 production [24]. Altogether, these studies suggest IL-27 could play a crucial role in the immunity and inflammation regulatory net in atherosclerosis. The human *IL-27p28* gene encodes the IL-27 alpha subunit, located in the 16p11 locus, spans 5 exons and is highly polymorphic [25]. Considering the important role of the IL-27 in the developing of atherosclerosis, the objective of this study was to evaluate whether *IL-27p28* polymorphisms are associated with premature CAD (pCAD) and/or cardiovascular risk factors, as well as to evaluate whether the associated polymorphisms have a functional effect.

RESULTS

Clinical characteristics and metabolic parameters

A total of 1107 controls with no tomographic evidence [coronary artery calcification (CAC) score = 0] of subclinical atherosclerosis (SA) and 1162 pCAD cases with complete clinical, demographic, anthropometric and biochemical information belonging

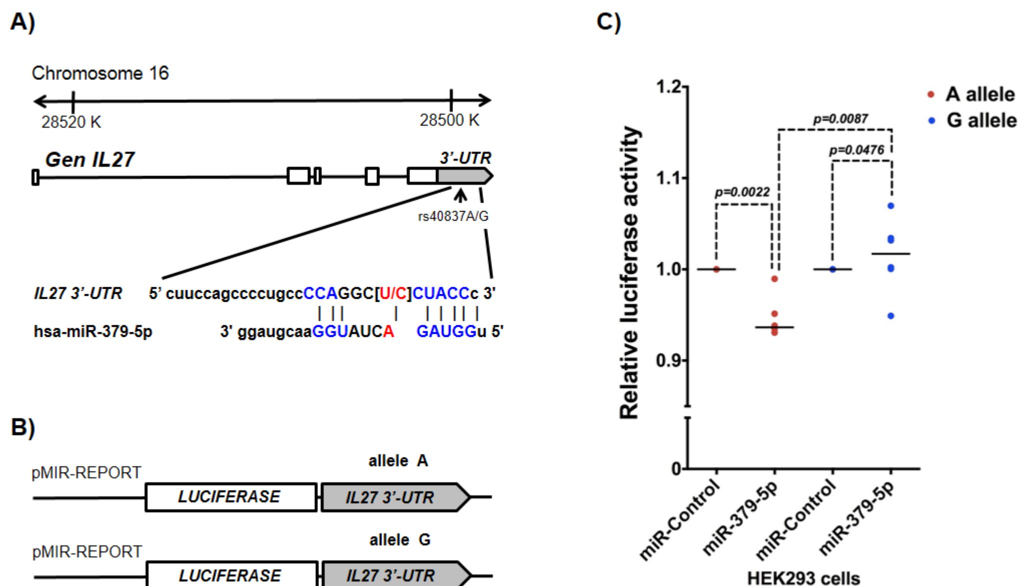

Figure 1: rs40837 affects luciferase expression levels in the presence of miR-379-5p. A. Schematic diagram of the IL27 gene indicating the location of rs40837 allelic variants (*A/G*) in the 3'-UTR region and its sequence complementarity with the miR-379-5p. **B.** Sequences with each allele were cloned in pMIR-REPORT vector to evaluate the expression of the luciferase reporter gene as described in Materials and Methods. **C.** The presence of allele *A* reduced luciferase expression in the cells co-transfected with miR-379-5p as compared to cells co-transfected with miR control ($P = 0.0022$), and cells with *G* allele co-transfected with miR-379-5p ($P = 0.0087$). n = 5 independent duplicate experiments; medians were compared using Mann Whitney's U test.

Table 1: Clinical characteristics and cardiovascular risk factors in the studied groups.

	Controls (*n* = 1107)	pCAD (*n* = 1162)	[a]*P*
Age (years)	51 ± 9	54 ± 8	<0.001
Sex (% male)	41.2	81.1	<0.001
Body mass index (kg/m2)	27.8 [25.4-30.8]	28.3 [25.9-31.1]	0.003
Waist circumference (cm)	94 ± 11	98 ± 10	<0.001
Systolic blood pressure (mmHg)	102 [104-122]	116 [106-127]	<0.001
Diastolic blood pressure (mmHg)	70 [65-76]	71 [66-78]	0.013
Visceral abdominal fat (cm2)	139 [10.4-180]	168 [129-215]	<0.001
ALT (IU/L)	24 [18-34]	26 [19-36]	0.031
AST (IU/L)	25 [21-30]	26 [22-31]	0.001
Total cholesterol > 200mg/dl (%)	36.5	20.3	<0.001
LDL-C ≥ 130 mg/dL (%)	29.6	16.1	<0.001
Hypoalphalipoproteinemia (%)	52.0	67.2	<0.001
Hypertriglyceridemia (%)	47.3	56.2	<0.001
Non HDL-C>160 mg/dL (%)	27.9	19.5	<0.001
Obesity (%)	30.1	35.0	0.003
Abdominal obesity (%)	81.0	83.6	0.060
Type 2 Diabetes mellitus (%)	10.1	35.4	<0.001
Insulin resistance (%)	54.2	77.0	<0.001
Metabolic syndrome (%)	40.7	71.9	<0.001
Hypertension (%)	6.7	68.1	<0.001
Increased VAF (%)	54.5	64.6	<0.001
Current smoking habit (%)	22.8	11.6	<0.001
Hypoadiponectinemia (%)	41.5	56.5	<0.001
hsCRP ≥ 3 mg/L (%)	26.6	21.3	0.002
Hyperuricemia (%)	19.9	35.9	<0.001

Abbreviations: pCAD, premature coronary artery disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL-C, low density lipoprotein cholesterol; Non HDL-C, non high density lipoprotein cholesterol; VAF, visceral abdominal fat; hsCRP, high sensitivity C reactive protein. Data are shown as average ± standard deviation, median [interquartile range] or percentage. aStudent's t test, Mann Whitney's U test or Chi square test.

to the Genetics of Atherosclerotic Disease (GEA) Mexican Study were selected for the analyses. Clinical characteristics of the pCAD cases and control subjects are shown in Table 1. Age, male percentage, body mass index (BMI), waist circumference, systolic and diastolic blood pressure, visceral abdominal fat (VAF), alanine aminotransferase (ALT), aspartate aminotransferase (AST), hypoalphalipoproteinemia, hypertriglyceridemia, general obesity, abdominal obesity, type 2 diabetes mellitus (T2DM), insulin resistance, metabolic syndrome, hypertension, high VAF, hyperuricemia and hypoadiponectinemia were significantly higher in pCAD cases than in controls (Table 1). On the other hand, hypercholesterolemia [total cholesterol (TC)>200 mg/dL or low density lipoprotein-cholesterol (LDL-C) ≥130 mg/dL], high non-HDL (high density lipoprotein) cholesterol, inflammation [defined as high sensitivity C reactive protein (hsCRP) levels ≥3mg/L] and current smoking habit were significantly more frequent in controls than in pCAD cases most likely due to the effect of statin treatment (Table 1). All differences were statistically significant.

Association of IL-27 polymorphisms with pCAD

Genotype distributions of all polymorphisms in pCAD cases and controls are described in Table 2. The polymorphisms were in Hardy-Weinberg equilibrium. While genotype distributions of rs17855750 and rs181206 were similar in cases and controls, rs26528 *T* and rs40837 *A* alleles were significantly associated with a lower risk of pCAD. The rs26528 *T* allele showed significant associations with lower risk of pCAD under dominant (OR = 0.794, P = 0.046), over-dominant (OR = 0.701, P = 0.002) and co-dominant 1 (OR = 0.718, P = 0.007) models, while the rs40837 *A* allele was significantly associated with a lower risk of pCAD under the over-dominant and co-dominant 1 models (OR = 0.740, P = 0.008 and OR = 0.768, P = 0.031, respectively). All associations were adjusted for age, gender, BMI, smoking habit, total

Table 2: Association of *IL-27p28* polymorphisms with premature coronary artery disease.

SNP	Genotype frequency			MAF	Model	OR [95% CI]	*P*
rs26528	*CC*	*CT*	*TT*				
					Additive	0.958 [0.817-1.122]	0.594
Control (*n* = 1107)	0.378	0.481	0.142	0.382	**Dominant**	**0.794 [0.634-0.996]**	**0.046**
Patients (*n* = 1162)	0.410	0.441	0.149	0.369	Recessive	1.302 [0.958-1.771]	0.092
					Over-dominant	**0.701 [0.562-0.875]**	**0.002**
					Co-dominant 1	**0.718 [0.564-0.913]**	**0.007**
					Co-dominant 2	1.086 [0.778-1.517]	0.627
rs17855750	*AA*	*AC*	*CC*				
					Additive	0.967 [0.777-1.205]	0.767
Control (*n* = 1107)	0.729	0.248	0.023	0.147	Dominant	0.932 [0.724-1.199]	0.582
Patients (*n* = 1162)	0.731	0.242	0.024	0.146	Recessive	1.240 [0.610-2.522]	0.553
					Over-dominant	0.900 [0.694-1.169]	0.431
					Co-dominant 1	0.906 [0.697-1.177]	0.460
					Co-dominant 2	1.211 [0.594-2.470]	0.599
rs181206	*AA*	*AG*	*GG*				
					Additive	1.003 [0.855-1.178]	0.967
Control (*n* = 1107)	0.451	0.426	0.123	0.336	Dominant	0.955 [0.768-1.187]	0.677
Patients (*n* = 1162)	0.442	0.441	0.117	0.337	Recessive	1.135 [0.810-1.590]	0.463
					Over-dominant	0.923 [0.740-1.151]	0.476
					Co-dominant 1	0.936 [0.742-1.182]	0.581
					Co-dominant 2	1.073 [0.749-1.536]	0.701
rs40837	*GG*	*GA*	*AA*				
					Additive	0.992 [0.847-1.162]	0.923
Control (*n* = 1107)	0.378	0.480	0.143	0.382	Dominant	0.847 [0.676-1.062]	0.150
Patients (*n* = 1162)	0.405	0.445	0.150	0.372	Recessive	1.316 [0.970-1.786]	0.078
					Over-dominant	**0.740 [0.593-0.923]**	**0.008**
					Co-dominant 1	**0.768 [0.604-0.977]**	**0.031**
					Co-dominant 2	1.140 [0.818-1.589]	0.440

Abbreviations: SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval. All models were adjusted by age, gender, body mass index, smoking habit, total abdominal fat, HOMA-IR, aspartate aminotransferase, adiponectin and uric acid.
Co-dominant 1: comparison of heterozygote vs major allele homozygote genotype frequencies.
Co-dominant 2: comparison of heterozygote vs minor allele homozygote genotype frequencies.

abdominal fat (TAF), homeostasis model assessment of insulin resistance (HOMA-IR), AST, adiponectin and uric acid levels (Table 2).

Associations with metabolic parameters

Tables 3 and 4 describe associations of *IL-27p28* polymorphisms with metabolic parameters in controls and pCAD cases, respectively. In the control group, rs26528 *T* and rs40837 *A* were significantly associated with a lower risk of insulin resistance (OR = 0.623, $P_{recessive}$ = 0.016; OR = 0.610, $P_{co\text{-}dominant2}$ = 0.021 for rs26528 *T*; OR = 0.646, $P_{additive}$ = 0.008; OR = 0.616, $P_{dominant}$ = 0.047; OR = 0.488, $P_{recessive}$ =0.014 and OR = 0.404, $P_{co\text{-}dominant2}$ = 0.006 for rs40837 *A*) and with a lower risk of increased AST activity (OR = 0.777, $P_{additive}$ = 0.016; OR = 0.654, $P_{recessive}$ = 0.046 and OR = 0.583, $P_{co\text{-}dominant2}$ = 0.018 for rs26528 *T*; OR = 0.771, $P_{additive}$ = 0.013 and OR = 0.603, $P_{co\text{-}dominant2}$ = 0.025 for rs40837 *A*); while rs181206 *G* was associated with a lower risk of hyperuricemia (OR = 0.788, $P_{additive}$ = 0.044) (Table 3). In pCAD cases, rs26528 *T* and rs40837 *A* were significantly associated with a lower risk of elevated AST activity (OR = 0.802, $P_{additive}$ = 0.014; OR = 0.689, $P_{dominant}$ = 0.003; OR = 0.737, $P_{over\text{-}dominant}$= 0.016 and OR = 0.678, $P_{co\text{-}dominant1}$ = 0.004 for rs26528 *T*; OR = 0.799, $P_{additive}$ = 0.013; OR = 0.720, $P_{over\text{-}dominant}$ = 0.009 and OR = 0.662, $P_{co\text{-}dominant1}$ = 0.002 for rs40837 *A*). Moreover, rs181206 *G*

Table 3: *IL27p28* gene polymorphism association with metabolic parameters in control subjects.

SNP	Genotype frequency			MAF	Model	OR [95% CI]	*P*
rs26528	*CC*	*CT*	*TT*				
Insulin resistance							
No (n=507)	0.358	0.466	0.176	0.408	Recessive	0.623 [0.423-0.916]	0.016
Si (n=600)	0.395	0.492	0.113	0.359	Co-dominant 2	0.610 [0.401-0.930]	0.021
Aspartate aminotransferase >p75							
No (n=705)	0.368	0.471	0.161	0.396	Additive	0.777 [0.633-0.954]	0.016
Si (n=402)	0.391	0.500	0.109	0.359	Recessive	0.654 [0.431-0.992]	0.046
					Co-dominant 2	0.583 [0.373-0.912]	0.018
rs181206	*AA*	*AG*	*GG*				
Hyperuricemia							
No (*n* = 887)	0.438	0.434	0.128	0.345	Additive	0.788 [0.624-0.994]	0.044
Si (*n* = 220)	0.495	0.405	0.100	0.302			
rs40837	*GG*	*GA*	*AA*				
Insulin resistance							
No (*n* = 506)	0.354	0.470	0.176	0.411	Additive	0.646 [0.468-0.892]	0.008
Si (*n* = 601)	0.399	0.486	0.115	0.358	Dominant	0.616 [0.381-0.994]	0.047
					Recessive	0.488 [0.274-0.867]	0.014
					Co-dominant 2	0.404 [0.210-0.775]	0.006
Aspartate aminotransferase >p75							
No (*n* = 705)	0.366	0.475	0.159	0.396	Additive	0.771 [0.628-0.947]	0.013
Si (*n* = 402)	0.339	0.489	0.112	0.357	Co-dominant 2	0.603 [0.388-0.938]	0.025

Abbreviations: SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval; p75, percentile 75. All models were adjusted by age, gender and body mass index.
Co-dominant 1: comparison of heterozygote vs major allele homozygote genotype frequencies.
Co-dominant 2: comparison of heterozygote vs minor allele homozygote genotype frequencies.

was associated with a lower risk of hyperuricemia (OR = 0.546, $P_{recessive}$ = 0.005 and OR = 0.566, $P_{co\text{-}dominant2}$ = 0.011) and rs40837 *A* was associated with a lower risk of insulin resistance (OR = 0.702, $P_{over\text{-}dominant}$ = 0.037) (Table 4).

Haplotype analysis

Only rs40837 and rs26528 polymorphisms were in high linkage disequilibrium (r^2>0.956). Four different haplotypes were observed, but none of them showed a significant association with pCAD (data not shown).

IL-27 plasma levels

In a subsample, pCAD cases showed significantly higher IL-27 levels than control subjects (2.9 pg/mL vs 0.94 pg/mL, respectively; P = 0.004,). Both pCAD cases and healthy controls were non-obese individuals with hsCRP levels < 3 mg/L. IL-27 plasma levels were not significantly associated with any of the *IL-27p28* polymorphisms analyzed here.

rs40837 *G/A* luciferase assays

Because rs40837 is a predicted 3'-UTR region target for miR-379-5p and/or miR-1225-5p, we examined the effect of both *G* and *A* alleles co-transfected with miR-379-5p or miR-1225-5p in a luciferase expression system. Cells co-transfected with the *A* allele construct and miR-379-5p showed a ~5% reduction in luciferase activity (P=0.0022) compared to those co-transfected with miR-Control. In contrast, cells co-transfected with the *G* allele construct (Figure 1B) and miR-379-5p showed higher mean luciferase activity (p = 0.0476) than those cotransfected with the miR-Control (Figure 1C). Moreover, in the presence of miR-379-5p, the *A* allele construct showed significantly lower luciferase activity

Table 4: **IL27p28 gene polymorphisms association with metabolic parameters in pCAD cases.**

SNP	Genotype frequency			MAF	Model	OR [95% CI]	*P*
rs26528	*CC*	*CT*	*TT*				
Aspartate aminotransferase>p75							
No (*n* = 710)	0.382	0.459	0.159	0.389	Additive	0.802 [0.673-0.957]	0.014
Si (*n* = 452)	0.454	0.407	0.139	0.343	Dominant	0.689 [0.539-0.882]	0.003
					Over-dominant	0.737 [0.576-0.944]	0.016
					Co-dominant 1	0.678 [0.521-0.883]	0.004
rs181206	*AA*	*AG*	*GG*				
Hyperuricemia							
No (*n* = 745)	0.439	0.426	0.135	0.348	Recessive	0.546 [0.359-0.830]	0.005
Si (*n* = 417)	0.455	0.460	0.085	0.314	Co-dominant 2	0.566 [0.365-0.878]	0.011
rs40837	*GG*	*GA*	*AA*				
Insulin resistance							
No (*n* = 316)	0.393	0.470	0.137	0.372	Over-dominant	0.702 [0.504-0.978]	0.037
Si (*n* = 846)	0.414	0.433	0.153	0.369			
Aspartate aminotransferase>p75							
No (*n* = 710)	0.375	0.469	0.156	0.391	Additive	0.799 [0.670-0.953]	0.013
Si (*n* = 452)	0.456	0.407	0.137	0.341	Over-dominant	0.720 [0.563-0.922]	0.009
					Co-dominant 1	0.662 [0.509-0.863]	0.002

Abbreviations: SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval; p75, percentile 75. All models were adjusted by age, gender and body mass index.
Co-dominant 1: comparison of heterozygote vs major allele homozygote genotype frequencies.
Co-dominant 2: comparison of heterozygote vs minor allele homozygote genotype frequencies.

than the *G* allele construct (p = 0.0087). Co-transfection of both constructs (*A* or *G* allele) with the miR-1225-5p did not significantly affect luciferase expression (data not shown).

DISCUSSION

As far as we know, this is the first study reporting the association of *IL-27p28* gene polymorphisms with pCAD. We analyzed the distribution of rs26528, rs17855750, rs181206 and rs40837 in pCAD cases and controls in order to determine whether they confer susceptibility to pCAD. Polymorphism selection was based on informatics analyses for informativity (minor allele frequency >5%) and/or predicted functional effects. Both rs26528 *T* and rs40837 *A* alleles were significantly associated with a decreased risk of developing pCAD. *IL-27p28* gene polymorphisms were also analyzed for associations with cardiometabolic risk factors, independently in cases and controls. rs26528 *T* and rs40837 *A* alleles were associated with a lower risk of high AST activity (>p75), rs181206 *G* was associated with a lower risk of hiperuricemia and rs40837 *A* with a lower risk of insulin resistance. The associations detected between the polymorphisms and cardiovascular parameters in both groups (cases and controls) were similar, suggesting that these associations are independent of the pathology present in these individuals.

Informatics analyses showed that rs40837 creates DNA binding sites for miR-379-5p and miR-1225-5p. In order to evaluate the functional effect of this polymorphism, luciferase assays were used to test the effect of each allele on gene expression in the presence of both miRNAs. In the presence of miR-379-5p, the rs40837 *A* allele showed significantly decreased luciferase gene expression. Several studies have provided evidence on the role of several miRNAs in atherosclerosis. They participate in the regulation of lipid metabolism, insulin biosynthesis, adipogenesis, endothelial dysfunction, neoangiogenesis, plaque development and rupture, as well as glucose homeostasis, among others [26-27]. Interestingly rs181206, associated with a lower risk of hyperuricemia, generates a binding site for SF/ASF proteins, which regulates alternative splicing [28]. This polymorphic site could regulate IL-27 isoforms relevant for the development of hiperuricemia.

IL-27 plays an important role in inflammation and atherosclerosis pathogenesis with dual effects, both pro

and anti-inflammatory [20, 21]. It promotes early Th1 cell differentiation [16] and suppresses Th2 [18] and Th17 [19] differentiation. The type of effect on inflammation exerted by IL-27 has been reported to differ in various diseases. While it is known to promote inflammation in hepatitis [29] and systemic sclerosis [30], it suppresses inflammation in autoimmune arthritis [31], allergic asthma [32] and autoimmune encephalomyelitis [19]. Moreover, studies on the role of IL-27 in atherosclerosis in animal models and humans have shown inconsistent results. In the murine model, IL-27 administration suppressed macrophage activation and atherosclerosis development [23] and mice deficient for IL-27 or its receptor showed increased atherosclerosis susceptibility [23], suggesting IL-27 has a protective role. In contrast, coronary patients showed significantly higher IL-27 levels, which correlated with the severity of stenosis [24], suggesting a pro-atherogenic role in humans. Similar to the findings of Jin et al. [24], significantly higher IL-27 plasma concentrations were observed in Mexican pCAD cases as compared to controls.

We consider that the main strengths of this study are the following: a) The study included a large cohort of Mexican cases and controls with thorough phenotyping, and with tomographic, clinical and biochemical data, allowing to adjust our analyses for a large number of potential confounders; b) Controls included only individuals without tomographic evidence of SA (CAC score=0); c) Population stratification was ruled out as a potential confounding factor, because the proportions of Caucasian, Native American and African ancestry were similar in cases and controls; and d) A functional effect of rs40837 polymorphism on luciferase expression was observed, which was in accordance with the observed associations. Nevertheless, results should be interpreted with caution, considering the following limitations. First, due to the transversal character of the study, conclusions on causality cannot be made. Second, because the selection of participants was not random, the findings may not be applicable to the general population. However, considering that the participants have no knowledge of their genotypes, the genotype distributions would be expected to be similar in a randomly selected sample. Third, insulin resistance was not evaluated using euglycemic/hyperinsulinemic clamp, nonetheless HOMA-IR index has proven to be a reliable measurement of insulin sensitivity [33].

This study shows for the first time, that *IL-27p28* gene polymorphisms are associated with pCAD, AST activity, hyperuricemia and insulin resistance in the Mexican population. Despite all the evidence on the role of IL-27 in atherosclerosis, to best of our knowledge, to date only one cross sectional analysis has evaluated the role of *IL-27p28* gene variants in cardiovascular disease. In this study, four *IL-27p28* tag SNPs, (rs181206, rs17855750, rs37833 and rs153109) were determined in a large number of CAD cases belonging to the GeneID Chinese Han population [34]. After adjusting for confounder's variables, the polymorphisms were not associated with CAD, age at disease onset or severity [34]. These results are consistent with the findings of the present study for the rs181206 and rs17855750 *IL-27p28* gene variants. Thus, replications of the associations here reported (rs26528, rs40837) should be sought in other cohorts to confirm these results. Our results suggest rs26528*T* and rs40837A alleles could be considered as potential susceptibility markers for pCAD and insulin resistance in our population. Although these polymorphisms were not significantly associated with IL-27 plasma levels, pCAD cases showed significantly higher IL-27 levels than control subjects as previously reported [24]. The fact that the IL-27 plasma levels were not significantly associated with any of the IL-27p28 polymorphisms analyzed here could be explained considering that like other molecules, the production of IL-27 include a complex mechanism that involve not only changes at DNA level but also epigenetic modifications. Moreover, is important to consider that in our study the levels of IL-27 were measured only in a subsample of pCAD cases and controls with specific characteristics.

Because the Mexican population has particular and different genetic characteristics to other ethnic groups [35-38], the *IL-27p28* polymorphism associations observed here should be sought in other populations in order to establish if they are specific for the Mexican population or are shared with other ethnic groups.

MATERIALS AND METHODS

Subjects

The GEA Mexican Study was designed to examine the genetic bases of pCAD and the relationship between traditional and emerging risk factors of SA in an adult Mexican population. This study included 1200 pCAD cases and 1500 healthy individuals as control group aged 30 to 75 years. All participants were unrelated and of self-reported Mexican mestizo ancestry for 3 generations. pCAD was defined as history of myocardial infarction, angioplasty, revascularization surgery or coronary stenosis >50% on angiography, diagnosed before age 55 in men and before age 65 in women. Patients with acute cardiovascular events 3 months prior to the selection were excluded. Controls were apparently healthy asymptomatic individuals without personal or family history of pCAD, recruited from blood bank donors and through brochures posted in Social Services centers. Exclusion criteria for controls included congestive heart failure; liver, renal, thyroid or oncological disease. Standardized questionnaires were applied to all participants to obtain demographic information, family medical history,

history of nutritional habits, physical activity, alcohol consumption and pharmacological treatment. The GEA study was approved by Bioethics Committee of the Instituto Nacional de Cardiología Ignacio Chávez (INCICH), and aligned to Helsinki's Declaration. All participants provided informed consent.

Anthropometric and biochemical measurements

BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured using a glass fiber measuring tape in the middle point of the distance between the lower side of the waist and the iliac crest. Blood pressure was measured at rest 3 times using a digital 5200 series Welch Allyn sphygmomanometer (Shaneateies Fails, N.Y., USA.) and the last two measurements were averaged. Venous blood samples were obtained after a 12-hour fast, and all biochemical measurements were performed at the Endocrinology Laboratory of the INCICH using standardized procedures as previously described [39-41].

Computed axial tomography study

Computed tomography of the chest and abdomen were performed using a 64-channel multi-detector helical computed tomography system (Somatom Sensation, Siemens) and interpreted by experienced radiologists. Scans were read to assess and quantify the following: 1) CAC score using the Agatston method [42]; 2) TAF, subcutaneous and visceral abdominal fat areas (SAF and VAF) as described by Kvist [43]; and 3) hepatic to splenic attenuation ratio as described by Longo et al [44]. SA was defined as the presence CAC score>0. All pCAD cases and healthy controls underwent computed tomography. Of the 1500 apparently healthy controls, 393 subjects had a CAC score above cero, therefore they were not considered for the present analysis and were thus considered as individuals with SA. The final control group included 1107 individuals (CAC scores = 0).

Definition of risk factors

Metabolic and cardiovascular risk factors were evaluated in both pCAD cases and controls and defined as previously described [39-41].

Genetic analysis and functional prediction

Functional prediction of *IL-27p28* single nucleotide polymorphisms (SNPs) was sought using bioinformatics tools including FastSNP, SplicePort: An Interactive Splice Site Analysis Tool (http://spliceport.cbcb.umd.edu/SplicingAnalyser.html), SNPs3D (http://www.snps3d.org/), PESX: Putative Exonic Splicing enhancers/Silencers (http://cubweb.biology.columbia.edu/pesx/), and ESEfinder release 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi).

Four IL-*27p28* gene polymorphisms with possible functional consequences and/or minor allele frequencies > 5% were selected for analysis: rs17855750 and rs181206 introduce binding sites for transcriptional factors SF2/ASF2; rs40837 modifies binding sites for miRNAs. rs26528, although not predicted as functional, was informative and in high linkage disequilibrium with rs40837 (r^2=0.956) and was thus included in the study. Genomic DNA was extracted from peripheral blood using standard methods. All SNPs were genotyped using TaqMan assays on a real-time PCR Prism 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and analyzed by the allelic exclusion program. Samples previously sequenced of the different genotypes of the polymorphisms studied were included as positive controls.

In order to gain insight into the possible functional implications of the 3'UTR rs40837 polymorphism, the TargetScan (http://www.targetscan.org), Diana-MicroT3.0 (http://diana.cslab.ece.ntua.gr) and miRanda (http://www.microrna.org) software's were used, revealing that this SNP could be a target for miR-379-5p and miR-1225-5p.

Estimation of ancestry

Because the Mexican-Mestizo population is admixed, in order to assess the possible influence of population stratification, a panel of 265 ancestry informative markers distinguishing mainly Amerindian, European and African ancestry were selected [45] and genotyped on Illumina BeadStation using the GoldenGate assay. Duplicate control samples were genotyped on each chip, which also served as internal controls for quality of clustering and reproducibility. The primary analysis of the genotyping data with the Illumina GenomeStudio software v.2011.1 was followed by visual inspection and assessment of data quality and clustering. Genotyping accuracy was also assessed by genotype clustering using the Illumina GeneTrain score, which is a measure of the clustering confidence of individual SNP alleles. Global Caucasian, Amerindian and African ancestry were determined using the ADMIXTURE software. Mean global ancestry was not significantly different between cases and controls (55.8% vs 54.0 % Amerindian ancestry, 34.3% vs 35.8% Caucasian and 9.8% vs 10.1% African mean ancestry for cases and controls respectively, P>0.05), strongly suggesting that population stratification was not a bias or confounding factor in this study.

Measurement of IL-27 plasma levels

IL-27 plasma concentrations were measured in a carefully selected subsample of 450 pCAD cases and 450 healthy controls (without obesity and hsCRP < 3 mg/L), using a Bioplex system (Bio-Rad, Contra Costa County, State of California, USA) according to manufacturer's instructions.

Reporter constructs, transfection, and luciferase assays

The 266 bp human *IL27* 3´ UTR containing the rs40837 *A* or *G* allele was amplified with the forward primer 5´-GCGCACGCGTCCCCCACCCTTTAGAACTTT-3´ and the reverse primer 5´-GCGCAAGCTTTGGATGAGAGTGCTTTATTGG-3´ from a homozygous human genomic DNA sample. PCR products were separated on agarose gels, purified and cloned into pMIR-REPORT plasmids (Applied Biosystems, Foster City, CA, USA) with MluI and HindIII digestion (Figure 1A and 1B). HEK293 cells were grown in Dulbecco's modify Eagle's medium (DMEM) (Invitrogen™) supplemented with 10% fetal bovine serum and 1% antibiotics-antimycotics (Invitrogen™) at 37°C with 5% CO_2. A total of 100,000 cells were plated into each well of 12-well plates in Opti-MEM® serum free medium (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA). Forty-eight hours after plating, cells were co-transfected using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Each co-transfection reaction contained 500 ng of pMIR-REPORT (rs40837) *A* or *G* allele vector plus 100 ng pRL/CMV *Renilla reniformis* luciferase vector plasmids that served as a transfection internal control. Twenty-four hours after co-transfection of the plasmids, 75 nM negative control (scrambled sequence) or miR-379-5p RNA (Dharmacon GE Life Sciences) were transfected with siPORT amine transfection agent (Applied Biosystems, Foster City, CA, USA). Forty-eight hours after miRNA transfection, both *firefly* and *Renilla* luciferase activities were quantified by a dual-luciferase reporter assay system (Promega, Madison, WI). The relative luciferase activity was calculated according to the manufacturer's instructions in a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA).

Statistical analysis

Categorical variables were compared between groups using the Chi square test, continuous variables were compared with Student's t test or Mann Whitney's U test for parametric and non-parametric variables, respectively. Allele and genotype frequencies were estimated by direct counting. Hardy-Weinberg's equilibrium was tested using the Chi square test. For all the studied variants, statistical power to detect association with pCAD was greater than 90% as estimated with QUANTO software [http:hydra.usc.edu/GxE/]. Multivariate logistic regression analysis was used to analyze associations with pCAD under different inheritance models: additive (major allele homozygotes vs. heterozygotes vs. minor allele homozygotes), co-dominant (major allele homozygotes vs. heterozygotes and major allele homozygotes vs. minor allele homozygotes), dominant (major allele homozygotes vs. heterozygotes + minor allele homozygotes), over-dominant (heterozygotes vs. major allele homozygotes + minor allele homozygotes), and recessive (major allele homozygotes + heterozygotes vs. minor allele homozygotes). The models were adjusted for age, gender, BMI, smoking habit, TAF, HOMA-IR, AST, adiponectin and uric acid levels as appropriate. Logistic regression analyses were performed to assess associations of *IL-27* SNPs with metabolic parameters and cardiovascular risk factors, under different inheritance models and adjusting for age, gender and BMI, as appropriate. Linkage disequilibrium and haplotype analysis were performed with Haploview software (version 4.1, Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA). $P < 0.05$ values were considered statistically significant. All analyses were performed using SPSS software v15.0 (SPSS Chicago, IL).

Abbreviations

ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMI: body mass index, CAC: coronary artery calcification, CAD: coronary artery disease, CI: confidence interval, CVD: cardiovascular disease, hsCRP: high sensitivity C reactive protein, HOMA-IR: homeostasis model assessment of insulin resistance, IL-27: interleukin 27, LDL-C: low density lipoprotein-cholesterol, MAF: minor allele frequency, OR= odds ratio, oxLDL: oxidized LDL, pCAD: premature coronary artery disease, SA: subclinical atherosclerosis, SAF: subcutaneous abdominal fat, SNP: single nucleotide polymorphism, VAF: visceral abdominal fat, TAF: total abdominal fat, TC: total cholesterol, T2DM: type 2 diabetes mellitus.

Author contributions

RPS and GVA designed the study, were responsible of the data analysis and extraction, and wrote the manuscript with final comment and approval by all the authors. NPH, JMRP, RMCV, BRR, LL and GL were responsible for the acquisition, analysis and interpretation of data and advice regarding study design. CFD, TVM

and CPR have been involved in the critical revision of the manuscript for important intellectual content. All authors were involved in the drafting of the manuscript. All authors read and approved the final manuscript version to be published.

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CONFLICTS OF INTEREST

No conflicts to disclose.

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Research Article

Interleukin 35 Polymorphisms Are Associated with Decreased Risk of Premature Coronary Artery Disease, Metabolic Parameters, and IL-35 Levels: The Genetics of Atherosclerotic Disease (GEA) Study

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Interleukin 35 (IL-35) is a heterodimeric cytokine involved in the development of atherosclerosis. The aim of the present study was to establish if the polymorphisms of *IL-12A* and *EBI3* genes that encode the IL-35 subunits are associated with the development of premature coronary artery disease (CAD) in Mexican individuals. The *IL-12A* and *EBI3* polymorphisms were determined in 1162 patients with premature CAD and 873 controls. Under different models, the *EBI3* rs428253 (OR = 0.831, P_{add} = 0.036; OR = 0.614, P_{rec} = 0.033; OR = 0.591, P_{cod2} = 0.027) and *IL-12A* rs2243115 (OR = 0.674, P_{add} = 0.010; OR = 0.676, P_{dom} = 0.014; OR = 0.698, P_{het} = 0.027; OR = 0.694, P_{cod1} = 0.024) polymorphisms were associated with decreased risk of developing premature CAD. Some polymorphisms were associated with clinical and metabolic parameters. Significant different levels of IL-35 were observed in *EBI3* rs4740 and rs4905 genotypes only in the group of healthy controls. In summary, our study suggests that the *EBI3* and *IL-12A* polymorphisms play an important role in decreasing the risk of developing premature CAD; it also demonstrates the relationship of the *EBI3* rs4740 and rs4905 genotypes with IL-35 levels in healthy individuals.

1. Introduction

Atherosclerosis is a progressive and multifactorial disease influenced by genetic and environmental factors. A major consequence of the atherosclerosis is the coronary artery disease (CAD). It is well known that inflammation plays an important role in the pathogenesis of atherosclerosis and its complications [1]. The inflammatory phenomenon begins when circulating low density lipoprotein (LDL) particles present in the subendothelial space are oxidized, acquiring proinflammatory properties [2]. Depositions of circulating monocytes/macrophages exacerbate the inflammatory response, because the arterial proteoglycans retain and modify the lipoproteins, increasing their phagocytosis into macrophages. In addition, cell recruitment, production of adhesion molecules, chemokines, and cytokines all cause increased atheroma volume [3]. Aside from the classic cytokines known to be involved in the inflammatory process, a new cytokine, interleukin 35, has recently been described, which also plays a significant role in this phenomenon [4].

Interleukin- (IL-) 35 is a heterodimeric cytokine composed of the Epstein-Barr virus-induced 3 (EBI3) and p35 subunits; it belongs to the IL-6/IL-12 cytokine family that includes IL-12, IL-23, IL-27, and IL-35 molecules [5]. Unlike TGFβ, but similar to IL-10 and IL-27, IL-35 is minimally expressed in human tissues and is mainly induced in inflammatory conditions [4]. Unlike the other members of the IL-12 family, IL-35 is predominantly secreted by regulatory T cells (Treg). As a matter of fact, it has been shown that this cytokine represses T-cell proliferation and function in several in vitro and in vivo disease models [6–8]. Some studies have reported that IL-35 inhibits several inflammatory disorders, such as inflammatory bowel disease [9], autoimmune encephalomyelitis [10], autoimmune diabetes [11], and collagen II-induced arthritis [12]. On the other hand, decreased levels of IL-35 have been reported in patients with acute coronary syndrome (unstable angina pectoris and acute myocardial infarction) compared with a chest pain syndrome group [13]. This finding and the fact that IL-35 is strongly expressed in atherosclerotic plaque [14] suggest that this cytokine could be involved in the development of atherosclerosis. In an animal model, Wang et al. [15] have recently demonstrated the role of IL-35 in the development of atherosclerosis. Apolipoprotein E-deficient (apoE$^{-/-}$) mice with an established atherosclerotic lesion displayed a lower level of IL-35 compared to the age-matched wild type C57BL/6 mice without plaque. On the other hand, the expression of the IL-35 increased significantly in apoE$^{-/-}$ mice with attenuated plaque.

The *IL-12A* gene encodes the p35 subunit of IL-35; it is located on chromosome 3q25.33 and consists of seven exons. Several polymorphisms have been described in the *IL-12A* gene and some of them have been associated with susceptibility to Graves' and Alzheimer's disease [16, 17]. The β subunit (EBI3) of IL-35 is encoded by *EBI3* gene located on chromosome 19q13.3 and contains 5 exons. Zhang et al. reported that the *EBI3* rs428253 polymorphism was associated with decreased risk of development of chronic rhinosinusitis and allergic rhinitis [18, 19]. Currently, no studies have examined the role of the polymorphisms present in the *IL-12A* and *EBI3* genes regarding the susceptibility or protection to the development of CAD. Thus, the aim of the present study was to establish the effect of these polymorphisms in the genetic susceptibility to development of premature CAD in Mexican individuals. Based on the results obtained with a functional prediction analysis, we decided to study four polymorphisms from the *IL-12A* gene (rs2243115, rs2243123, rs583911, and rs568408) and three from the *EBI3* gene (rs428253, rs4740, and rs4905) with possible functional consequences and/or with minor allele frequency > 5%. The *IL-12A* rs2243115 polymorphism produces binding sites for the transcription factors AP2, LRH1, and SF1, whereas the *IL-12A* rs568408 polymorphism produces binding sites for microRNAs. Further, the *EBI3* rs428253 produces a binding site for LEF1 factor and rs4740 for SR proteins. In spite of the fact that the rs4905 (*EBI3* gene), rs2243123, and rs583911 (*IL-12A* gene) polymorphisms were not functional, they were informative (minor allele frequency > 5%) and were therefore included in the study.

2. Materials and Methods

2.1. Subjects. The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of the Instituto Nacional de Cardiología Ignacio Chávez (INCICH). All participants provided written informed consent. The study included 1162 patients with premature CAD and 873 healthy controls belonging to the Genetics of Atherosclerotic Disease (GEA) Mexican Study. Premature CAD was defined as history of myocardial infarction, angioplasty, revascularization surgery, or coronary stenosis > 50% on angiography, diagnosed before age of 55 in men and before age of 65 in women. Controls were apparently healthy asymptomatic individuals without family history of premature CAD, recruited from blood bank donors and through brochures posted in Social Service centers. Chest and abdomen computed tomographies were performed using a 64-channel multidetector helical computed tomography system (Somatom Sensation, Siemens) and interpreted by experienced radiologists. Scans were read to assess and quantify the following: (1) coronary artery calcification (CAC) score using the Agatston method [20] and (2) total adipose tissue (TAT) and subcutaneous and visceral adipose tissue areas (SAT and VAT) as described by Kvist et al. [21]. For the present study, the control group only included individuals with CAC = 0, who were nondiabetic, and with normal glucose levels ($n = 873$). In the whole sample, the demographic, clinical, anthropometric, and biochemical parameters and cardiovascular risk factors were evaluated and defined as previously described [22–24]. Briefly, hypercholesterolemia was defined as total cholesterol (TC) levels ≥ 200 mg/dL. Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or the use of oral antihypertensive therapy. Type 2 diabetes mellitus (T2DM) was defined with a fasting glucose ≥ 126 mg/dL and was also considered when participants reported glucose-lowering treatment or a physician diagnosis of T2DM. Obesity was defined as body mass index (BMI) ≥ 30 kg/m^2. Hypoalphalipoproteinemia, hypertriglyceridemia, and metabolic syndrome (MS) were defined using the criteria from the American Heart Association, National Heart, Lung, and Blood Institute Scientific Statement [25], except for central obesity that was considered when waist circumference was 90 cm in men and 80 cm in women [26]. Hyperuricemia was considered with a serum uric acid > 6.0 mg/dL and >7.0 mg/dL for women and men, respectively [27]. Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR). The presence of insulin resistance was considered when the HOMA-IR values were ≥75th percentile (3.66 in women and 3.38 in men). Hyperinsulinemia was defined when insulin concentration was ≥75th percentile (16.97 μIU/mL in women and 15.20 μIU/mL in men). Hypoadiponectinemia was defined when adiponectin concentration was ≤25th percentile (8.67 μg/mL in women and 5.30 μg/mL in men). Increased VAT was defined as VAT ≥ 75th percentile (122.0 cm^2 in women and 151.5 cm^2 in men) and increased SAT as SAT ≥ 75th percentile (335.5 cm^2 in women and 221.7 cm^2 in men). Elevated alanine aminotransferase (ALT) was defined as ALT activity ≥ 75th percentile

(21.0 IU/L in women and 24.5 IU/L in men). Elevated aspartate aminotransferase (AST) was defined as AST activity ≥ 75th percentile (25 IU/L in women and 28 IU/L in men) and elevated gamma glutamyltransferase (GGT) was defined as GGT ≥ 75th percentile (21.0 IU/L in women and 27.5 IU/L in men). These cutoff points were obtained from a GEA study sample of 131 men and 185 women without obesity and with normal values of blood pressure, fasting glucose, and lipids.

All GEA participants are unrelated and of self-reported Mexican-Mestizo ancestry (three generations). In order to establish the ethnical characteristics of the studied groups, we analyzed 265 ancestry informative markers (AIMs). Using the ADMIXTURE software, the Caucasian, Amerindian, and African backgrounds were determined. Similar background in premature CAD patients and healthy controls was found ($P > 0.05$). Patients showed 55.8% of Amerindian ancestry, 34.3% of Caucasian ancestry, and 9.8% of African ancestry, whereas controls showed 54.0% of Amerindian ancestry, 35.8% of Caucasian ancestry, and 10.1% of African ancestry.

2.2. IL-35 Levels Determination. Considering that obesity is frequently associated with a chronic low grade inflammatory process, which could modify the cytokine levels, plasma concentration of IL-35 was determined in a subsample of nonobese subjects with normal values (<3 mg/L) of high sensitivity C reactive protein (hsCRP) (451 premature CAD patients and 458 healthy controls) using a Bioplex system (Bio-Rad, Contra Costa County, State of California, USA) according to manufacturer's instructions.

2.3. Genetic Analysis. The 5′ exonuclease TaqMan genotyping assays were used to determine the *IL-12A* (rs2243115, rs568408, rs2243123, and rs583911) and *EBI3* (rs428253, rs4740, and rs4905) polymorphisms. The determinations were made on an ABI Prism 7900HT Fast Real-Time PCR system, according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Samples previously sequenced of the different genotypes of the polymorphisms studied were included as positive controls.

2.4. Functional Prediction Analysis. In order to predict the potential effect of the *IL-12A* and *EBI3* polymorphisms, we used the following bioinformatics tools: FastSNP [28], SNP Function Prediction (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html), Human-transcriptome Database for Alternative Splicing (http://www.h-invitational.jp/h-dbas/), Splice Port: An Interactive Splice Site Analysis Tool (http://spliceport.cbcb.umd.edu/SplicingAnalyser.html), ESE finder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi), HSF (http://www.umd.be/HSF/), and SNPs3D (http://www.snps3d.org/).

2.5. Statistical Analysis. The analysis was made using the SPSS version 15.0 statistical package (SPSS, Chicago, Il). Means, medians, interquartile ranges, and frequencies were calculated as the case may be. Continuous and categorical variables were analyzed by t-Student's test, Mann–Whitney U test, Kruskal-Wallis, and Chi square or Fisher test as appropriate. The polymorphism associations with premature CAD and other variables were analyzed using logistic regression under the following inheritance models: additive (major allele homozygotes versus heterozygotes versus minor allele homozygotes), codominant 1 (major allele homozygotes versus heterozygotes), codominant 2 (major allele homozygotes versus minor allele homozygotes), dominant (major allele homozygotes versus heterozygotes + minor allele homozygotes), heterozygous (heterozygotes versus major allele homozygotes + minor allele homozygotes), and recessive (major allele homozygotes + heterozygotes versus minor allele homozygotes). For the *EBI3* polymorphisms all the inheritance models were adjusted for age, gender, BMI, current smoking status, ALT, AST, and uric acid. For the *IL12A* polymorphisms, models were adjusted for age, gender, BMI, and current smoking status. Genotype frequencies did not deviate from Hardy-Weinberg equilibrium in any case (HWE, $P > 0.05$).

3. Results

Tables 1 and 2 exhibit the clinical and demographic characteristics of the studied individuals. As we can see, a number of differences were observed between premature CAD patients and healthy controls. As shown in Table 1, the systolic and diastolic blood pressure are both low and within normal limits; however, some of our patients have hypertension (Table 2). The reason for this discrepancy is that some patients with hypertension are under treatment and in consequence their pressure levels were within normal range. As expected, hypercholesterolemia [TC > 200 mg/dL or low density lipoprotein cholesterol (LDL-C) ≥ 130 mg/dL], inflammation [defined as hsCRP levels ≥ 3 mg/L], and current smoking habit were significantly more frequent in controls than in premature CAD patients most likely due to the effect of statin treatment and a life style changes advice after the cardiovascular event.

3.1. Association of the EBI3 and IL-12A Polymorphisms with Premature CAD. The distribution of the *EBI3* (rs4740 and rs4905) and *IL-12A* (rs2243123, rs568408, and rs583911) polymorphisms was similar in premature CAD and healthy controls. However, under additive, recessive, and codominant 2 models, the *EBI3* rs428253 polymorphism was associated with decreased risk of developing premature CAD ($P_{add} = 0.036$, $P_{rec} = 0.033$, and $P_{cod2} = 0.027$). The models were adjusted for age, gender, BMI, current smoking status, ALT, AST, and uric acid. In the same way, the *IL-12A* rs2243115 ($P_{add} = 0.010$, $P_{dom} = 0.014$, $P_{het} = 0.027$, and $P_{cod1} = 0.024$) polymorphism was associated with diminished risk of developing premature CAD (Table 3) under different models adjusted for age, gender, BMI, and current smoking status.

3.2. Association of the EBI3 and IL-12A Polymorphisms with Metabolic Parameters. In premature CAD patients under different models, the *EBI3* rs428253 polymorphism was associated with high levels of ALT > p75 ($P_{add} = 0.006$, $P_{dom} = 0.004$, $P_{het} = 0.010$, and $P_{cod1} = 0.006$) and AST > p75 ($P_{cod2} = 0.042$) and with decreased risk of developing T2DM ($P_{dom} = 0.033$, $P_{het} = 0.022$, and $P_{cod1} = 0.022$). The *EBI3* rs4905 polymorphism was associated with high levels of ALT > p75

Table 1: Clinical and metabolic characteristics of the studied groups.

	Control (n = 873)	Premature CAD (n = 1162)	P
Age (years)	51 ± 9	54 ± 8	<0.001
Gender (% male)	40.7	81.1	<0.001
Body mass index (kg/m^2)	27.3 [24.9–30.2]	28.3 [25.9–31.1]	<0.001
Waist circumferences (cm)	92 ± 11	98 ± 10	<0.001
Systolic blood pressure (mmHg)	111 [103–121]	116 [106–127]	<0.001
Diastolic blood pressure (mmHg)	70 [65–76]	71 [66–78]	0.001
Total adipose tissue (cm^2)	416 [330–514]	425 [340–523]	0.147
Visceral adipose tissue (cm^2)	130 [98–172]	168 [129–215]	<0.001
Subcutaneous adipose tissue (cm^2)	280 [209–356]	245 [193–313]	<0.001
Total cholesterol (mg/dL)	190 [167–210]	160 [132–193]	<0.001
High density lipoprotein cholesterol (mg/dL)	46 [37–56]	37 [32–44]	<0.001
Low density lipoprotein cholesterol (mg/dL)	116 [95–133]	91 [68–116]	<0.001
Triglycerides (mg/dL)	138 [102–190]	162 [119–219]	<0.001
Non-HDL-cholesterol (mg/dL)	141 [119–162]	120 [93–151]	<0.001
Alanine aminotransferase (IU/L)	23 [17–32]	26 [19–36]	<0.001
Aspartate aminotransferase (IU/L)	24 [20–30]	26 [22–31]	<0.001
Glucose (mg/dL)	87 [82–92]	95 [87–117]	<0.001
Insulin (μIU/mL)	16 [12–21]	20 [15–28]	<0.001
Homeostasis model assessment of insulin resistance	3.3 [2.4–4.7]	5.1 [3.5–7.7]	<0.001
High sensitivity C reactive protein (mg/L)	1.4 [0.7–2.9]	1.2 [0.6–2.6]	0.005
Adiponectin (μg/mL)	8.5 [5.3–13.6]	5.2 [3.2–8,1]	<0.001
Uric acid (mg/dL)	5.3 [4.3–6.3]	6.5 [5.4–7.4]	<0.001

Data are shown as mean ± standard deviation, median [interquartile range], or percentage. Comparisons were made using Student's t-test or Mann–Whitney U test, as appropriate, for continuous variables, and by Chi square analysis for categorical variables. CAD: coronary artery disease.

(P_{add} = 0.023, P_{dom} = 0.024, and P_{cod1} = 0.045). Additionally, the *IL-12A* rs2243123 polymorphism was associated with increased risk of T2DM (P_{rec} = 0.021, P_{cod2} = 0.028), while the rs2243115 polymorphism correlated with reduced risk of metabolic syndrome (P_{add} = 0.015, P_{dom} = 0.017, P_{het} = 0.022, and P_{cod1} = 0.021). The rs583911 polymorphism was linked

Table 2: Cardiovascular risk factors prevalence in the study population.

	Control (n = 873)	Premature CAD (n = 1162)	*P
Total cholesterol > 200 mg/dL (%)	36.3	20.3	<0.001
LDL-cholesterol ≥ 130 mg/dL (%)	29.2	16.1	<0.001
Hypoalphalipoproteinemia (%)	49.3	67.2	<0.001
Hypertriglyceridemia (%)	42.8	56.2	<0.001
Non-HDL-cholesterol > 160 mg/dL (%)	26.0	19.5	<0.001
Obesity (%)	26.1	35.0	<0.001
Abdominal obesity (%)	77.6	83.6	<0.001
Type 2 diabetes mellitus (%)	0	35.4	<0.001
Hyperinsulinemia (%)	45.8	71.4	<0.001
Insulin resistance (%)	44.2	77.0	<0.001
Metabolic syndrome (%)	29.7	71.9	<0.001
Hypertension (%)	5.7	68.1	<0.001
High visceral adipose tissue (%)	49.8	64.6	<0.001
Current smoking status (%)	23.5	11.6	<0.001
Hypoadiponectinemia (%)	40.0	56.5	<0.001
High sensitivity C reactive protein ≥ 3 mg/L (%)	23.6	21.3	0.114
Hyperuricemia (%)	16.8	35.9	<0.001

Data is shown as percentage. *Comparisons were made using Chi square analysis. CAD: coronary artery disease, LDL: low density lipoprotein, and HDL: high density lipoprotein.

with diminished levels of inflammation (hsCRP ≥ 3 mg/L, P_{rec} = 0.017), high levels of AST > p75 (P_{add} = 0.013, P_{dom} = 0.046, P_{rec} = 0.035, and P_{cod2} = 0.013), and high levels of GGT > p75 (P_{rec} = 0.042) (Table 4).

In healthy controls, the *EBI3* rs428253 polymorphism was associated with the presence of hyperuricemia (P_{het} = 0.024, P_{cod1} = 0.032), the *EBI3* rs4740 was associated with decreased risk of central obesity (P_{het} = 0.035, P_{cod2} = 0.038) and with increased risk of high levels of AST > p75 (P_{add} = 0.046, P_{dom} = 0.014, P_{het} = 0.015, and P_{cod1} = 0.011), and the *EBI3* rs4905 was linked with reduced risk of central obesity (P_{rec} = 0.040, P_{cod2} = 0.046) and increased risk of high levels of AST > p75 (P_{dom} = 0.020, P_{het} = 0.020, and P_{cod1} = 0.016). In addition, we found that the *IL-12A* rs568408 correlated with decreased risk of metabolic syndrome (P_{add} = 0.042) and the *IL-12A* rs583911 was associated with high levels of SAT (P_{het} = 0.004, P_{cod1} = 0.017) (Table 5).

3.3. Association of the EBI3 and IL-12A Genotypes with IL-35 Levels. The levels of IL-35 were determined in 451 premature CAD patients and in 458 healthy controls. Individuals with extreme outliers values were not included in the analysis (4 patients and 11 controls). Figure 1 shows that premature CAD patients have significantly higher IL-35 levels than control

Table 3: Association between *EBI3* and *IL-12A* gene polymorphisms and premature coronary artery disease.

Polymorphism	Genotype frequency n (%)			MAF	Model	OR [95% CI]	P
(i) *EBI3*[*]							
rs428253		*G>C*					
	GG	*GC*	*CC*				
Control (n = 873)	536 (0.614)	277 (0.317)	60 (0.069)	0.227	*Additive*	*0.831 [0.699–0.988]*	*0.036*
					Dominant	0.842 [0.681–1.042]	0.115
					Recessive	*0.614 [0.392–0.963]*	*0.033*
pCAD (n = 1162)	740 (0.637)	371 (0.319)	51 (0.044)	0.204	Heterozygote	0.935 [0.750–1.167]	0.553
					Codominant 1	0.895 [0.715–1.120]	0.334
					Codominant 2	*0.591 [0.375–0.933]*	*0.027*
(ii) *IL-12A*[**]							
rs2243115		*T>G*					
	TT	*TG*	*GG*				
Control (n = 873)	746 (0.855)	120 (0.137)	7 (0.008)	0.077	*Additive*	*0.674 [0.499–0.909]*	*0.010*
					Dominant	*0.676 [0.494–0.925]*	*0.014*
					Recessive	0.294 [0.048–1.785]	0.183
pCAD (n = 1162)	1048 (0.902)	112 (0.096)	2 (0.002)	0.050	*Heterozygote*	*0.698 [0.508–0.956]*	*0.027*
					Codominant 1	*0.694 [0.505–0.954]*	*0.024*
					Codominant 2	0.282 [0.046–1.712]	0.169

[*]Models were adjusted for age, gender, body mass index, current smoking status, alanine aminotransferase, aspartate aminotransferase, and uric acid. [**]Models were adjusted for age, gender, body mass index, and current smoking status. Italic numbers indicate significant associations. The control group subjects were normoglycaemic nondiabetic. MAF: minor allele frequency; pCAD: premature coronary artery disease.
Only the significant associated polymorphisms are shown.

subjects (3.2 [1.6–6.7] pg/mL versus 2.7 [0.8–5.2] pg/mL, respectively, $P = 0.001$, Figure 1). Additionally, we found that, in the healthy control group, significant different levels of IL-35 were observed in *EBI3* rs4740 (*AA* = 3.40 [0.88–7.90] pg/mL, *GA* = 3.00 [1.63–5.23] pg/mL, and *GG* = 2.52 [0.88–4.50] pg/mL; $P = 0.020$) and rs4905 (*GG* = 3.78 [0.88–7.90] pg/mL, *AG* = 3.00 [1.63–5.23] pg/mL, and *AA* = 2.52 [0.88–4.40] pg/mL; $P = 0.017$) genotypes (Table 6).

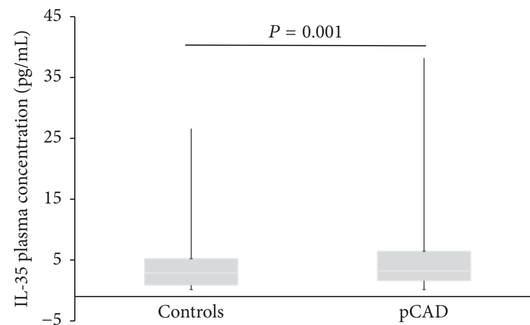

Figure 1: Interleukin 35 plasma concentration in 477 control subjects and 447 premature CAD (pCAD) patients. Comparisons were made using Mann–Whitney U test. Premature CAD patients have significantly higher IL-35 levels than control subjects (3.2 [1.6–6.7] pg/mL versus 2.7 [0.8–5.2] pg/mL, respectively, $P = 0.001$).

4. Discussion

Interleukin-35 is a heterodimeric cytokine that belongs to the IL-6/IL-12 family and is composed of two chains (p35 and EBI3): one encoded by the *IL-12A* (p35) gene and the other by the *EBI3* gene. This cytokine has been associated with the development of several inflammatory diseases. In fact, a recent study on this molecule points out its probable protective role against atherosclerosis [15]. The role of the IL-35 in the inflammatory diseases suggests that the genes that encode its different subunits could be candidates in the study of atherosclerosis and its complications (e.g., CAD). To the best of our knowledge, this is the first study that evaluates the role of *IL-12A* and *EBI3* polymorphisms in premature CAD. In this report, we found that two polymorphisms, namely, *EBI3* rs428253 and *IL-12A* rs2243115, were associated with reduced risk of developing premature CAD. These polymorphisms were also associated with decreased risk of T2DM (*EBI3* rs428253) and metabolic syndrome (*IL-12A* rs2243115) in premature CAD patients. However, in healthy controls only the *EBI3* rs428253 correlates with increased risk of hyperuricemia. The polymorphisms that were not linked with risk of premature CAD were associated with other clinical and metabolic parameters. In premature CAD patients, the *EBI3* rs4905 was related to high levels of ALT, the *IL-12A* rs2243123 was associated with increased risk of T2DM, and *IL-12A* rs583911 correlated with inflammation, high levels of AST, and GGT. In healthy controls, the *EBI3* rs4905 and *EBI3* rs4740 were associated with low risk of central obesity and increased risk of high levels of AST, whereas the *IL-12A* rs583911 correlated with high risk of increased SAT and *IL-12A* rs568408 with diminished risk of metabolic syndrome.

Table 4: Association between *EBI3* and *IL-12A* gene polymorphisms and metabolic abnormalities in premature coronary artery disease patients.

Polymorphism	Genotype frequency *n* (%)			MAF	Model	OR [95% CI]	*P*
(i) *EBI3*							
rs428253		*G*>*C*					
	GG	*GC*	*CC*				
Alanine aminotransferase > p75					Additive	1.330 [1.083–1.632]	0.006
No ($n = 590$)	401 (0.679)	167 (0.283)	22 (0.038)	0.179	Dominant	1.429 [1.121–1.821]	0.004
Si ($n = 572$)	340 (0.594)	204 (0.357)	28 (0.049)	0.227	Heterozygote	1.392 [1.084–1.787]	0.010
					Codominant 1	1.425 [1.107–1.835]	0.006
Aspartate aminotransferase > p75							
No ($n = 752$)	489 (0.650)	238 (0.316)	25 (0.034)	0.191	Codominant 2	1.823 [1.022–3.250]	0.042
Si ($n = 410$)	251 (0.613)	135 (0.328)	24 (0.059)	0.223			
Type 2 diabetes mellitus					Dominant	0.753 [0.580–0.978]	0.033
No ($n = 750$)	458 (0.611)	259 (0.345)	33 (0.044)	0.217	Heterozygote	0.727 [0.554–0.954]	0.022
Si ($n = 412$)	282 (0.684)	112 (0.272)	18 (0.044)	0.180	Codominant 1	0.726 [0.522–0.955]	0.022
rs4905		*A*>*G*					
	AA	*AG*	*GG*				
Alanine aminotransferase > p75					Additive	1.241 [1.031–1.495]	0.023
No ($n = 752$)	400 (0.532)	302 (0.402)	50 (0.066)	0.267	Dominant	1.309 [1.037–1.653]	0.024
Si ($n = 410$)	212 (0.517)	162 (0.394)	36 (0.089)	0.285	Codominant 1	1.284 [1.006–1.640]	0.045
(ii) *IL-12A*							
rs2243123		*T*>*C*					
	TT	*TC*	*CC*				
Type 2 diabetes mellitus							
No ($n = 750$)	290 (0.387)	358 (0.477)	102 (0.137)	0.375	Recessive	1.148 [1.061–2.063]	0.021
Si ($n = 412$)	150 (0.365)	182 (0.441)	80 (0.194)	0.415	Codominant 2	1.511 [1.048–2.178]	0.028
rs2243115		*T*>*G*					
	TT	*TG*	*GG*				
Metabolic syndrome					Additive	0.591 [0.386–0.905]	0.015
No ($n = 327$)	286 (0.875)	40 (0.122)	1 (0.003)	0.064	Dominant	0.590 [0.381–0.912]	0.017
Si ($n = 835$)	762 (0.913)	72 (0.086)	1 (0.001)	0.044	Heterozygote	0.599 [0.386–0.929]	0.022
					Codominant 1	0.592 [0.385–0.927]	0.021
rs583911		*A*>*G*					
	AA	*AG*	*GG*				
Inflammation							
No ($n = 930$)	255 (0.274)	433 (0.466)	242 (0.260)	0.493	Recessive	0.633 [0.435–0.921]	0.017
Si ($n = 232$)	67 (0.287)	123 (0.532)	42 (0.181)	0.446			
Aspartate aminotransferase > p75					Additive	1.236 [1.046–1.460]	0.013
No ($n = 712$)	211 (0.296)	343 (0.482)	158 (0.222)	0.463	Dominant	1.318 [1.004–1.730]	0.046
Si ($n = 450$)	109 (0.242)	217 (0.483)	124 (0.275)	0.517	Recessive	1.344 [1.021–1.769]	0.035
					Codominant 2	1.529 [1.096–2.133]	0.013
GGT > p75							
No ($n = 625$)	178 (0.285)	309 (0.494)	138 (0.221)	0.468	Recessive	1.329 [1.011–1.748]	0.042
Si ($n = 537$)	141 (0.263)	251 (0.468)	145 (0.269)	0.504			

Table shows the models with significant associations. Models were adjusted for age, gender, and body mass index. MAF: minor allele frequency; GGT: gamma-glutamyl transferase.

Table 5: Association between *EBI3* and *IL-12A* gene polymorphisms and metabolic abnormalities in the control group.

Polymorphism	Genotype frequency *n* (%)			MAF	Model	OR [95% CI]	*P*
(i) *EBI3*							
rs428253		*G>C*					
	GG	*GC*	*CC*				
Hyperuricemia							
No (*n* = 726)	454 (0.625)	220 (0.303)	52 (0.072)	0.223	Heterozygote	1.595 [1.064–2.389]	0.024
Si (*n* = 147)	82 (0.555)	57 (0.390)	8 (0.055)	0.248	Codominant 1	1.567 [1.038–2.365]	0.032
rs4740		*G>A*					
	GG	*GA*	*AA*				
Central obesity							
No (*n* = 196)	99 (0.503)	78 (0.400)	19 (0.097)	0.296	Heterozygote	0.391 [0.163–0.937]	0.035
Si (*n* = 677)	377 (0.557)	251 (0.371)	49 (0.072)	0.258	Codominant 2	0.386 [0.157–0.949]	0.038
AST > p75					Additive	1.250 [1.004–1.557]	0.046
No (*n* = 567)	327 (0.576)	197 (0.348)	43 (0.076)	0.250	Dominant	1.430 [1.076–1.899]	0.014
Si (*n* = 306)	148 (0.485)	133 (0.433)	25 (0.082)	0.299	Heterozygote	1.433 [1.073–1.913]	0.015
					Codominant 1	1.473 [1.093–1.985]	0.011
rs4905		*A>G*					
	AA	*AG*	*GG*				
Central obesity							
No (*n* = 196)	99 (0.503)	78 (0.400)	19 (0.097)	0.296	Recessive	0.404 [0.170–0.960]	0.040
Si (*n* = 677)	374 (0.552)	253 (0.371)	50 (0.074)	0.261	Codominant 2	0.403 [0.165–0.983]	0.046
AST > p75					Dominant	1.399 [1.054–1.858]	0.020
No (*n* = 567)	324 (0.571)	199 (0.352)	44 (0.078)	0.253	Heterozygote	1.410 [1.056–1.882]	0.020
Si (*n* = 306)	148 (0.485)	133 (0.433)	25 (0.082)	0.299	Codominant 1	1.445 [1.072–1.946]	0.016
(ii) *IL-12A*							
rs568408		*G>A*					
	GG	*GA*	*AA*				
Metabolic syndrome							
No (*n* = 614)	542 (0.883)	69 (0.112)	3 (0.005)	0.061	Additive	0.583 [0.347–0.981]	0.042
Si (*n* = 259)	237 (0.915)	21 (0.081)	1 (0.004)	0.044			
rs583911		*A>G*					
	AA	*AG*	*GG*				
SAT > p75							
No (*n* = 483)	129 (0.268)	222 (0.459)	132 (0.273)	0.503	Heterozygote	1.776 [1.203–2.622]	0.004
Si (*n* = 390)	88 (0.226)	220 (0.563)	82 (0.211)	0.492	Codominant 1	1.776 [1.107–2.849]	0.017

Table shows the models with significant associations. Models were adjusted for age, gender, and body mass index. MAF: minor allele frequency, AST: aspartate aminotransferase, and SAT: subcutaneous adipose tissue.

According to the informatics tools, the two polymorphisms, which were associated with decreased risk of developing premature CAD, have a possible functional effect. Specifically, the *EBI3* rs428253 modifies a binding site for the lymphoid enhancer-binding factor 1 (LEF1) that is a decisive transcription factor in the control of the granulopoiesis proliferation, proper lineage commitment, and granulocytic differentiation [29]. Furthermore, the *IL-12A* rs2243115 polymorphism, located in the promoter region, produces binding sites for the transcription factors AP2, LRH1, and SF1. Thus, after considering that the studied polymorphisms could have an effect in the production of IL-35, we analyzed the molecule serum levels in a group of premature CAD patients and healthy controls. Coronary patients showed significantly higher IL-35 levels than control subjects; however, the difference was small. We cannot define whether these differences could have an effect on the development of atherosclerosis. As we know, atherosclerosis is a multifactorial disease and multiple cytokines, both pro- and anti-inflammatory, play a role in the genesis and progression of the inflammatory process. In this analysis, neither *EBI3* rs428253 nor *IL-12A* rs2243115 (the polymorphisms associated with premature CAD with

Table 6: Interleukin 35 plasma concentrations in the study groups according to the *EBI3* and *IL-12A* polymorphisms.

Polymorphism	Genotype	Controls (n = 447)		P^*	pCAD (n = 447)		P
		n	Concentration (pg/mL)		n	Concentration (pg/mL)	
(i) *EBI3*							
rs428253	*GG*	275	2.72 [0.88–4.97]		286	3.16 [1.03–6.51]	
	GC	151	3.00 [1.62–5.23]	0.273	140	3.40 [1.19–7.76]	0.433
	CC	21	1.98 [0.19–5.10]		21	2.20 [1.63–3.78]	
rs4740	*GG*	*250*	*2.52 [0.88–4.50]*		233	3.23 [1.62–7.61]	
	GA	*160*	*3.00 [1.63–5.23]*	*0.020*	185	3.16 [0.95–6.15]	0.311
	AA	*37*	*3.40 [0.88–7.90]*		29	3.16 [1.62–5.23]	
rs4905	*AA*	*248*	*2.52 [0.88–4.40]*		233	3.23 [1.62–7.61]	
	AG	*164*	*3.00 [1.63–5.23]*	*0.017*	186	3.16 [0.95–6.15]	0.338
	GG	*35*	*3.78 [0.88–7.90]*		28	3.08 [1.62–5.23]	
(ii) *IL12A*							
rs2243115	*TT*	389	2.72 [0.88–4.97]		407	3.16 [1.62–6.44]	
	TG + *GG*	58	3.08 [0.83–5.23]	0.376	40	3.56 [0.38–7.95]	0.985
rs568408	*GG*	398	2.81 [0.88–4.97]		396	3.23 [1.62–6.74]	
	GA + *AA*	49	2.32 [0.31–7.17]	0.763	51	2.72 [0.95–6.44]	0.254
rs2243123	*TT*	188	2.72 [0.59–4.97]		183	3.40 [1.03–7.09]	
	TC	198	2.90 [0.88–5.23]	0.702	190	3.00 [1.62–6.31]	0.714
	CC	61	3.00 [0.88–5.23]		74	3.32 [1.47–8.38]	
rs583911	*AA*	113	3.00 [0.88–5.23]		130	3.23 [1.30–8.56]	
	AG	224	2.90 [0.88–5.23]	0.570	194	3.00 [0.95–6.19]	0.215
	GG	110	2.46 [0.88–4.82]		123	3.40 [1.62–7.09]	

Data are shown as median [interquartile range]. Comparisons were made using Mann–Whitney U test or Kruskal-Wallis test as appropriate. Italic numbers indicate significant associations.
pCAD: premature coronary artery disease.

a possible functional effect) showed a correlation with IL-35 serum levels. The fact that the associated polymorphisms with decreased risk of developing premature CAD did not correlate with IL-35 levels could be explained considering that the production of IL-35 and other molecules is a complex mechanism that involves not only changes at DNA level but also epigenetics modifications. Moreover, it is important to considered that in our study the levels of IL-35 were measured in circulation and not at the lesion site. On the other hand, the *EBI3* rs4740 and rs4905 polymorphisms were associated with different levels of IL-35. Furthermore, this association was observed only in the healthy control groups. From these two polymorphisms, only *EBI3* rs4740 was functional according to the informatics tools. Interestingly, this polymorphism produces binding sites for Srp40, and SRp55, which belong to the family of SR proteins that regulate alternative splicing [30].

IL-35 is a heterodimeric cytokine that belongs to the IL-6/IL-12 cytokine family, which includes IL-12, IL-23, IL-27, and IL-35 molecules. These cytokines share subunits that are encoded by *EBI3*, *IL-12A*, *IL-12B*, *IL-23A*, and *IL27p28* genes. Our research group is studying several polymorphisms located in these genes in order to establish its role in the genetic susceptibility to developing premature CAD and cardiovascular risk factors. At the moment, we have analyzed the polymorphisms of the *IL27p28* gene that encode the p28 subunit of the IL-27. This analysis showed that two polymorphisms of this gene (rs26528 and rs40837) were significantly associated with a lower risk of premature CAD. Using the luciferase assay we demonstrate that the rs40837 polymorphism has a functional effect. In this study, we also determined independently the levels of IL-27. None of the studied polymorphisms were associated with IL-27 levels (personal communication).

IL-12A polymorphisms have been associated with the development of several diseases, such as rheumatoid arthritis [31], Alzheimer's disease [17], Graves' disease [16], and asthma [32]. In contrast, *EBI3* polymorphisms have been associated with ulcerative colitis [33], pulmonary tuberculosis [34], chronic rhinosinusitis [19], and allergic rhinitis [18]. In these studies, *IL-12A* and *EBI3* genes were analyzed independently. To the best of our knowledge, no studies so far have reported an analysis, in which both genes have been analyzed in concert for any disease.

As for the limitations, herein, we have only included the study of four polymorphisms of *IL-12A* and three of the *EBI3* gene, which seem to be functional and/or informative based on the analysis of the prediction software results. Since this is the first work that documents the correlation of the *IL-35* polymorphisms with premature CAD, and cardiovascular parameters, further studies in an independent group of patients are mandatory to validate the results. It is important

to note that one strength of our work is that the control group only included individuals without subclinical atherosclerosis (i.e., individuals without coronary artery calcification).

5. Conclusion

In summary, our results indicate that there exists a statistically significant association between the *EBI3* rs428253 and *IL-12A* rs2243115 polymorphisms and a reduced risk of developing premature CAD. Some of the studied polymorphisms were associated with cardiovascular parameters. The *EBI3* rs4740 and *EBI3* rs4905 genotypes were associated with a variation in IL-35 serum levels in healthy controls. To the best of our knowledge, this is the first study that evaluates the role of *IL-12A* and *EBI3* polymorphisms in premature CAD. For this reason, the detected associations are not yet definitive, and replicate studies in independent populations are warranted to confirm these findings.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this article.

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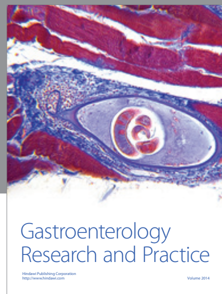
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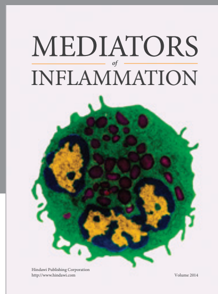
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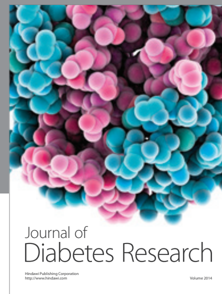
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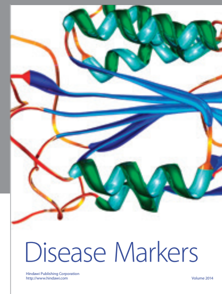
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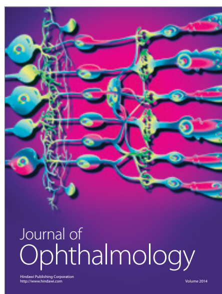
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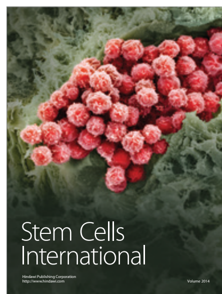
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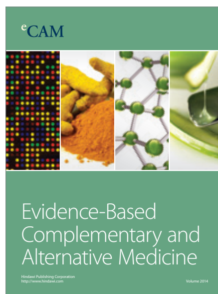
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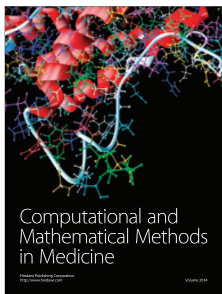
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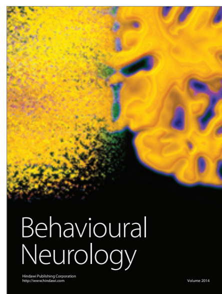
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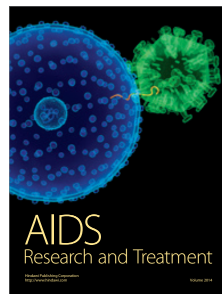
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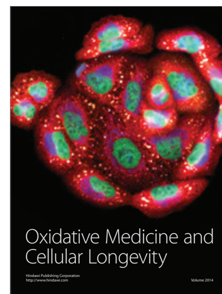
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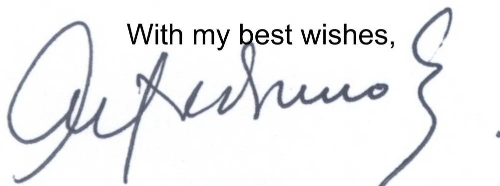

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Innate Immunity in coronary disease.

The role of Interleukin-12 family cytokines in atherosclerosis.

Running title: IL-12 family and atherosclerosis

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CONFLICTS OF INTEREST

No conflicts to disclose.

Abstract

Atherosclerosis is a chronic, progressive and multifactorial disease modulated by genetic and environmental factors. In recent years, the paradigm that explained the atherosclerosis as result of a complex interaction between non-accessible factors to medical intervention and modifiable risk factors have changed. In this paradigm, alterations of lipid metabolism were the pivot concept of atherosclerosis as a chronic degenerative disease. In the last years, an increasing number of observations have shown that the innate and adaptive immune responses to lipoprotein deposition and oxidation in the arterial wall significantly influenced the atherosclerosis. Actually, it is well recognized that the pathogenesis of atherosclerosis and its complications involves the inflammatory process, which includes the participation of several cytokines. Besides the classic cytokines involved in this process, the role of the family of interleukin 12 has been recently shown. This review describes our current understanding of the role of the interleukin 12 family in atherosclerosis considering the participation of the genes that encodes these cytokines in the genetic susceptibility to developing this disease.

Keywords: Atherosclerosis; inflammation; interleukin 12 cytokine family; genes; genetic susceptibility.

Immunology of atherosclerosis

Inflammation is a process that plays an important role in the initiation and progression of atherosclerosis. In this process multiple cell types, including macrophages, T-lymphocytes, endothelial cells, smooth muscle cells and mast cells are involved [1]. It is well known that the innate and adaptive immune systems participate in atherosclerosis from its early stages to plaque erosion [2]. The innate immune response in atherosclerosis is represented by the immune-inflammatory cells, mainly monocytes and macrophages, which respond to the excessive uptake of lipoproteins, while adaptive immune response is represented by antigen-specific T cells [3]. In the first studies, only the presence of macrophages in the atherosclerosis lesion was reported; however, later studies reported the presence of other cells, such as mast cells, myeloid cells, CD4+ and regulatory T cells. CD4+ cells are the most abundant T cells present in atherosclerotic lesions and play an important role in the development and progression of the disease, whereas regulatory T cells have a protective effect against the development of atherosclerosis [4]. Using atherosclerosis-prone models, it has been demonstrated that the development and progression of atherosclerosis is related to an imbalance of anti- and pro-inflammatory cytokines [5,6]. Anti- and pro-inflammatory cytokines produced by vascular and immune cells participate in atherosclerosis development through modulating cellular functions in the arterial wall in a paracrine and autocrine fashion [6]. Cytokines are involved in all stages of atherosclerosis and have a relevant influence on the pathogenesis of this disease. Cytokines participate in the foam cell and fatty streak formation, in development of complex lesions and in the plaque stability and rupture [7]. Inflammatory cells and pro-inflammatory cytokines have been detected in early and severe lesions, as well as in plaque rupture and thrombosis.

Interleukin 12 family

The interleukin-12 family is evolutionarily linked to the IL-6 cytokine superfamily and consists of a single group of α/β heterodimeric cytokines composed of one out of three possible α chains (p19, p28, or p35) and one out of two β chains [p40 or Epstein-Barr virus induced gene 3 (EBI3)] [8] (Figure 1). The α subunit is a four-helix bundle structurally similar to the type I cytokine IL-6, whereas the β chains are composed of two tandem fibronectin type II domains that form a cytokine-binding homology region and a N-terminal immunoglobulin domain homologous to the soluble IL-6 receptor form [9]. Different combinations between the α and β subunits result in six heterodimeric cytokines (Figure 1). The IL-12 family members are key players in both the promotion and suppression of multiple immune responses under physiological, as well as pathological conditions. The IL-12 family members signal through cell surface heterodimer receptors [8]. Each chain binds individually to its corresponding receptor subunit.

Interleukin-12 was independently discovered by both Kobayashi et. al. [10] as a “natural killer-stimulating factor” and Stern et. al. [11] as a “cytotoxic lymphocyte maturation factor”. Interleukin-12 is a disulphide-linked 75 kDa heterodimeric protein composed of p40 and p35 subunits [12] that bind the heterodimeric receptor complex IL-12Rβ1/IL-12Rβ2. Subunit β p40 also binds p19 via a disulfide bridge to form IL-23 [13], which engages the receptor IL-12Rβ1/IL-23R. Interleukin-27 is formed by p28 and EBI3 subunits, which are not covalently linked and signals via a receptor complex WSX-1/gp130 [14]. The binding of p35 and EBI3 subunits forms IL-35; this cytokine has a unique feature of signaling, not shared by other members of the family. It signals via four receptor complexes: IL-12Rβ2/gp130, IL-12Rβ2/IL12Rβ2, gp130/gp130 and IL-12Rβ2/WSX-1 [15,16]. Recently discovered interleukin-39 consists of p19 and EBI3 chains and signals via IL-23R/gp130 [17]. The heterodimer p28/p40, tentatively called IL-Y, has been demonstrated to antagonize the signaling by IL-12 and IL-27 [18]. Signaling via the above-mentioned receptors is mediated by Janus kinase-signal transducer members and activators of transcription (JAK-STAT) family [19]. IL-12 mediates signaling via STAT4 [20], IL-23 via STAT3/STAT4 [21], IL-27 and IL-39 via STAT1/STAT3 [9, 22], IL-35 via STAT1/STAT4 [16] and IL-Y via STAT3 [9] (Figure 1).

The co-expression of the α and β chains is a pre-requisite for secretion of the bioactive cytokine [23]. Expression of the α chain limits the production of each heterodimeric cytokine. Compared with EBI3 and p40, p35 is expressed at lower levels thus limiting the assembly of IL-12 and IL-35. Similarly, the tissue and cell-restricted expression of p19, limits the secretion of IL-23 [13]. The antigen presenting cells, such as dendritic and macrophages, express and secrete IL-12, IL-23, and IL-27. Their expression is induced by the activation of innate receptors. IL-35 is mainly produced by FoxP3+ Treg cells, but can also be produced by γδT cells, CD8+ T cells and placental trophoblast [24]. Additionally, it has been demonstrated that activated B cells secreted IL-39 [17].

IL-12 family cytokines can directly induce the development of T cell subpopulations and alter the fate and function of many cell populations that dictate disease outcome; they thus act as an immunological playmaker, shaping immune responses [8]. The cytokine IL-12 family members exhibit immunoregulatory properties due to their effects on T cell differentiation and function. Two examples of pro-inflammatory cytokines are IL-12 and IL-23; the former is able to induce Th1 cells while

the latter has a major role in the induction of Th17 cells. Further, IL-27 exhibits a dual functional phenotype capable of increased pro- and anti-inflammatory responses [14]. IL-35 is a suppressive cytokine inhibiting the development of Th17 cells, the proliferation of effectors T cell [25], and inhibiting effectors T cells responses [8, 24] in a IL-10 dependent way [26]. Alternatively, IL-39 was shown to induced differentiation and/or expansion of neutrophils [27] (Figure 1).

In early-stage atherosclerosis, the inflammatory response is initiated by lipid accumulation in the vessel wall. The pro-inflammatory response is driven by Th17 and Th1 cells, associated with increased production of IL-6, IL-17, IFN-γ and IgG2a antibodies against modified low-density lipoprotein [28, 29]. Indeed, the IL-12 cytokine family plays a critical role in priming dendritic cell-mediated differentiation of CD4+ naïve T cells.

Interleukin-12

Interleukin-12 is recognized as a master regulator of adaptive type 1 cell-mediated immunity, the critical pathway involved in protection against neoplasia and many viruses. It binds to naïve CD4+ T cells by its IL-12β1/IL-12β2 subunits [30] and promotes the generation of pro-inflammatory Th1 [31] and Th17 cells [32]. It also inhibits IL-4, antagonizes Th2 responses and can also limit IL-2 production [33]; consequently, it has been shown to have a negative impact on nTregs cells [34]. In addition to its noted effects on the priming of Th1 cell responses and IFN-γ production by T and natural killer (NK) cells, more recent studies support not only its critical role as a third signal for CD8+ T cell differentiation [35], but also its ability to serve as an important factor in the reactivation and survival of memory CD4+ T cells [36]. This finding is particularly relevant in the repolarization of CD4+ T cells from dysfunctional antitumor Th2 into Th1 cells in the cancer setting [37]. Besides its regulatory function, it has been reported that IL-12 expression is increased in several inflammatory diseases such as rheumatoid arthritis (RA) [38, 39].

IL-12, known as the Th1 response master controller, stimulates T and NK cells to produce IFN-γ, which induces multiple proatherogenic processes in the atherosclerotic lesion [40]. A marked expression of both IL-12 mRNA and protein has been demonstrated in human atherosclerotic plaques [41]. Similarly, it has been suggested that the production of IL-12 contributes to their progression [42]. Experimental data have shown an association between circulating IL-12 levels and arterial stiffness in healthy individuals [43]. In an observational study, Opstad et al. showed that the IL-12 levels were not associated with cardiovascular events [44]; however, when the levels of IL-12 and IL-18 were analyzed simultaneously, they were found to be independently and significantly associated with a higher risk of cardiovascular events [44]. Increased IL-12 production has been shown in animal models of atherosclerosis [45, 46] and in newly diagnosed type 2 diabetic patients with cardiovascular complications [47]. Actually, a correlation between advanced inflammatory responses and IL-12 serum levels has been demonstrated in murine models of atherosclerosis, as well as in coronary artery disease patients' [48].

Interleukin 23

IL-23 is mainly produced by macrophages and dendritic cells after toll like receptor activation. IL-23 expression is further augmented by interactions between dendritic cells and T cells after CD40-CD40L binding [49]. In addition, its secretion is limited by the tissue and cell-restricted expression of p19 subunit [13, 23]. The action of the IL-23 is generated after its interaction with the heterodimeric IL-23 receptor (IL-23R), composed of the IL-12Rβ1 unit and the specific IL-23R chain. IL-23 plays a central role in inflammation, since it promotes Th1 cell differentiation, stimulates the proliferation of the T memory cells and is critical in the development of pro-inflammatory Th17 populations [50]. Moreover, IL-23 is able to switch IL-17 secreting FoxP3+RORγt Tregs to the pro-inflammatory Th17 phenotype [51]. Naïve FoxP3+ Tregs start differentiation into Th17 cells in presence of IL-1β and IL-2, and IL-23 is able to enhance this process [52, 53]. IL-23 promotes the survival of Th17 cells induced by TGFβ and IL-6 [54]. In fact, acting in concert with IL-12, IL-18, and IL-2, IL-23 promotes the production of INFγ by NK cells [21]. Other innate immune cells, termed "type 17" cells respond to IL-23; these cells compromise γδT cells, natural killer T cells and innate lymphoid cells, which are important for resistance to infection and autoimmune pathology [55]. These capacities give to IL-23 strong pro-inflammatory and proatherogenic properties. Indeed, IL-23 has been related to several inflammatory disorders, such as RA [56], ulcerative colitis [57], psoriasis [58], systemic lupus erythematosus [59], and inflammatory bowel diseases [60], as well as cancer [61].

Interestingly, IL-23 has also been implicated in atherogenesis [62]. In IL-23p19$^{-/-}$ mice, Savvatis et al. showed that after myocardial infarction, IL-23 deficiency results in myocardial inflammation, lower cardiac fibroblast activation with impaired scar formation, adverse remodeling, ventricular rupture, and increased mortality [63]. In 25 consecutive individuals defined as having CAD after a coronary angiography, the expression of *IL-23* gene was evaluated in unstimulated peripheral blood lymphocytes (PBMCs) and compared with the *IL-23* gene expression in unstimulated PBMCs of 25 consecutive individuals with normal coronary angiography studies. The results showed a significant lower *IL-23* gene expression in unstimulated PBMCs of patients with CAD+ compared to those without CAD after adjustment for age, sex, hypertension and diabetes mellitus [64]. These results are in accordance with the data obtained in murine models. In addition, in a model of ischemic stroke, levels of IL-23 were upregulated in both the brain and the circulation [65]. Peripheral arterial disease patients also showed an increase of IL-23 serum levels and higher IL-23 expression in human carotid lesions [66, 67]. Furthermore, in a follow-up study (mean 3.5 years) of 177 patients with carotid atherosclerosis and 24 healthy control subjects, the IL-23 levels were significantly higher in patients when compared with control subjects, and higher levels of the cytokine were associated with disease progression and increased mortality [68]. The inconsistent results regarding the role of IL-23 in atherosclerosis may be partly due to different experimental setups in animal model and human studies. As for the animal model studies, wild type mice with different genetic background are used with a selection of different time points making comparisons difficult as the inflammatory reaction in atherosclerosis and myocardial infarction is a continuously changing process. Concerning human studies, population sample size, diagnosis criteria, gender, and

presence of other clinical phenotypes such as obesity and type 2 diabetes mellitus can generate different results. In sum, the role for IL-23 in atherosclerosis is still unclear.

Interleukin 27

Interleukin-27 is mainly produced by dendritic cells, monocytes and macrophages after their activation by recognition of pathogen-specific patterns [22, 69]. Moreover, LPS-activated macrophages express the highest level of p28 and EBI3 in any tissue or cell type described [22, 69, 70]. CD40-CD40L interactions between dendritic cells and T cells increase production of IL-27, IL-23 and IL-12 [49]. IL-27 is considered a pleiotropic cytokine as it modulates anti-inflammatory and pro-inflammatory responses depending on the predominant type of immune response, disease type and severity. The pleiotropic effects of this cytokine suggest that its wide participation in several inflammatory diseases could be due to its effect in common mechanisms involved in these pathologies [71]. Initially it was reported that IL-27 together with IL-12 initiate clonal expansion of naïve T cells and increased IFNγ production via this cell type and NK cells [22, 69], suggesting that IL-27 sensitizes T cells to IL-12 effects. As a consequence, it is critical for the early events leading to Th1 cell proliferation and differentiation [72]. Later studies have demonstrated that IL-27 also has anti-inflammatory activity. In fact, IL-27 can inhibit Th1-driven infections [73], developments of Th17 cells in several inflammatory settings [74], Treg formation induced by transforming growth factor-β [75] and, secretion of IL-4. Interestingly, it also antagonizes IL-2 production, hence limiting Th2 cell differentiation and effector cell function [76]. IL-27 converts activated inflammatory CD4+ T cells into IL-10-producing Th1 or Tr1 cells [74, 77]. It seems that IL-27 has a bidirectional function: while it induces a pro-inflammatory response in naïve cells, it does the opposite in activated cells. In addition, it has been suggested an enhanced IL-27 expression during fetal development, for instance IL-27 like IL-35, could have a role in fetal-maternal tolerance [78]. In this regard, IL-27 is the only IL-12 family member to have both inflammatory and immunomodulatory activities. The IL-27 p28 subunit (IL-30) alone acts as an agonist of gp130-mediated signaling [79]. IL-30 blocks signaling mediated by IL-6, IL-12 and IL-27, including IL-6-dependent Th17 responses.

The interleukin 27 receptor is a complex formed by a WSX-1 chain and a glycoprotein 130 (gp130) subunit [80]. WSX-1 and gp130 are co-expressed on different cell types, such as dendritic cells, mast cells, monocytes, macrophages, NK cells, endothelial cells, and B and T lymphocytes [69, 81, 82]. Indeed, the complexity of its receptor could explain the wide-ranging immunomodulatory function of this cytokine. The promotion or suppression of inflammation by IL-27 may vary within different pathologies. Actually, the available evidence shows that IL-27 suppresses inflammation in *Leishmania donovani* infection [83], intraocular inflammation [84], chronic inflammation of the central nervous system [85], autoimmune arthritis [86], experimental autoimmune encephalitis [87], allergic asthma [88]. In contrast, it promotes inflammation in systemic sclerosis [89], experimental crescentic glomerulonephritis [90], experimental colitis [91], and hepatitis [92].

The participation of IL-27 in the regulation of the innate and adaptive immunity supports its part in atherosclerosis. IL-27 is expressed in atherosclerotic plaques [93], and its role in atherosclerosis has been reported in cultured cells, animal models and coronary patients with inconsistent findings. In IL-27-deficient (*Ldlr*$^{-/-}$*Ebi3*$^{-/-}$) and IL-27

receptor-deficient (*Ldlr*$^{-/-}$*WSX-1*$^{-/-}$) *Ldlr*$^{-/-}$ mice, Hirase et al. demonstrated that the deficiency of this cytokine and its receptor accelerates atherosclerosis, and that IL-27 recombinant treatment inhibits atherosclerosis *in vivo* and macrophage activation *in vitro* [94]. Additionally, in a rat model of ischemia/reperfusion injury, IL-27 administrated 5 minutes before reperfusion reduced tissue damage and markedly improved post-ischemia recovery in isolated perfused hearts; this finding suggests that this cytokine protects the myocardium against ischemia/reperfusion injury, thus facilitating the recovery of damaged cardiomyocites [95]. In an *in vitro* study, it has been demonstrated that IL-27 decreased lipid accumulation in THP-1 derived macrophages and markedly enhanced cholesterol efflux through increasing the expression of both ABCA1 mRNA and protein [96]. These findings suggest that IL-27 reduces lipid accumulation of foam cell by upregulating ABCA1 expression. In contrast, in a clinical study of 136 CAD patients and 29 controls, Jin et al. found that IL-27 plasma concentration was higher and correlated with the severity of the coronary atherosclerosis lesion in CAD patients [97]. Similarly, significantly higher IL-27 plasma concentrations were observed in Mexican premature CAD patients compared to controls [98]. Moreover, dendritic cells incubated with oxidized low-density lipoprotein produced IL-27, suggesting that these modified lipoproteins could play an important role in dendritic cell activation and IL-27 production [97]. Altogether, these studies suggest IL-27 could play a crucial role in the immunity and inflammation regulatory net in atherosclerosis.

Interleukin-35

Interleukin 35 (IL-35) is considered an anti-inflammatory and immunosuppressive cytokine [99]. IL-35 is constitutively secreted by CD4+ regulatory T (Treg) cells instead of CD4+ effectors T cells [15]. In human and mice, IL-35 is able to induce the conversion of conventional Tregs cell into a suppressive IL-35-producing Treg-cell population (termed iTr35) [100]. IL-35 does not have a constitutive expression in tissue [101]. The genes encoding IL-35 are also transcribed by activated B cells and to a lower degree by monocytes, smooth muscle cells and vascular endothelial cells after activation with lipopolysaccharide and pro-inflammatory cytokines [101]. Although IL-35 shares structural features and binding partners with the IL-12 siblings, its function appears to be strictly regulatory. Contrary to the inflammatory effects of the IL-12 and IL-23, IL-35 can efficiently inhibit Th1 and Th17 cells through the expansion of Treg cells and IL-10 production; indeed, this cytokine is also important for optimal Treg cell function [15]. Devergne et al. [102] reported a high expression of EBI3 and p35 (subunits of IL-35) in placental trophoblast, suggesting that, like IL-27, IL-35 may be an immunomodulator at the fetal-maternal border. Thus, IL-35 plays critical roles in preventing autoimmunity, maintaining self-tolerance, and suppressing antitumor immune responses. In experimental models, IL-35 has been demonstrated to suppress development of collagen-induced arthritis [25], autoimmune diabetes [103], autoimmune encephalomyelitis [104], inflammatory bowel disease [105], and IL-17-dependent allergic airway disease [106].

As mentioned before, the gene that codifies IL-35 is transcribed by vascular endothelial cells, smooth muscle cells and monocytes when activated by stimulation [101]. Then it may play a role in atherosclerosis development. In ApoE$^{-/-}$ mice previously fed with high-fat diet, it has been reported that IL-35 treatment reduces the

atherosclerotic lesion area by improving Treg-mediated suppression [107]. In addition, in these mice with an established atherosclerotic lesion, Wang et al. [108] have recently demonstrated that they displayed a lower level of IL-35 compared to the age-matched wild type C57BL/6 mice without plaque. On the other hand, the expression of the IL-35 increased significantly in apoE$^{-/-}$ mice with attenuated plaque. In fact, the co-expression of the two subunits of IL-35 has been demonstrated in human advanced atherosclerotic plaque [93]. Decreased levels of IL-35 have been reported in patients with acute coronary syndrome (unstable angina pectoris and acute myocardial infarction) compared with a chest pain syndrome group; IL-35 levels also positively correlated with left ventricular ejection fraction whose reduction is associated with heart failure [109]. In contrast, it has been recently reported that premature CAD patients have significantly higher IL-35 plasma levels compared with healthy control with no personal o familiar history of premature CAD and without subclinical atherosclerosis evaluated by computed tomography (coronary calcium score = 0) [110].

Genes encoding α and β subunits of the interleukin-12 family members and their association with cardiovascular and other diseases.

The genes encoding the IL-12 family subunits are polymorphic. In fact, some of the polymorphisms could have a functional effect that changes the expression of these subunits and, in consequence, the expression and production of the IL-12 family cytokines. Functional genetic polymorphisms that altered cytokine gene expression are candidate genetic factors that could modulate the development and progression of atherosclerosis and cardiovascular disease. Indeed, the genotypes of the polymorphisms of the α and β chain genes of the IL-12 family members could influence cytokine production and activities, and may define the balance in Th response in atherosclerosis.

IL-12B gene

Beta subunit p40 (present in IL-12, IL-23 and IL-Y) is encoded by *IL-12B* gene on chromosome 5. Genome wide association (GWA) studies have become a powerful approach to identify genes involved in complex pathologies. Recent GWA studies have pointed out a set of polymorphisms in *IL-12B* gene that are consistently associated with chronic inflammatory disorders. The rs3212227 ($A \rightarrow C$) polymorphism present in the 3'UTR region of *IL-12B* has been associated with psoriasis [111], type 1 [112] and type 2 diabetes mellitus [113], multiple sclerosis [114], ankylosing spondylitis [115], allergic rhinitis [116], asthma [117] and lepromatous leprosy [118]. However, the information regarding the association of polymorphisms present in the *IL-12B* gene with coronary artery disease (CAD) is scarce. In a small Japanese cohort, Momiyama et al. failed to show an association between rs3212227 *IL-12B* polymorphism and the presence or severity of CAD diagnosed by angiography. Although an important limitation in this study is the lack of a healthy control group [119], these results are in agreement with those reported by Mangino et al. in a British cohort [120]. In conclusion, the data available so far do not support the association between the rs3212227 *IL12B* polymorphism and the developing of cardiovascular disease.

IL-23A gene

The α subunit p19 (present in IL-23 and IL-39) is encoded by the *IL-23A* gene located on chromosome 3. Considering that IL-23 cytokine has an important role in the atherosclerosis process, the gene that encodes this cytokine could be candidate for genetic association studies. Genetic studies have associated the presence of the rs2066808, rs2371494, rs11575248 polymorphisms in the *IL23A* gene with susceptibility to multiple sclerosis [121]. Alternatively, the rs11171806 polymorphism in the *IL-23A* gene has been significantly associated with the susceptibility to Graves' disease in Han Chinese [122], but not in the northern Italian population [123]. In a Brazilian cohort, the *GG* haplotype of rs11171806 and 2066808 polymorphisms in the *IL-23A* gene was more frequent in control subjects than in diabetic patients, thus conferring protection for this disease [124]. In German and Chinese cohorts, the intronic rs2066808 *IL-23A* variant has been associated with psoriasis [125, 126], whereas in Northern Spaniards it has been associated with psoriatic arthritis [127]. Lastly, these associations have been confirmed in a Romanian cohort [128], and in a Caucasian cohort (British and Irish patients) [129]. Unfortunately, at present, no studies have evaluated the association of the *IL23A* polymorphisms with the development of cardiovascular disease.

IL27p28 gene

The human *IL-27p28* gene that encodes the IL-27 and IL-Y alpha subunit is located in the 16p11 locus, spans 5 exons and is highly polymorphic [130]. Polymorphisms in the *IL-27p28* gene have been associated with risk of inflammatory bowel disease [131], rheumatoid arthritis [132], asthma [130], allergic rhinitis [133], chronic obstructive pulmonary disease [134], and with protection of ulcerative colitis [135]. In a Chinese case-control study, an association of atrial septal defects and ventricular septal defect with the *G* allele of rs153109 polymorphism was reported; no association was found with the r*s17855750* polymorphism [136]. In a large number of CAD cases belonging to the GeneID Chinese Han population, four *IL-27p28* tag polymorphisms (rs181206, rs17855750, rs37833 and rs153109) were determined and after adjusting for confounding variables, the polymorphisms were not associated with CAD, age at disease onset or severity [137]. These results concur well with the findings of the GEA Study in a Mexican population, in which the rs181206 and rs17855750 *IL-27p28* gene variants were not significantly associated with premature CAD [98]. However, Posadas-Sánchez et al. reported a significant association (after adjusting for age, gender, body mass index, smoking habit, total abdominal fat, HOMA-RI, aspartate aminotransferase, adiponectin and uric acid) of the both rs26528 *T* and rs40837 *A* alleles with a decreased risk of developing premature CAD [98]. Additionally, luciferase assays showed that co-transfection of the rs40837 *A* allele and miR-379-5p significantly decreased luciferase gene expression. To the best of our knowledge, this is the first and only study reporting a significant association and with functional approach of *IL-27p28* gene polymorphisms with premature CAD. In consequence, the study reporting the associations (rs26528, rs40837) should be repeated in other cohorts to confirm these results.

IL-12A gene

The α subunit p35 present in IL-12 and IL-35 is encoded by *IL-12A;* this gene is located on chromosome 3q25.33 and consists of seven exons. Previous studies have

indicated the association of *IL-12A* gene polymorphisms with inflammatory diseases. In a Chinese population, the *IL-12A* rs2243115 polymorphism was associated with genetic susceptibility to chronic obstructive pulmonary disease [138]. Chen et al. [117] reported the association of the *IL-12A* rs568404 polymorphism with the risk of asthma development in a Chinese population. In a case-control association study, 5 tag polymorphisms (rs2243115, rs2243123, rs583911, rs568408 and rs2243143) in the *IL-12A* gene were determined in two independent Chinese cohorts (a pilot cohort conducted in Shanghai and a replicate cohort in the Xiamen Island); the haplotype analysis showed that the haplotype of the five polymorphisms (*TTAAG*) was associated with a significant risk of Graves' disease in both cohorts [139]. Recently, Shen et al. reported that the *IL-12A* rs2243115 *GG* genotype may increase the risk of rheumatoid arthritis in Chinese individuals negative for rheumatoid factor [140]. A case-control study showed that the haplotype *TA* (rs582054 and rs2243151) in the *IL-12A* gene was significantly associated with the atopic dermatitis phenotype in a Korean population [141]. Only one study has heretofore evaluated the participation of *IL-12A* gene polymorphisms in the susceptibility to premature cardiovascular disease. A very recent report of the Mexican GEA study determined four *IL-12A* polymorphisms (rs2243115, rs2243123, rs583911 and rs568408) in 1162 patients with premature CAD and 873 control subjects [110]. In this study, after adjustment for age, gender, body mass index and current smoking status, it was described that the *IL-12A* rs2243115 polymorphism was significantly associated with reduced risk of developing premature CAD under different inheritance models. Considering that this is the first study that evaluates the role of *IL-12A* polymorphisms in premature CAD, the detected associations are not yet definitive, and replicate studies in independent populations are warranted to confirm these findings.

EBI3 gene

The β subunit (EBI3) of IL-35 and IL-27 is encoded by *EBI3* gene; it is located on chromosome 19q13.3 and contains 5 exons. A Chinese population-based case-control association study design was used to assess the risk of allergic rhinitis conferred by polymorphisms in *FOXP3* and *EBI3* gene regions. Logistic regression analyses, adjusted for age and gender, showed a significant association of the *EBI3* rs428253 polymorphism with decreased risk of developing allergic rhinitis [142]. In another Chinese cohort, the *EBI3* gene rs428253 polymorphism was also associated with chronic rhinosinusitis [143]. In a Mexican study, the *EBI3* rs428253, rs4740, and rs4905 polymorphisms were associated with decreased risk of developing ulcerative colitis [135]. Furthermore, Zheng et al. [144] found that the *EBI3* rs4740 polymorphism is closely associated with the susceptibility to pulmonary tuberculosis in Chinese subjects. As was described for *IL-12A* gene, only one study has addressed the participation of *EBI3* gene polymorphisms in the susceptibility to premature cardiovascular disease [110]. In this study, three *EBI3* gene polymorphisms (rs428253, rs4740 and rs4905) were evaluated; the results showed that the *EBI3* rs428253 polymorphism was associated with reduced risk of developing premature CAD. They also found that coronary patients showed significantly higher IL-35 levels than control subjects, and that the *EBI3* rs4740 and rs4905 polymorphisms were associated with different levels of IL-35 [110]. Nevertheless, considering that this is the first report that evaluates the role of

EBI3 polymorphisms in premature CAD, the detected associations are not yet definitive, and replicate studies in independent populations are required.

Conclusions

The IL-12 family cytokines act as the immunological playmaker, shaping immune responses by directly inducing the development of T cell subpopulations and altering the function and fate of many cell populations that dictate disease outcome. They are a unique group of heterodimeric cytokines composed of one out of the three α subunits p19, p35, or p28 and one out of two β subunits p40 or Epstein-Barr virus induced gene 3 (EBI3). Despite their common structures, the biological function of the members of this family is very diverse. Whilst IL-12 and IL-23 are pro-inflammatory, IL-27 is bi-directional in terms of being both pro- and anti-inflammatory; alternatively, IL-35 is anti-inflammatory. Considering their effects in the immune response, these cytokines have been associated with the developing of cardiovascular diseases in both animal models and humans. In the former, some of these cytokines have been administered and variation in the development of atherosclerosis has been observed. Moreover, the variation in the levels of these cytokines in humans has been detected in both patients and healthy controls. The association studies of the polymorphisms present in the genes that encode the IL-12 family subunits are recent and many of them need confirmation. From point of view of etiopathology and genetics, it is important to consider that the information about the role of the IL-12 family of cytokines in the development of atherosclerosis is limited. Indeed, future studies could help to define the true role of these cytokines in the development of this complex disease.

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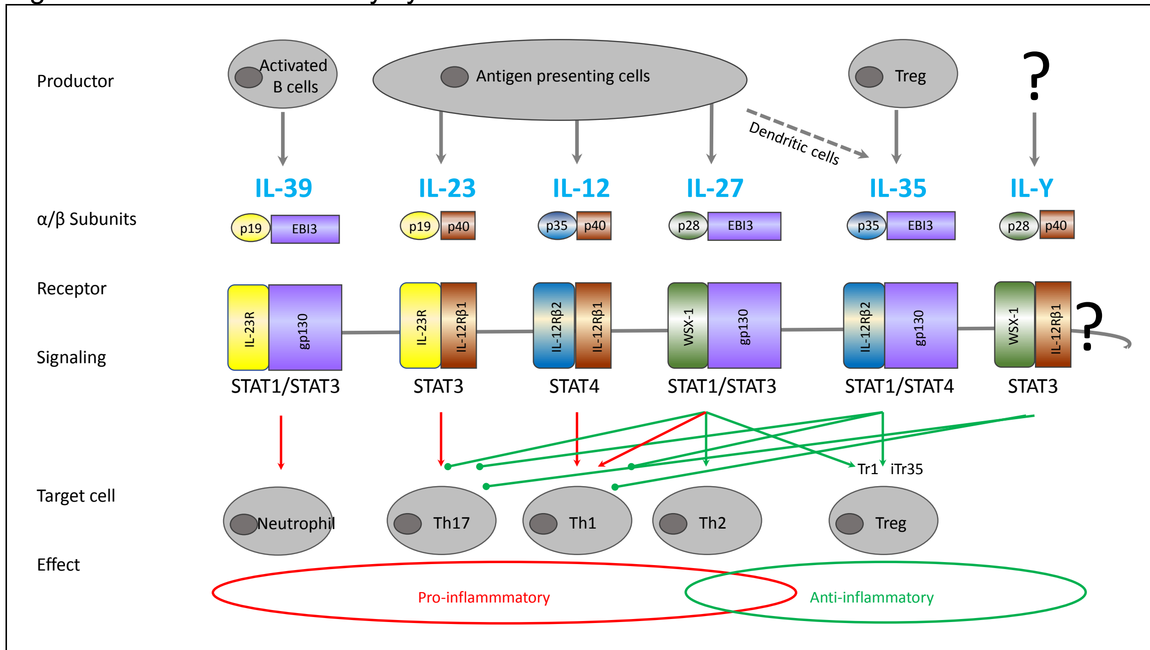
Table 1 Studies and their findings on interleukin-12 family members in atherosclerosis

Study	Finding	Ref
IL-12		
Animal model	Production of IL-12 contributes to atherosclerotic plaque progression	42
	Increased IL-12 production in models of atherosclerosis	45, 46
Human	Increased expression of IL-12 mRNA and protein in human atherosclerotic plaques	41
	Circulating IL-12 levels are associated with arterial stiffness in healthy individuals	43
	IL-12 and IL-18 elevated levels are significantly associated with higher risk of cardiovascular events	44
	Increased production of IL-12 in newly diagnosed type 2 diabetic patients with cardiovascular complications	47
	Correlation between increased inflammatory responses and IL-12 serum levels in coronary artery disease patients	48
IL-23		
Cell culture	Lower IL-23 gene expression in unstimulated peripheral blood lymphocytes of coronary patients compared with subjects with normal coronary angiography	64
Animal model	In IL-23p19-/- mice, after myocardial infarction, IL-23 deficiency results in myocardial inflammation, lower cardiac fibroblast activation, impaired scar formation, adverse remodeling, ventricular rupture and increased mortality	63
	IL-23 levels are upregulated in brain and circulation in a murine model of stoke	65
Human	Increased IL-23 serum levels and higher IL-23 expression in human carotid lesions of peripheral arterial disease patients	66, 67
IL-27		
Cell culture	Interleukin 27 reduces lipid accumulation in THP-1 derived macrophages and enhances cholesterol efflux	96
Animal model	The deficiency of IL-27 and its receptor accelerates atherosclerosis. Treatment with IL-27 recombinant inhibits atherosclerosis *in vivo* and macrophage activation *in vitro*	94
	In rat model of ischemia/reperfusion injury, the administration of IL-27 reduces damaged tissue and improves post-ischemia recovery	95
Human	Interleukin 27 is expressed in atherosclerotic plaques	93
	Compared with controls, coronary patients have higher IL-27 plasma concentrations, that correlated with the severity of the coronary atherosclerotic lesion	97, 98
IL-35		
Animal model	Interleukin 35 treatment reduces the atherosclerotic lesion in $ApoE^{-/-}$ mice.	107
	Apo E-/- mice with established atherosclerotic lesion have lower IL-35 levels compared with control mice	108
Human	The co-expression of the IL-35 α and β subunits was demonstrated in human advanced atherosclerotic plaque	93
	Patients with acute coronary syndrome have decreased levels compared with subjects with chest pain syndrome IL-35 levels significantly correlated with left ventricular ejection fraction.	109
	Premature coronary patients have lower IL-35 levels compared with healthy controls	110

Table 2 Studies and their findings on genes encoding α and β subunits of the interleukin-12 family members and atherosclerosis

Gene	Finding	Ref
IL-12B		
	Lack of association between rs3212227 *IL-12B* polymorphism and the presence or severity of CAD evaluated by angiography.	119
	Lack of association between myocardial infarction and rs3212227 *IL-12B* polymorphism	120
IL-23A		
	So far, no studies have evaluated the association of the *IL23A* polymorphisms with cardiovascular disease	
IL-27p28		
	rs153109 G variant was associated with atrial septal defects and ventricular septal defects	136
	rs181206, rs17855750, rs37833 and rs153109 single nucleotide polymorphisms were not associated with CAD, age at disease onset or severity	136
	rs26528 *T* and rs40837 *A* alleles were associated with a decreased risk of developing premature CAD after adjusting for confounding variables	98
IL-12A		
	IL-12A rs2243115 polymorphism was significantly associated with reduced risk of developing premature CAD after adjusting for confounding variables	110
EBI3		
	The *EBI3* rs428253 was associated with reduced risk of developing premature CAD The *EBI3* rs4740 and rs4905 polymorphisms were associated with different levels of IL-35	110

Figure 1. Interleukin 12 family cytokines.



This interesting and fascinating family is composed by heterodimeric cytokines sharing three α-chains (p19, p28, p35) and two β-chains (p40 and EBI3). Their receptors are also heterodimeric and they share their subunits too. Signaling is mediated by the Jak-STAT family having different effects on T-eff cell subsets. Red arrows indicate pro-inflammatory effects, green arrows represent anti-inflammatory properties.
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Receptor-interacting protein 2 (*RIP2*) gene polymorphisms are associated with increased risk of subclinical atherosclerosis and clinical and metabolic parameters. The Genetics of Atherosclerotic Disease (GEA) Mexican study

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ABSTRACT

The receptor-interacting protein 2 (Rip2) is a serine/threonine kinase involved in multiple nuclear factor-κB (NFκB) activation pathways and is a key regulator of cellular lipid metabolism and cardiovascular disease. The aim of the present study was to evaluate the role of *RIP2* gene polymorphisms as susceptibility markers for subclinical atherosclerosis (SA). Using an informatics analysis, four *RIP2* gene polymorphisms with predicted functional effects (rs2293808, rs43133, rs431264, and rs16900627) were selected. The polymorphisms were genotyped in 405 individuals with SA (calcium score > 0 assessed by computed tomography) and 1099 controls (calcium score = 0). Clinical, anthropometric, tomographic and biochemical traits were measured. The association between the *RIP2* polymorphisms and SA was evaluated using logistic regression analyses. Pair wise linkage disequilibrium (LD, D′) estimations between polymorphisms and haplotype reconstruction were performed with Haploview version 4:1. Under different models adjusted by age, gender, body mass index, hypertension, diabetes mellitus, smoking habit, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride levels, rs43133 (OR = 1.43, 95% CI: 1.05–1.94, P = 0.022), and rs16900627 (OR = 1.59, 95% CI: 1.00–2.54, P_{dom} = 0.048 and OR = 1.60, 95% CI: 1.05–2.54, P_{add} = 0.028) were associated with increased risk of developing SA. Moreover, rs2293808, and rs431264 were associated with clinical or metabolic parameters in SA individuals and in healthy controls. The four polymorphisms were in high linkage disequilibrium and the *GAAG* haplotype was associated with increased risk of developing SA (OR = 1.47, P = 0.027). This study shows for the first time, that *RIP2* polymorphisms are associated with increased risk of SA and with some clinical and metabolic parameters.

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1. Introduction

Coronary artery disease (CAD) is a multifactorial disorder where inflammation plays a crucial role in disease initiation and progression (Sorci-Thomas and Thomas, 2016; Lubrano and Balzan, 2015). Multiple cytokines, mainly pro-inflammatory, have been detected in atherosclerotic plaques (Ramji and Davies, 2015; van der Vorst et al., 2015). Being a multifactorial disorder, both genetic and environmental factors play and important role in its etiology (Dai et al., 2016; Roberts, 2014), and many candidate gene and genome-wide association studies have reported the association of polymorphisms with CAD en various populations (Prins et al., 2012; Dichgans et al., 2014; Girelli et al., 2009).

The receptor-interacting protein 2 (Rip2) is a serine/threonine kinase involved in nuclear factor-κB (NFκB) activation pathways,

Abbreviations: ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; CAC, coronary artery calcification; CAD, coronary artery disease; CT, computed tomography; CRP, high-sensitivity C-reactive protein; GGT, gamma-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment of insulin resistance; LD, linkage disequilibrium; MS, metabolic syndrome; PCR, polymerase chain reaction; RIP2, receptor-interacting protein 2; SA, subclinical atherosclerosis; SNP, single nucleotide polymorphism; T2DM, type 2 diabetes mellitus.

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including Toll-like receptors. The gene encoding this protein is therefore an interesting potential candidate for study in CAD. Recently, Levin et al. (2011) reported that lipid accumulation and inflammation are dissociated in the vessel wall in mice with *Rip2* −/− macrophages. Rip2 was also shown to modify vascular endothelial growth factor (VEGF)-induced signaling and vascular permeability in myocardial ischemia (Andersson et al., 2015). This suggests that Rip2 regulates cellular lipid metabolism and could be involved in cardiovascular disease. The *RIP2* gene is polymorphic and while some of these variants have been associated with asthma and inflammatory bowel disease (Nakashima et al., 2006; Thiébaut et al., 2011), to date no associations with cardiovascular disease have been reported. Thus, the aim of the present study was to evaluate whether potentially functional *RIP2* gene polymorphisms are associated with subclinical atherosclerosis (SA) in Mexican patients. Four *RIP2* gene polymorphisms predicted to be functional with minor allele frequencies > 5% (rs2293808, rs43133, rs431264, and rs16900627) were selected. The functional analysis showed that rs2293808 produces binding sites for CIZ, ETS, HMGIY, IK and NFAT transcription factors; rs43133 produces binding sites for BRCA, CEBPA, CEBP, E2A and MYB factors; and rs16900627 produces binding sites for miR-217 and miR-367. Rs431264 was not functional according to this analysis, however it was informative and included in the study.

2. Material and methods

2.1. Subjects

The study included 1504 unrelated asymptomatic subjects with no family and personal history of premature CAD, recruited from blood banks and Social Services centers. All study subjects identified themselves as Mexican-mestizos, defined as people born in Mexico, with an ancestry comprised of both indigenous inhabitants and individuals of African and/or Caucasian origin (mainly Spaniards), who had migrated to the Americas from the sixteenth century onward. Exclusion criteria included congestive heart failure, liver, renal, thyroid or oncological disease. Demographic, anthropometric, biochemical, tomographic parameters and cardiovascular risk factors were assessed in all subjects. Height, weight, and waist circumference were measured and body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Type 2 diabetes mellitus (T2DM) was defined by the American Diabetes Association criteria (American Diabetes Association, 2009), reported glucose-lowering treatment or previous T2DM diagnosis by a physician. Systolic and diastolic blood pressures were measured after rest for at least 10 min, and the average of the second and third measurements was recorded for analyses. Hypertension was defined as systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure > 90 mm Hg or the current use of antihypertensive medication. The metabolic syndrome (MS) was defined using the criteria from the American Heart Association/National Heart, Lung, and Blood Institute scientific statement on the MS (Grund et al., 2005), except for central obesity that was considered when waist circumference was >90 cm in men and >80 cm in women (Sánchez-Castillo et al., 2003). Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR). Hyperinsulinemia was defined as insulin values ≥ 75th percentile (16.97 μU/mL in women and 15.20 μU/mL in men), insulin resistance as HOMA-IR values ≥ 75th percentile (3.66 in women and 3.38 in men), while low adiponectin was defined as serum adiponectin levels ≤ 25th percentile (≤8.67 μg/mL in women and ≤5.30 μg/mL in men). These cutoff points were obtained from a Genetics of Atherosclerotic Disease (GEA) Mexican study subsample of 131 men and 185 women without obesity and with normal blood pressure, fasting glucose and lipids measurements. LDL particle size was estimated with the LDL-cholesterol/apoB ratio, LDL pattern B was considered with a LDL-cholesterol/apoB ratio ≤ 1.2, which corresponded to an LDL diameter of 25.5 nm, which is the cut-off value to distinguishing LDL pattern A from pattern B (Hirano et al., 2005).

Total, subcutaneous and visceral abdominal fat and coronary artery calcification (CAC) were quantified by computed tomography in each participant. Computed tomography (CT) of the chest and abdomen was performed using a 64-channel multidetector helical computed tomography system (Somatom Cardiac Sensation, 64, Forcheim, Germany) and interpreted by experienced radiologists. Scans were read to assess and quantify the following: a) total abdominal, subcutaneous, and visceral abdominal fat areas as described by Kvist et al. (1988); b) CAC score using the Agatston method (Mautner et al., 1994); c) liver to spleen attenuation ratio (L:SAR) as described by Longo et al. (Longo et al., 1993). SA was defined as the presence of a CAC score > 0, healthy controls with CAC score = 0 and hepatic steatosis as L:SAR ≤ 1.0 (McKimmie et al., 2008). Elevated visceral abdominal fat was defined as visceral abdominal fat ≥ 75th percentile (127 cm^2 in women; 152.7 cm^2 in men). This cut-off point was also estimated in the sub-group of GEA participants without cardiometabolic risk factors. Four hundred and five individuals had SA and 1099 were considered healthy controls. All participants provided written informed consent, and the study complies with the Declaration of Helsinki and was approved by the Ethics Committee of the Instituto Nacional de Cardiología Ignacio Chávez.

2.2. Biochemical analyses

Venous blood samples were collected from subjects after a 12 h fast. Plasma glucose, total cholesterol (TC), triglycerides, high density lipoprotein (HDL) cholesterol, apolipoprotein A1 (apo A), apolipoprotein B (apo B), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP) and gamma-glutamyl transpeptidase (GGT), were measured in fresh samples, using standardized enzymatic procedures in a Hitachi 902 analyzer (Hitachi Ltd, Tokyo, Japan). Accuracy and precision of lipid measurements in our laboratory are under periodic surveillance by the Centers for Disease Control and Prevention service (Atlanta, GA, USA). Low-density lipoprotein (LDL) cholesterol was estimated (DeLong et al., 1986). Total high-sensitivity C-reactive protein (CRP) levels were determined by immunonephelometry on a BN Pro Spec nephelometer (Dade Behring Marburg GmbH, Germany). Interassay coefficient of variation (CV) values were <6% for all of these assays. Serum total adiponectin was determined by ELISA (R&D Systems, Minneapolis, USA) Quantine kit. The intra- and interassay CV were <10%. Plasma insulin concentrations were determined by a radioimmunoassay (Millipore; RIA Kit, Cat. No. HI-14 K, MO, USA) and the intra- and interassay CV values were 2.1 and 6.8%, respectively.

2.3. Genetic analysis

Genomic DNA was isolated from whole blood using standard techniques. Four *RIP2* gene polymorphisms (rs2293808, rs43133, rs431264, and rs16900627) were determined on an ABI Prism 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using 5′ exonuclease TaqMan assays following the manufacturer's instructions. Genotyping call rate surpassed 95% for all SNPs tested, with no discordant genotypes in 10% of duplicate samples.

2.4. Statistical analysis

The SPSS version 18.0 statistical package (SPSS, Chicago, Il) was employed for the analysis. Medians, interquartile range and frequencies were calculated as appropriate. Mann-Whitney *U* test and Chi-square or Fisher test analysis were used to test continuous and categorical variables respectively. Logistic regression analysis was used to test for associations of polymorphisms with SA and other variables under different inheritance models. Genotype frequencies did not deviate from Hardy-Weinberg equilibrium in any case (HWE, P > 0.05). Haploview version 4.1 (Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA) was used to calculate

Table 1
Clinical characteristics of the study population.

		Controls (n = 1099)	SA (n = 405)	P-value
Age (years)		51 [45–57]	59 [53–64]	<0.0001
Body mass index (Kg/m^2)		27.96 [25.43–31.02]	28.23 [25.89–31.30]	0.047
Waist circumference (cm)		94 [86–101.5]	96.5 [90.7–104.6]	<0.0001
Total abdominal fat (cm^2)		434 [349–538]	452.00 [363–564]	0.050
Subcutaneous abdominal fat (cm^2)		287 [218–369]	262.00 [198–341]	0.002
Visceral abdominal fat (cm^2)		140 [105–181]	176.00 [137–227]	<0.0001
Visceral/subcutaneous abdominal fat ratio		0.47 [0.35–0.66]	0.68 [0.48–0.88]	<0.0001
Blood pressure (mm Hg)	Systolic	112 [104–122]	122 [112–135]	<0.0001
	Diastolic	71 [65–76]	75 [69–82]	<0.0001
Heart rate (bpm)		66 [60–72]	66 [60–72]	0.887
Gender n (%)	Female	653 (59.4)	105 (25.9)	
	Male	446 (40.6)	300 (74.1)	<0.0001
Weight n (%)	Normal weight	240 (21.8)	69 (17.0)	
	Overweight	518 (47.1)	199 (49.1)	0.074
	Obesity	341 (31.0)	137 (33.8)	0.056
Central obesity n (%)		895 (81.6)	339 (83.7)	0.191
Tobacco smoking n (%)	Current	242 (22.0)	86 (21.2)	0.401
	Former	364 (42.4)	191 (60.1)	<0.0001
Use of alcohol n (%)		808 (73.5)	314 (77.5)	0.064
Hypertension n (%)		74 (6.7)	80 (19.8)	<0.0001

Data are expressed as median (interquartile range) or percentage.
P values were estimated using Mann-Whitney *U* test for continuous variables and Chi-square or Fisher's test analysis for categorical values.
SA: subclinical atherosclerosis.

pair wise linkage disequilibrium (LD, D′) between polymorphisms and haplotype reconstruction.

2.5. Functional prediction analysis

The functional effect of *RIP2* SNPs was predicted using the following bioinformatics software: FastSNP (Yuan et al., 2006), SNP Function Prediction (http://snpinfo.niehs.nih.gov/snpfunc.htm), Human-transcriptome Database for Alternative Splicing (http://www.h-invitational.jp/h-dbas/), Splice Port: An Interactive Splice Site Analysis Tool (http://www.spliceport.cs.umd.edu/SplicingAnalyser2.html), ESE finder
(http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi), HSF (http://www.umd.be/HSF/), and SNPs3D (http://www.snps3d.org/).

3. Results

3.1. Clinical and metabolic characteristics

General characteristics of the study population are shown in Tables 1 and 2. Individuals with SA had higher BMI, waist circumference, visceral abdominal fat, visceral/subcutaneous abdominal fat ratio, systolic and diastolic blood pressure, prevalence of hypertension (Table 1), total-cholesterol, LDL-cholesterol, triglyceride, apolipoprotein B, glucose, HOMA-IR and gamma-glutamyl transpeptidase levels, but lower levels of HDL-cholesterol, alkaline phosphatase as compared to healthy controls (Table 2). As expected, the prevalence of TC > 200 mg/dL, hypertriglyceridemia, T2DM, MS, hyperinsulinemia and fatty liver observed in SA group was higher than in control subjects (Table 2).

Table 2
Comparison of biochemical parameters in individuals with SA and controls.

	Control (n = 1099)	SA (n = 405)	P-value
Total cholesterol (mg/dl)	190.0 [166.9–211]	198 [168.9–220.2]	0.005
HDL-cholesterol (mg/dl)	45 [36.2–55]	43 [36.2–50]	0.008
LDL-cholesterol (mg/dl)	115.5 [95.4–133.9]	123.7 [101.2–145.1]	<0.0001
Triglycerides (mg/dl)	145.70 [108–202.3]	157.1 [118–205.9]	0.020
Apolipoprotein A1 (mg/dl)	134.10 [115.4–155.6]	132.8 [114.3–156.5]	0.776
Apolipoprotein B (mg/dl)	94.00 [76–113]	98 [80.5–122]	0.002
Glucose (mg/dl)	90 [84–97]	94 [87–105]	<0.0001
HOMA-IR	3.82 [2.64–5.53]	4.5 [2.92–6.82]	<0.0001
Alanine aminotransferase (IU/L)	24 [18.34]	23.5 [18–32]	0.276
Aspartate aminotransferase (IU/L)	25 [21–30]	25 [21–30]	0.546
Alkaline phosphatase (IU/L)	81 [68–96]	77.5 [64.5–93]	0.008
Gamma-glutamyl transpeptidase (IU/L)	26 [18–42]	30 [21.5–42]	<0.0001
High sensitivity CRP (mg/L)	1.55 [0.81–3.25]	1.65 [0.86–3.42]	0.188
Total cholesterol > 200 mg/dL n (%)	409 (37.2)	192 (47.4)	<0.0001
Hypo-α-lipoproteinemia n (%)	574 (52.3)	186 (46.0)	0.018
Hypertriglyceridemia n (%)	523 (47.6)	215 (53.2)	0.031
Type 2 diabetes mellitus n (%)	114 (10.4)	90 (22.2)	<0.0001
Metabolic syndrome n (%)	453 (41.2)	215 (53.1)	<0.0001
Hyperinsulinemia n (%)	578 (52.6)	252 (62.2)	0.001
Fatty liver n (%)	356 (32.5)	154 (38.4)	0.020

Data are expressed as median (interquartile range) or percentage.
P values were estimated using Mann-Whitney *U* test for continuous variables and Chi-square or Fisher test analysis for categorical values.
SA: subclinical atherosclerosis, HDL: high density lipoprotein, LDL: low density lipoprotein, HOMA-IR: homeostasis model assessment of insulin resistance, CRP: C reactive protein.

Table 3
Associations of *RIP2* polymorphisms with SA.

SNP	Genotypes			RAF[a]	Model	OR (95% CI)	P
rs2293808	*GG*	*GA*	*AA*				
Control (n = 1099)	1020 (0.928)	76 (0.069)	3 (0.003)	0.037			
SA (n = 405)	375 (0.926)	28 (0.069)	2 (0.005)	0.040	Dominant	1.10 (0.62–1.98)	0.726
					Recessive	2.44 (0.25–23.35)	0.437
					Additive	1.14 (0.67–1.94)	0.610
rs43133	*TT*	*TA*	*AA*				
Control (n = 1099)	118 (0.107)	473 (0.430)	508 (0.462)	0.677			
SA (n = 405)	43 (0.106)	160 (0.395)	202 (0.499)	0.696	Dominant	1.43 (1.05–1.94)	0.022
					Recessive	1.08 (0.67–1.72)	0.755
					Additive	1.23 (0.98–1.54)	0.070
rs431264	*GG*	*GA*	*AA*				
Control (n = 1099)	380 (0.346)	528 (0.480)	191 (0.174)	0.414			
SA (n = 405)	144 (0.356)	184 (0.454)	77 (0.190)	0.417	Dominant	0.86 (0.62–1.19)	0.381
					Recessive	1.09 (0.75–1.60)	0.630
					Additive	0.96 (0.78–1.19)	0.758
rs16900627	*AA*	*AG*	*GG*				
Control (n = 1099)	985 (0.896)	111 (0.101)	3 (0.003)	0.053			
SA (n = 405)	351 (0.867)	50 (0.123)	4 (0.010)	0.072	Dominant	1.59 (1.00–2.54)	0.048
					Recessive	3.84 (0.72–20.41)	0.113
					Additive	1.60 (1.05–2.54)	0.028

Data are expressed as odds ratios (OR) and 95% confidence interval (95% CI) as assessed by multivariate logistic regression analyses. All models were adjusted for age, gender, BMI, hypertension, diabetes mellitus, smoking history, TC, HDL-cholesterol, LDL-cholesterol and triglycerides.
SA: subclinical atherosclerosis.
[a] RAF, risk allele frequency.

3.2. Association of RIP2 polymorphisms with SA

The distribution of the rs2293808 and rs431264 polymorphisms was similar in SA and healthy individuals. However, rs43133 was associated with an increased risk of SA under a dominant model (OR = 1.43, 95% CI: 1.05–1.94, P = 0.022), whereas, rs16900627 was associated with increased risk of SA under both dominant and additive models (OR = 1.59, 95% CI: 1.00–2.54, $P_{dom} = 0.048$ and OR = 1.60, 95% CI: 1.05–2.54, $P_{add} = 0.028$) (Table 3). The models were adjusted by age, gender, BMI, hypertension, diabetes mellitus, smoking habit, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides.

3.3. Association of RIP2 polymorphisms with cardiovascular risk factors

Within the SA group, rs2293808 was associated with decreased risk of insulin resistance (OR = 0.417, 95% CI: 0.177–0.980, $P_{het} = 0.045$) and visceral abdominal fat (OR = 0.406, 95% CI: 0.166–0.995, $P_{add} = 0.049$). Moreover, rs431264 was associated with an increased risk of central obesity (OR = 4.92, 95% CI: 1.237–19.56, $P_{rec} = 0.024$), hypertriglyceridemia (OR = 1.648, 95% CI: 1.079–2.518, $P_{dom} = 0.021$), LDL pattern B (OR = 1.793, 95% CI: 1.074–2.995, $P_{rec} = 0.026$) and with a decreased risk of low adiponectin levels (OR = 0.573, 95% CI: 0.367–0.892, $P_{dom} = 0.014$) (Table 4).

Table 4
Association of the *RIP2* polymorphisms with cardiometabolic risk factors in SA individuals.

				RAF[a]	Model	OR (95% CI)	P
rs2293808	*GG*	*GA*	*AA*				
Increased visceral abdominal fat					Dominant	0.394 [0.155–1.000]	0.050
No (n = 118)	105 (0.890)	12 (0.102)	1 (0.008)	0.059	Additive	0.406 [0.166–0.995]	0.049
Yes (n = 276)	260 (0.942)	15 (0.054)	1 (0.001)	0.031	Heterozygote	0.406 [0.157–1.046]	0.062
Insulin resistance					Dominant	0.493 [0.215–1.132]	0.095
No (n = 137)	123 (0.898)	14 (0.102)	0	0.051	Additive	0.607 [0.281–1.312]	0.204
Yes (n = 247)	242 (942)	13 (0.051)	2 (0.008)	0.035	Heterozygote	0.417 [0.177–0.980]	0.045
rs431264	*GG*	*GA*	*AA*				
Central obesity					Dominant	1.380 [0.612–2.795]	0.488
No (n = 66)	26 (0.394)	36 (0.545)	4 (0.061)	0.333	Recessive	4.920 [1.237–19.56]	0.024
Yes (n = 328)	114 (0.348)	141 (0.430)	73 (0.223)	0.438	Additive	1.610 [0.918–2.822]	0.096
Hypertriglyceridemia					Dominant	1.648 [1.079–2.518]	0.021
No (n = 186)	77 (0.414)	73 (0.392)	36 (0.194)	0.390	Recessive	0.979 [0.586–1.636]	0.937
Yes (n = 208)	63 (0.303)	104 (0.500)	41 (0.197)	0.447	Additive	1.239 [0.935–1.642]	0.135
LDL pattern B					Dominant	1.204 [0.973–1.830]	0.384
No (n = 209)	79 (0.378)	99 (0.474)	31 (0.148)	0.385	Recessive	1.793 [1.074–2.995]	0.026
Yes (n = 185)	62 (0.335)	77 (0.416)	46 (0.248)	0.457	Additive	1.291 [0.977–1.704]	0.072
Low adiponectin (n = 368)					Dominant	0.573 [0.367–0.892]	0.014
No (n = 223)	67 (0.300)	114 (0.511)	42 (118)	0.444	Recessive	1.015 [0.587–1.755]	0.957
Yes (n = 145)	62 (0.428)	55 (0.379)	28 (0.193)	0.382	Additive	0.780 [0.577–1.057]	0.105

Data are expressed as odds ratios (OR) and 95% confidence interval (95% CI) as assessed by multivariate logistic regression analyses. All models were adjusted by age, gender and body mass index.
[a] RAF = risk allele frequency; LDL = low density lipoprotein.

Table 5
Association of the *RIP2* polymorphisms with cardiometabolic risk factors in healthy controls.

				RAF[a]	MODEL	OR (95% CI)	P
rs2293808	*GG*	*GA*	*AA*				
Metabolic syndrome					Dominant	0.562 [0.334–0.947]	0.030
No (n = 650)	591 (0.910)	56 (0.086)	3 (0.004)	0.048	Additive	0.579 [0.353–0.951]	0.031
Yes (n = 449)	424 (0.944)	24 (0.053)	1 (0.002)	0.029	Heterozygote	0.568 [0.335–0.963]	0.036
LDL pattern B					Dominant	0.521 [0.322–0.834]	0.008
No (n = 579)	524 (0.905)	52 (0.089)	3 (0.005)	0.050	Additive	0.509 [0.320–0.809]	0.004
Yes (n = 520)	492 (0.946)	28 (0.054)	0	0.027	Heterozygote	0.562 [0.346–0.913]	0.020
rs431264	*GG*	*GA*	*AA*				
Hypo-α-lipoproteinemia					Dominant	1.326 [1.026–1.713]	0.031
No (n = 528)	194 (0.367)	246 (0.466)	88 (0.167)	0.400	Recessive	1.209 [0.880–1.662]	0.241
Yes (n = 571)	182 (0.318)	283 (0.496)	106 (0.186)	0.433	Additive	1.205 [1.013–1.434]	0.036

Data are expressed as odds ratios (OR) and 95% confidence interval (95% CI) as assessed by multivariate logistic regression analyses. All models were adjusted by age, gender and body mass index.
[a] RAF = risk allele frequency; LDL = low density lipoprotein.

In healthy controls, under different models, rs2293808 was associated with a decreased risk of metabolic syndrome (OR = 0.562, 95% CI: 0.334–0.947, $P_{dom} = 0.030$; OR = 0.579, 95% CI: 0.353–0.951, $P_{add} = 0.031$; OR = 0.568, 95% CI: 0.335–0.963, $P_{het} = 0.036$) and LDL patterns B (OR = 0.521, 95% CI: 0.322–0.834, $P_{dom} = 0.008$; OR = 0.509, 95% CI: 0.320–0.809, $P_{add} = 0.004$; OR = 0.562, 95% CI: 0.346–0.913, $P_{het} = 0.020$); while rs431264 was associated with an increased risk of hypo-α-lipoproteinemia (OR = 1.326, 95% CI: 1.026–1.713, $P_{dom} = 0.031$; OR = 1.205, 95% CI: 1.013–1.434, $P_{add} = 0.036$) (Table 5). All models were adjusted by age, gender and BMI.

3.4. RIP2 haplotype analysis

The four polymorphisms included in the analysis were in strong linkage disequilibrium (D′ > 0.85) and five haplotypes were observed: *GAGA, GTAA, GAAG, AAAA, GAAA*. The *GAAG* haplotype was significantly associated with increased risk of SA (OR = 1.47, 95% CI: 1.01–2.16, P = 0.027) (Table 6).

4. Discussion

In the present study, after a functional prediction analysis, four *RIP2* gene polymorphisms were tested for association with the risk of developing SA. To the best of our knowledge, this is the first study that evaluates the role of *RIP2* polymorphisms reporting the association of rs43133 and rs16900627 with SA. Rip2 is a serine/threonine kinase that participates in pro-inflammatory transcription factor nuclear-κB (NF-κB) activation and pro-inflammatory molecule expression (Loppnow et al., 2008; Lundberg and Hansson, 2010), suggesting it could be a proatherogenic molecule. In a model analyzing mice transplanted with *Rip2* −/− bone marrow, Rip2 was found to have a protective role during atherogenesis (Levin et al., 2011). These mice showed decreased levels of several circulating proinflammatory mediators such as interleukin 6 and CXCL1, reduced systemic inflammation, and reduced local inflammation in the vessel wall as shown by a marked reduction in ICAM-1 expression in atherosclerotic lesions. Unexpectedly, these mice also showed increased subendothelial lipids in the aorta and an increased macrophage lipid uptake. Thus, these mice developed atherosclerosis despite having reduced local and systemic inflammation.

Table 6
RIP2 haplotype frequencies in SA and healthy controls.

Haplotypes	SA	Control	X^2	OR (95% CI)	P
G-A-G-A	0.576	0.577	0.005	0.99 (0.83–1.18)	0.944
G-T-A-A	0.299	0.318	0.934	0.91 (0.75–1.10)	0.333
G-A-A-G	0.068	0.047	4.887	1.47 (1.01–2.16)	0.027
A-A-A-A	0.037	0.035	0.110	1.05 (0.66–1.69)	0.740
G-A-A-A	0.011	0.013	0.209	0.84 (0.37–1.89)	0.647

SA, subclinical atherosclerosis; OR, odds ratio; CI, confidence interval.
The order of the polymorphisms in the haplotypes is according to their positions in the chromosome (rs2293808, rs43133, rs431264, rs16900627).

The informatics analyses showed that the rs43133 produces binding sites for several transcription factors (BRCA, CEBPA, CEBP, E2A and MYB), some of which participate in the regulation of the immune system and in different process during the liver, adipocyte and hematopoietic cell development (Miyazaki et al., 2014; Ohlsson et al., 2016). Moreover, rs16900627 produces binding sites for microRNAs (miR-217 and miR-367). MiR-217 is known to be expressed in human carotid plaques and to regulate the expression of silent information regulator 1 (Sirt1), suggesting a role in the pathogenesis of atherosclerosis (Menghini et al., 2009). In addition, several reports suggest that miR-367 plays a significant role in regulation of cellular proliferation, differentiation and reprogramming (Gao et al., 2015).

RIP2 polymorphisms have been associated with other disorders with immune involvement, such as systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD) and atopic asthma (Nakashima et al., 2006; Thiébaut et al., 2011; Li et al., 2012). While Li et al. (2012) reported an association of two *RIP2* polymorphisms (rs16900617 and rs16900627) with SLE in 590 Chinese patients and 660 healthy controls, the Nakashima group did not observe association of 31 *RIP2* polymorphisms with atopic asthma in a study involving 300 patients and 637 healthy controls (Nakashima et al., 2006). The association of rs16900627 with SA observed in the present study is consistent with the association with immune diseases like SLE reported by Li et al. (2012). Interestingly, we observed association of *RIP2* polymorphisms with metabolic traits that differed when analyzed separately in individuals with SA and healthy controls. In SA individuals, *RIP2* variants were associated with a decreased risk of insulin resistance and visceral abdominal fat (rs2293808), and with an increased risk of central obesity, hypertriglyceridemia and LDL pattern B and with decreased risk of hypoadiponectinemia (rs431264). In healthy controls, *RIP2* variants were associated with a decreased risk of metabolic syndrome and LDL pattern B (rs2293808), and with increased risk of hypo-α-lipoproteinemia (rs431264). At the present time, no studies seeking associations of *RIP2* polymorphisms with clinical and metabolic parameters have been reported. However, previous studies in our group of patients have demonstrated association of the *interleukin 24* polymorphisms with some clinical and metabolic characteristics in individuals with SA (Vargas-Alarcón et al., 2014), and of the interleukin 17 (Vargas-Alarcón et al., 2015) and monocyte chemoattractant protein-1 (Angeles-Martínez et al., 2015) polymorphisms with clinical and metabolic characteristics in CAD patients.

The *RIP2* polymorphisms were in strong linkage disequilibrium (D′ > 0.85), and haplotype *GAAG* was found to be significantly associated with an increased risk of SA. This haplotype is defined by the presence of

the rs16900627 *G* allele, which is consistent with the individual analysis of *RIP2* polymorphisms, where this allele was significantly associated with an increased risk of SA.

Some limitation of the study should be pointed out: firstly, the number of individuals with SA is reduced. Studies in other populations and in a higher number of individuals could help to define the effect of the *RIP2* polymorphisms as SA susceptibility markers. Moreover, only 4 polymorphisms were analyzed. However, these polymorphisms were selected using informatics tools, which allowed us to select only functionally predicted or informative polymorphisms. Informatics approaches should be demonstrated using specific experimental designs. Since this is the first work that documents the correlation of the *RIP2* polymorphisms with SA and metabolic parameters, further studies in an independent group of patients are required to validate the results.

In summary, our study shows that the *RIP2* rs43133 and rs16900627 polymorphisms are associated with an increased risk of developing SA. Some polymorphisms were associated with clinical or metabolic parameters in the SA group and in control individuals. The four *RIP2* polymorphisms were in high linkage disequilibrium and one haplotype (*GAAG*) was significantly associated with SA. Because the Mexican population has a distinct genetic background (Lisker et al., 1986; Lisker et al., 1988a; Lisker et al., 1988b; Juárez-Cedillo et al., 2008), these associations with SA and with clinical and metabolic parameters should be explored in other populations. We considered it is very important the study several polymorphisms within a specific gene, as each polymorphism may be show different associations with disease or other clinical and metabolic traits.

Author disclosure statement

No competing financial interests exist.

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RESEARCH ARTICLE

The rs7044343 Polymorphism of the Interleukin 33 Gene Is Associated with Decreased Risk of Developing Premature Coronary Artery Disease and Central Obesity, and Could Be Involved in Regulating the Production of IL-33

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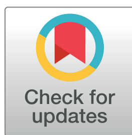

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Abstract

Aim

The effect of interleukin 33 (IL-33) in the inflammatory process generates significant interest in the potential significance of IL-33 as a biomarker for coronary artery disease (CAD). Here, our objective was to analyze whether IL-33 gene polymorphisms are associated with premature CAD in a case-control association study.

Methods

Four *IL-33* polymorphisms (rs7848215, rs16924144, rs16924159 and rs7044343) were genotyped by 5' exonuclease TaqMan assays in 1095 patients with premature CAD and 1118 controls.

Results

The rs7044343 *T* allele was significantly associated with a diminished risk of premature CAD (OR = 0.81, 95% CI: 0.69–0.97, P_{dom} = 0.020; OR = 0.85, 95% CI: 0.75–0.96, P_{add} = 0.019) and central obesity (OR = 0.74, 95% CI: 0.58–0.93, P_{dom} = 0.0007), respectively. When patients were divided into groups with and without type 2 diabetes mellitus (T2DM), the rs7044343 *T* allele was associated with a reduced risk of premature CAD in patients

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without (OR = 0.85, 95% CI: 0.73–0.99, P_{add} = 0.038) and with T2DM (OR = 0.61, 95% CI: 0.38–0.97, P_{dom} = 0.039; OR = 0.69, 95% CI: 0.49–0.97, P_{add} = 0.035). In order to establish the functional effect of the rs7044343 polymorphism, the production of IL-33 was determined in monocytes of selected individuals. Monocytes from individuals with rs7044343 *CC* genotype produced higher levels of IL-33 than monocytes from individuals with other genotypes.

Conclusion

The results suggest that the *IL-33* rs7044343 *T* allele could be a susceptibility marker for premature CAD and central obesity. The rs7044343 polymorphism could be involved in regulating the production of IL-33.

Introduction

Coronary artery disease (CAD) is a complex multifactorial disorder. This polygenic disease is caused by an inordinate inflammatory response to different forms of injuries to the arterial wall endothelium [1–3]. Admittedly, inflammation is as leading cause of atherogenesis since it disturbs lipoprotein metabolism and arterial wall biology. Infiltrates of T cells and activated macrophages are salient in atherosclerotic lesions of both humans and murines [4, 5]. The majority of T cells present in human atherosclerotic plaques belong to the CD4+ subset and produce predominantly cytokines of the Th1 subtype that have a critical pathogenic role in murine atherosclerosis models [6–10]. In contrast, it has been reported that the Th2 cells have an atheroprotective effect [11, 12].

IL-33 is a cytokine member of the IL-1 family, which includes IL-1 and IL-18 [13]. Unlike IL-1 and IL-18, which mainly promote Th1-associated responses, IL-33 predominantly induces the production of Th2 cytokines (IL-5 and IL-13) [14]. Miller et al. showed that IL-33 administration to ApoE-/- mice induced Th2 cytokines and protective ox-LDL antibodies, which significantly reduced atherosclerotic plaque development in the aortic sinus [15]. These data suggest that the gene that encodes IL-33 could be an important candidate gene for study in atherosclerosis. Recently, Tu et al. studied three IL-33 Tag SNPs (rs7025417, rs10975514, and rs10975519) in patients with CAD from the Chinese Han population [16]. In this study, the rs7025417 polymorphism was associated with CAD, with altered regulation of *IL-33* gene expression and with high plasma IL-33 levels. Results of association studies may vary between populations due to genetic differences amongst them, including differences in allele frequencies and linkage disequilibrium (LD) structures. Therefore, it is important to examine multiple ethnic populations for the identification of ethnicity-specific loci as well as common susceptibility loci. The objective of our study was to evaluate whether *IL-33* gene polymorphisms are associated with premature CAD in the Genetics of Atherosclerotic Disease (GEA) case-control association study. Also, the aim was to establish the possible effect of the associated polymorphism in the production of IL-33 in monocytes of individuals with different genotypes. After a functional prediction analysis, we selected four IL-33 gene polymorphisms (rs7848215, rs169 24144, rs16924159, and rs7044343) with possible functional consequences and with minor allele frequency $>$ 5% to be analyzed in the present study. The functional analysis showed that the rs7848215 produces a DNA binding site for the PBX1 transcription factor, the rs16924144 for SF/ASF, the rs16924159 for the SRp40 protein and the rs7044343 polymorphism produces binding site for the transcription factors SC35 and SF/ASF.

Material and Methods

Subjects

Every participant signed a written informed consent document. This protocol complies with the Declaration of Helsinki and was approved by the Ethics Committee of the Instituto Nacional de Cardiología Ignacio Chávez (INCICH). The GEA study focuses on the Mexican population and its main objective is to establish genetic factors linked with premature CAD and other coronary risk factors. All GEA study subjects are not blood related and are Mexican mestizos, who are defined as people born in Mexico, with an ancestry comprised of both indigenous inhabitants and individuals of African and/or Caucasian origin (mainly Spaniards), who had migrated to the Americas from the sixteenth century onward. A total of 2213 individuals were recruited, 1095 diagnosed with premature CAD and 1118 apparently healthy controls. History of myocardial infarction, angioplasty, revascularization surgery or coronary stenosis $>$50% on angiography (diagnosed before age 55 in men and before age 65 in women) was used to characterize premature CAD. Controls were seemingly healthy asymptomatic subjects without premature CAD family history, recruited from blood banks and Social Services centers. Congestive heart failure, liver, renal, thyroid or oncological disease were the exclusion criteria for controls. In an earlier report, we documented the selection of patients and controls of the GEA study [17]. Demographic, clinical, anthropometric, biochemical parameters and cardiovascular risk factors were assessed in all subjects. Qualified staff measured waist circumference, body mass index (BMI, kg/m^2) and other anthropometric parameters. A sphygmomanometer was used to determine blood pressure (the average of the last two of three assessments). Patients with BMI $\geq$30 kg/m^2 were classified as obese. Adult Treatment Panel III (ATP-III) criteria 2002 (Third report of the National Cholesterol Education Program) definitions were followed for central obesity, hypoalphalipoproteinemia, hypertriglyceridemia, and metabolic syndrome [18]. Total cholesterol (TC) levels $\geq$200 mg/dL defined hypercholesterolemia. Patients with systolic blood pressure $\geq$140 mmHg and/or diastolic blood pressure $\geq$90 mmHg, or the use of oral antihypertensive therapy were labeled as hypertense. And finally, we followed World Health Organization criteria to diagnose type 2 Diabetes mellitus (T2DM).

Computed tomography of the chest and abdomen

Experienced radiologists interpreted the computed tomography of the chest and abdomen, performed using a 64-channel multi-detector helical computed tomography system (Somatom Sensation, Siemens). Coronary artery calcification (CAC) score was calculated using the Agatston method [19]. Total abdominal, subcutaneous and visceral adipose tissue areas (as described by Kvist et al.) were measured to assess the visceral to subcutaneous adipose tissue ratio (VAT/SAT) [20]. The hepatic to splenic attenuation ratio (LSAR) was estimated as described by Longo et al. [21]. CAC, VAT/SAT and LSAR were quantified using tomography scans. All patients and 1523 healthy controls underwent tomography. 405 controls were not considered for analysis, since their CAC score was positive, and they were thus considered as individuals with subclinical atherosclerosis (SA). The final control group included individuals (n = 1118) with only negative CAC scores.

Genetic analysis

We isolated genomic DNA from whole blood containing EDTA using standard techniques. The rs7848215, rs16924144, rs16924159, and rs7044343 *IL-33* single nucleotide polymorphisms (SNPs) were genotyped using 5' exonuclease TaqMan assays on an ABI Prism 7900HT

Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Genotyping call rate surpassed 95% for all SNPs tested, with no discordant genotypes in 10% of duplicate samples. We adhered to the manufacturer's instructions to perform the assays.

Because the Mexican-Mestizo population is admixed, in order to assess the possible influence of population stratification, a panel of 265 ancestry informative markers (AIMs) distinguishing mainly Amerindian, European and African ancestry were selected [22] and genotyped on Illumina BeadStation using the GoldenGate assay. Duplicate control samples were genotyped on each chip, which also served as internal controls for quality of clustering and reproducibility. The primary analysis of the genotyping data with the Illumina Genome Studio software v.2011.1 was followed by visual inspection and assessment of data quality and clustering. Genotyping accuracy was also assessed by genotype clustering using the Illumina GeneTrain score, which is a measure of the clustering confidence of individual SNP alleles. Global Caucasian, Amerindian and African ancestry were determined in each individual using the ADMIXTURE software.

Functional prediction analysis

The effect of the *IL-33* SNPs was predicted using the following bioinformatics software: FastSNP [23], SNP Function Prediction (http://snpinfo.niehs.nih.gov/snpfunc.htm), Human-transcriptome Database for Alternative Splicing (http://www.h-invitational.jp/h-dbas/), Splice Port: An Interactive Splice Site Analysis Tool (http://www.spliceport.cs.umd.edu/SplicingAnalyser2.html), ESE finder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi), HSF (http://www.umd.be/HSF/), and SNPs3D (http://www.snps3d.org/).

Monocyte isolation

A sample of venous blood (40 mL) was obtained from 61 healthy controls selected according to the rs7044343 polymorphism (21 with *CC*, 21 with *TC* and 21 with *TT* genotypes). The peripheral blood mononuclear cell (PBMC) population was isolated by gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Monocytes were isolated by positive selection with CD14-mAb-coated micro beads (Miltenyi Biotec, Bergisch Gladbach; Germany) following the manufacturer's instructions (purity of 95–98%).

Monocyte cultures and IL-33 detection

Monocytes were counted in a Neubauer hemocytometer (Propper MFG Company, NY USA) chamber using 0.4% Trypan blue stain (Cambrex Bio Science, MD USA) to exclude dead cells. Monocyte density in culture was adjusted to 1 $x10^6$ per milliliter. Monocytes were cultured in RPMI-1640 medium (Sigma, Poole, UK), supplemented with 10% (volume/volume) heat-inactivated fetal bovine serum (Sigma), 0.1 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were stimulated with 100 ng/ml *E coli* lipopolysaccharide (from strain 0111:B4, Sigma) and 100 ng/ml of *P gingivalis* lipopolysaccharide (Invivogen, Calne, UK) for 6 hours in a humidified atmosphere with 5% CO2 at 37˚C. Necrosis was induced by subjecting stimulated cells to five cycles of freezing to -70˚C and thawing at 38˚C. Necrotic cell preparations were centrifuged at 10,000 for 5 min and supernatants were kept at -70˚C. The IL-33 levels were detected using specific ELISA kit for IL-33 (Biolegend, San Diego, CA); the sensitivity for the ELISA was 4.14 pg/ml.

Statistical analysis

The SPSS version 18.0 statistical package (SPSS, Chicago, Il) was employed for the statistical estimation of means ± SD and frequencies of baseline characteristics. We compared frequencies

using Chi-square tests, and means using the ANOVA and Students t-test. To determine the association between the polymorphisms and metabolic variables, we used ANCOVA and adjusted for age, gender, BMI, smoking history and alcohol consumption. The correlation of polymorphisms with premature CAD under dominant, recessive and additive inheritance models was analyzed with logistic regression analysis. Also, we utilized age, gender, BMI, smoking history, alcohol consumption and ancestry to adapt the models. They were constructed including one variable at time, and final models included variables with biological relevance or with statistical significance or both. Confounding bias was accepted when changes in estimated odds ratios (ORs) were equal or greater than 10%. When a principal effect model was reached, effect modification was also tested and interactions terms were constructed between the polymorphisms and different variables; the terms were included in the model when the significance of the p-value was greater or equal to 0.20. Hosmer–Lemeshow Goodness of Fit test was performed for each multiple logistic model. Bonferroni correction was used as appropriate. Statistical power to detect association with CAD was 0.80 as estimated with QUANTO software [http://hydra.usc.edu/GxE/]. The obtained genotype frequencies did not deviate from Hardy-Weinberg equilibrium (HWE, $P > 0.05$). Haploview version 4:1 (Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA) was used to calculate pairwise linkage disequilibrium (LD, D´) between polymorphisms and haplotype reconstruction.

Results

Tables 1 and 2 illustrate the general characteristics of the study population. Global ancestry was similar in patients and controls, showing 55.8% and 54.0% of Native American ancestry respectively; and 34.3% and 35.8% of Caucasian ancestry, respectively.

Association of polymorphisms with premature CAD

Genotype frequencies in the polymorphic sites were in HWE. In all the evaluated models, the distribution of rs16924144, rs16924159, and rs7848215 polymorphisms was comparable in premature CAD patients and healthy controls. Conversely, the distribution of rs7044343 was not the same in the investigated groups. The rs7044343 *T* allele was associated with diminished risk of premature CAD when contrasted with to healthy controls (OR = 0.81, 95% CI = 0.69–0.97, $P_{dom} = 0.020$; OR = 0.85, 95% CI: 0.75–0.96, $P_{add} = 0.019$)) under dominant and additive models adjusted for age, gender, BMI, smoking history, alcohol consumption and ancestry (Table 3).

Association of the polymorphisms with cardiovascular risk factors

We considered the association of rs16924144, rs16924159, rs7848215 and rs7044343 polymorphisms with cardiovascular risk factors by comparing CAD patients and healthy controls. Under dominant model adjusted by age, gender, BMI, smoking history and alcohol consumption, the rs7044343 polymorphism was associated with reduced risk of central obesity (OR = 0.74, 95% CI = 0.58–0.93, $P_{dom} = 0.0007$) (Table 4).

Association of the polymorphisms with metabolic parameters

We evaluated the effect of rs16924144, rs16924159, rs7848215 and rs7044343 polymorphisms on numerous metabolic parameters separately in controls (CAC score = 0), and premature CAD subjects. None of the studied polymorphisms was associated with metabolic parameters in the groups.

Table 1. Demographic characteristics of the population.

		Premature CAD (n = 1095)			Controls (n = 1118)			P value
		P25	Median	P75	P25	Median	P75	
Age (years)		49	54	59	45.0	51.0	57.0	<0.0001
Body Mass Index (Kg/m^2)		26.0	28.3	31.3	25.4	27.9	31.0	0.006
Waist circumference (cm)		91.2	97.5	105.4	86.0	94.0	101.5	<0.0001
Total Abdominal Fat (cm2)		340	426	530.25	346	443	545	0.186
Subcutaneous Abdominal Fat (cm2)		193	245.5	316	219.3	288.5	373.0	<0.0001
Visceral Abdominal Fat (cm2)		130	171	218.5	104	142	183	<0.0001
Visceral/Subcutaneous adipose tissue ratio		0.95	1.29	1.81	0.53	1.26	2.29	0.031
Blood Pressure (mmHg)	Systolic	107	116.3	128	105.3	114.6	125.0	0.002
	Diastolic	66	71.7	78.5	66.0	71.3	77.3	0.235
Heart Rate (bpm)		57.5	64.3	72.7	59.5	65.3	71.0	0.158
Gender n (%)	Male	905 (82.6)			456 (40.8)			
	Female	192 (17.4)			662 (59.2)			<0.0001
Weight n (%)	Normal weight	190 (17.4)			259 (23.2)			
	Overweight	515 (47)			508 (45.4)			0.005
	Obesity	390 (35.6)			351 (31.4)			<0.000
Central obesity n (%)		866 (79.2)			868 (77.7)			0.263
Tobacco smoking n (%)	Current	135 (12.3)			250 (22.4)			<0.0001
	Former	702 (65.4)			346 (35.2)			<0.0001
Use of alcohol n (%)		610 (55.7)			823 (73.8)			<0.0001
Hypertension n (%)		740 (67.6)			302 (27.0)			<0.0001
Hypertensive Medication n (%)		737 (71.3)			170 (15.2)			<0.0001

Data are expressed as median and percentiles 25 and 75.

*P values were estimated using Mann-Whitney U-test continuous variables and Chi-square or Fisher test for categorical values.

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Association of the polymorphisms with premature CAD in patients with and without diabetes mellitus

Considering the high frequency of diabetes mellitus in our group of patients with CAD, we carried out an analysis in patients with and without this pathology in order to establish if the polymorphisms are associated with CAD or with T2DM. The rs7044343 *T* allele was associated with decreased risk of CAD in patients without T2DM (OR = 0.85, 95% CI = 0.73–0.99, P_{add} = 0.038) (Table 5) and with T2DM (OR = 0.61, 95% CI = 0.38–0.97, P_{dom} = 0.039; OR = 0.69, 95% CI = 0.49–0.97, P_{add} = 0.035) (Table 6). The models were adjusted for age, gender, BMI, smoking history, alcohol consumption and ancestry.

Haplotype analysis and SNP functional prediction

Even though the *IL-33* polymorphisms were in high linkage disequilibrium (D'>0.8 and r^2>0.9), the distribution of the haplotypes in premature CAD patients and healthy controls was comparable (data not included).

Interestingly, SNP functional prediction software results suggest that the rs7044343 polymorphism is functional. The variation in the rs7044343 polymorphism produces a DNA binding site for the transcription factors SC35 and SF/ASF with possible consequences in the expression of the *IL-33*.

Table 2. Comparison of biochemical parameters in individuals with premature CAD and healthy controls.

	CAD premature			Controls			P value
	P25	Median	P75	P25	Median	P75	
Total cholesterol (mg/dl)	132.50	160.70	193.60	168.08	190.05	210.00	<0.0001
HDL-C (mg/dl)	32.50	38.30	45.05	36.93	46.00	56.00	<0.0001
LDL-C (mg/dl)	68.70	91.00	116.00	96.16	115.76	133.67	<0.0001
Triglycerides (mg/dl)	119.00	162.80	221.60	108.10	143.20	199.50	<0.0001
ApoA1 (mg/dl)	63	79	102	73	90	108	<0.0001
ApoB (mg/dl)	102.00	120.00	136.90	114	134	157	<0.0001
Glucose (mg/dl)	87	95	120	84	90	97	<0.0001
Insulin	14.84	20.04	28.19	12.59	17.51	24.04	<0.0001
HOMA	3.53	5.15	7.86	2.70	3.94	5.68	<0.0001
Alanine transaminase (IU/L)	19.0	26.0	36.0	17.0	23.0	33.0	0.002
Aspartate transaminase (IU/L)	22.0	26.0	31.0	21.0	25.0	30.0	0.002
Alkaline Phosphatase (IU/L)	64.0	77.0	95.0	68.0	81.0	97.8	0.001
Gamma-glutamyl transpeptidase (IU/L)	23.0	33.0	50.0	17.0	25.0	41.0	<0.0001
TC > 200 mg/dL n (%)	228 (20.8)			401 (35.9)			<0.0001
Hypo-a-lipoproteinemia n (%)	696 (63.6)			565 (50.6)			<0.0001
Hypertriglyceridemia n (%)	626 (57.2)			520 (46.6)			<0.0001
Type 2 Diabetes Mellitus n (%)	414 (37.8)			109 (9.7)			<0.0001
Metabolic Syndrome n (%)	551 (50.3)			450 (40.3)			<0.0001

Data are expressed as median and percentiles 25 and 75.

*P values were estimated using Mann-Whitney U-test continuous variables and Chi-square or Fisher test for categorical values.

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Table 3. Associations of *IL33* polymorphisms with premature CAD.

Polymorphism	Alleles	MAF[a]	MAF[a]	Genotypes	Genotypes	P_{hwe}	OR (95% CI)
		CAD	Control	Premature CAD	Control	CAD/Control	P_{dom} value
rs16924144	*C/T*	0.48	0.48	281/564/250	294/569/255	0.33/0.55	0.85 (0.62–1.18); 0.34
				0.25/0.52/0.23	0.26/0.51/0.23		1.00 (0.73–1.38); 0.97[b] 0.94 (0.77–1.15); 0.57[c]
rs16924159	*A/G*	0.49	0.49	226/448/224	238/471/227	0.95/0.90	1.07 (0.74–1.55); 0.69
				0.25/0.50/0.25	0.25/0.50/0.24		1.06 (0.72–1.56); 0.75[b] 1.05 (0.83–1.32); 0.66[c]
rs7848215	*C/T*	0.14	0.14	827/238/30	832/263/23	0.01/0.71	0.91 (0.67–1.24); 0.58
				0.75/0.22/0.03	0.74/0.24/0.02		1.60 (0.66–3.84); 0.29[b] 0.98 (0.75–1.27); 0.88[c]
rs7044343	*C/T*	0.33	0.37	481/492/122	437/540/141	0.84/0.22	**0.81 (0.69–0.97); 0.020**
				0.44/0.45/0.11	0.39/0.48/0.13		0.84 (0.56–1.27); 0.420 [b]
							0.85 (0.75–0.96); 0.019 [c]

Adjusted for age, gender, BMI, smoking history, alcohol consumption and ancestry.

[a]: MAF, minor allele frequency.

[b]: recessive model.

[c]: additive model.

CAD premature: Coronary artery disease premature.

Phwe: p value from Hardy-Weinberg equilibrium tests.

NS: Not significant.

*Underlined letter denotes the minor allele in the control samples.

Significant values are in bold.

The p values were corrected multiplying by 4, number of SNPs tested.

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Table 4. Association of the rs7044343 polymorphism with metabolic risk factors.

	Dominant model	Premature CAD	Control	OR (95% CI)	P value
Obesity *n* (%)	*C/C*	162 (0.15)	124 (0.11)		
	C/T+TT	221 (0.20)	179 (0.16)	NS	-
Central obesity	*C/C*	372 (0.34)	286 (0.26)		
	C/T+TT	470 (0.43)	475 (0.42)	**0.74 (0.58–0.93)**	**0.0007**
Hypo-α-lipoproteinemia *n* (%)	*C/C*	287 (0.26)	199 (0.18)		
	C/T+TT	386 (0.35)	287 (0.26)	NS	-
Hypercholesterolemia *n* (%)	*C/C*	102 (0.09)	120 (0.11)		
	C/T+TT	121 (0.11)	225 (0.20)	NS	-
Hypertriglyceridemia *n* (%)	*C/C*	264 (0.24)	173 (0.15)		
	C/T+TT	346 (0.32)	271 (0.24)	NS	-
Metabolic syndrome *n* (%)	*C/C*	229 (0.21)	163 (0.15)		
	C/T+TT	307 (0.28)	228 (0.20)	NS	-
Type 2 diabetes mellitus *n* (%)	*C/C*	176 (0.16)	51 (0.05)		
	C/T+TT	226 (0.21)	49 (0.04)	NS	-

The model was adjusted by age, gender, BMI, smoking history and alcohol consumption.

NS: Not significant.

OR: Odds ratio.

CI: Confidence intervals.

Significant values are in bold.

The dominant model was analyzed considering 7 metabolic risk factors, so the p values were corrected multiplying by 7.

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Table 5. Associations of *IL33* polymorphisms in patients with premature CAD without diabetes mellitus.

Polymorphism	Alleles	MAF[a]	MAF[a]	Genotypes	Genotypes	P_{hwe}	OR (95% CI)
		CAD	Control	Premature CAD	Control	CAD/Control	P_{dom} value
rs16924144	*C/T*	0.49	0.48	168/355/159	240/460/211	0.45/0.38	0.86 (0.60–1.25); 0.45
				0.24/0.52/0.23	0.26/0.51/0.23		0.99 (0.69–1.42); 0.96[b] 0.94 (0.75–1.18); 0.62[c]
rs16924159	*A/G*	0.50	0.50	142/296/145	192/400/192	0.87/0.50	1.17 (0.77–1.78); 0.44
				0.24/0.51/0.25	0.25/0.51/0.24		1.06 (0.68–1.65); 0.77[b] 1.09 (0.83–1.42); 0.51[c]
rs7848215	*C/T*	0.15	0.14	500/162/20	678/213/20	0.09/0.52	0.97 (0.68–1.37); 0.88
				0.73/0.24/0.03	0.74/0.23/0.02		1.30 (0.51–3.35); 0.57[b]1.01 (0.74–1.35); 0.95[c]
rs7044343	*C/T*	0.34	0.38	300/306/76	343/447/121	0.87/0.29	1.03 (0.74–1.45); 0.826
				0.44/0.45/0.11	0.38/0.49/0.13		0.81 (0.50–1.32); 0.404[b]
							0.85 (0.73–0.99); 0.038[c]

Adjusted for age, gender, BMI, smoking history, alcohol consumption and ancestry.

[a]: MAF, minor allele frequency.

[b]: recessive model.

[c]: additive model.

CAD premature: Coronary artery disease premature.

Phwe: p value from Hardy-Weinberg equilibrium tests.

NS: Not significant.

*Underlined letter denotes the minor allele in the control samples.

Significant values are in bold.

The p values were corrected multiplying by 4, number of SNPs tested.

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Table 6. Associations of *IL33* polymorphisms in patients with premature CAD and diabetes mellitus.

Polymorphism	Alleles	MAF[a]	MAF[a]	Genotypes	Genotypes	P_{hwe}	OR (95% CI)
		CAD	Control	Premature CAD	Control	CAD/Control	P_{dom} value
rs16924144	C/T	0.47	0.49	109/207/86	167/358/162	0.55/0.13	0.77 (0.46–1.31); 0.34
				0.27/0.52/0.21	0.24/0.52/0.24		0.95 (0.56–1.61); 0.84[b]0.89 (0.64–1.23); 0.48[c]
rs16924159	A/G	0.50	0.48	83/156/85	148/315/130	0.51/0.18	1.14 (0.64–2.04); 0.65
				0.26/0.48/0.26	0.25/0.53/0.22		1.31 (0.71–2.42); 0.39[b] 1.16 (0.80–1.67); 0.42[c]
rs7848215	C/T	0.12	0.15	316/75/11	500/172/15	0.03/1.00	0.68 (0.41–1.13);0.145
				0.79/0.19/0.03	0.73/0.25/0.02		0.96 (0.24–3.72); 0.95[b]
							0.75 (0.49–1.16); 0.202[c]
rs7044343	C/T	0.33	0.40	176/185/41	235/351/101	0.66/0.26	**0.61 (0.38–0.97); 0.039**
				0.44/0.46/0.10	0.34/0.51/0.15		0.65 (0.34–1.27); 0.216[b]
							0.69(0.49–0.97); 0.035[c]

Adjusted for age, gender, BMI, smoking history, alcohol consumption and ancestry.

[a]: MAF, minor allele frequency.

[b]: recessive model.

[c]: additive model.

CAD premature: Coronary artery disease premature.

Phwe: p value from Hardy-Weinberg equilibrium tests.

NS: Not significant.

*Underlined letter denotes the minor allele in the control samples.

In this analysis the control group only included individuals without diabetes.

Significant values are in bold.

The p values were corrected multiplying by 4, number of SNPs tested.

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IL-33 levels in monocytes

In order to establish the functional effect of the rs7044343 polymorphism, the production of IL-33 was determined in monocytes of selected individuals (Fig 1). Monocytes from individuals with rs7044343 *CC* genotype produced higher levels of IL-33 (32.08+24.30) than those from patients with *CT* (16.32 ± 6.23) (P = 0.005) and *TT* (17.10 ± 5.48) (P = 0.007) genotypes.

Discussion

IL-33 is a cytokine with an important role in the inflammatory process and in the pathogenesis of atherosclerosis [15]. Animal studies have indicated that IL-33 reduces macrophage foam cell formation [24] and inhibits the development of atherosclerosis in apolipoprotein E-deficient mice [15]. In spite of the important role of IL-33 in the development of atherosclerosis, too few studies have explored the possible role of the gene that encodes this cytokine in the genetic susceptibility to coronary artery disease. Tu et al. reported the association of the *IL-33* rs7025417 polymorphism with the risk of developing CAD in a Chinese Han population, thus demonstrating an effect of this polymorphism in the *IL-33* gene expression and plasma levels [16]. In another work, four *IL-33* polymorphisms (rs1929992, rs10975520, rs11792633 and rs16924159) were studied in Chinese patients with CAD and none of them was associated with the disease [25]. Due to the effect of IL-33 in the inflammatory process, other polymorphisms in this gene have been associated with asthma, inflammatory bowel disease and Alzheimer's disease [26–28]. In our study, four *IL-33* gene polymorphisms (rs7848215, rs16924144, rs16924159, and rs7044343) were analyzed in order to establish their role as a susceptibility marker for premature CAD. We selected these polymorphisms considering their possible

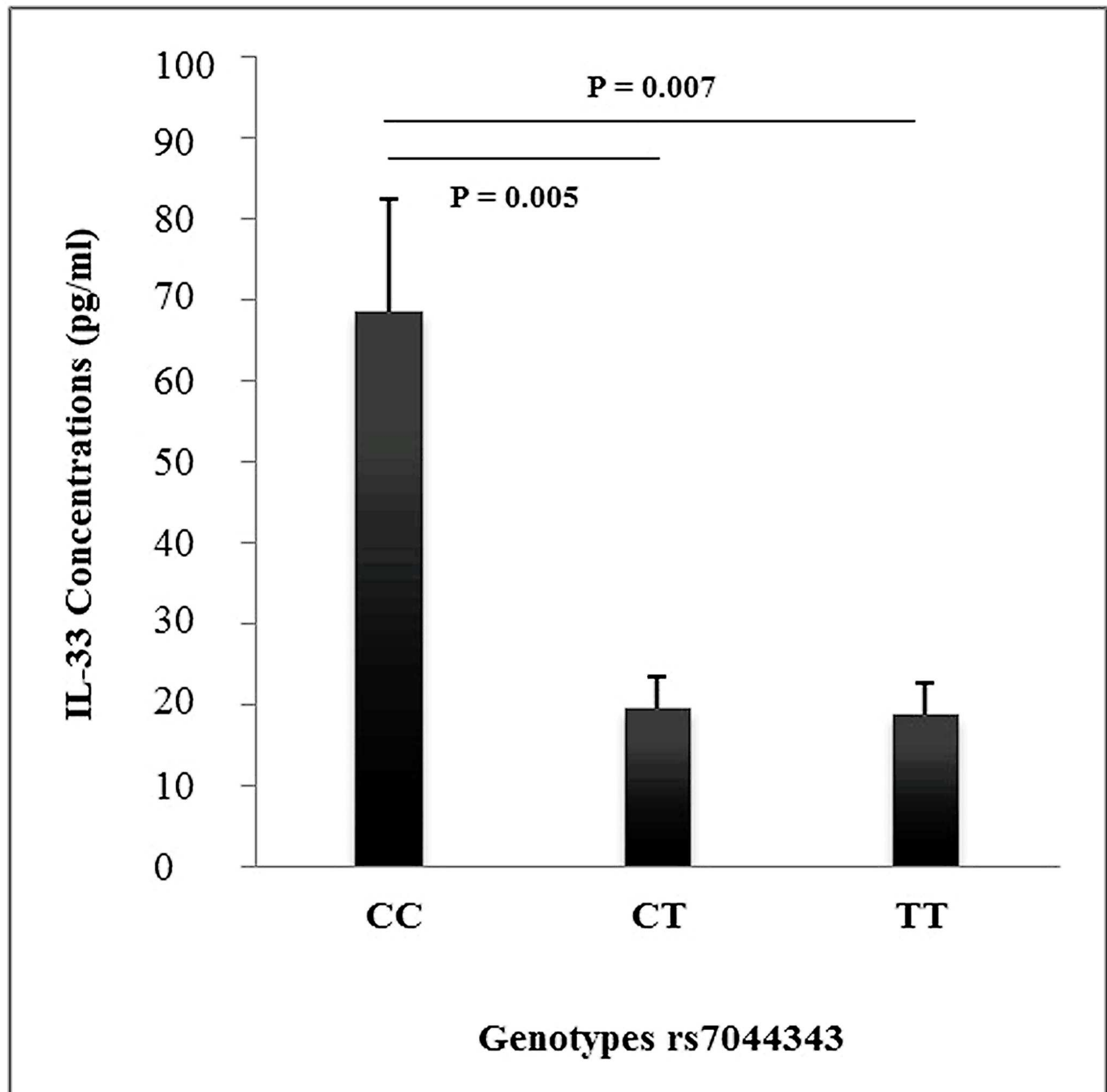

Fig 1. Detection of IL-33 in monocytes from healthy individuals with rs7044343 *CC*, *TC* and *TT* genotypes (21 participants were included for each genotype). Data are presented as mean ± SD. *CC* vs *CT* (P = 0.005) and *CC* vs *TT* (P = 0.007).

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functional effect after an informatics analysis and considering that they have a minor allele frequency major to 5%. In our study, the rs7044343 was associated with reduced risk of developing CAD in patients with and without diabetes mellitus. This polymorphism was also associated with reduced risk of developing central obesity. The association of the rs7044343 genotypes in some diseases is contradictory. In the study of Li et al., the rs7044343 *CC* genotype was associated with decreased risk of developing rheumatoid arthritis (RA) and with low serum IL-33

levels [29]. Contrary to that detected in RA, in systemic sclerosis, the rs7044343 *CC* genotype was associated with increased risk of developing this disease [30]. Our result of rs7044343 polymorphism contrasts with that reported by Li et al., [29] in RA, however, are in line with that reported by Koca et al., [30] in systemic sclerosis, because in our study, the rs7044343 *T* allele was significantly associated with a diminished risk of premature CAD. Also, in the study of Li et al., [29] the rs7044343 *CC* genotype was associated with low serum IL-33 levels in AR patients. This result contrasts with our report, because, we detected a higher production of IL-33 in monocytes of individuals with the *CC* genotype, compared to those carrying CT and TT genotypes. Some methodological differences between the two studies could explain the apparent contradictory results. In the study by Li et al., [29] the measures were made in serum of RA patients, whereas in our study, the measures were made in monocytes cultures of healthy controls. The functional prediction software used here predicted that this polymorphism is functional. The presence of the *C* allele in this polymorphism produces a binding site for the transcription factors SC35 and SF/ASF proteins. These proteins belong to the family of SR proteins that regulate alternative splicing [31]. This polymorphism could have functional effects increasing the production of IL-33 isoforms with the consequent increase of the anti-atherogenic effect of this cytokine. In order to establish the functional effect of the rs7044343 polymorphism, the production of IL-33 was determined in monocytes of selected individuals. Monocytes from individuals with rs7044343 *CC* genotype produced higher levels of IL-33 than monocytes from individuals with other genotypes, suggesting a role of this polymorphism in the production of IL-33. It should be mentioned that monocytes used for this analysis were obtained from healthy individuals (without CAD or CAC). Therefore, the fact that individuals with the *CC* genotype produce more IL-33 does not necessarily mean the development of CAD. Alternatively, the inflammatory process in CAD includes the participation of several both pro- and anti-inflammatory cytokines. This is a complex phenomenon in which IL-33 may be playing a very important role. The functional analysis on monocytes only was made considering the rs7044343 polymorphism because the main objective of the study was to analyze whether IL-33 gene polymorphisms are associated with premature CAD. The analysis in the whole group of CAD patients and in the group of CAD patients with and without T2DM confirms the association of the rs7044343 polymorphism with CAD.

As for the limitations herein, we only included the study of four polymorphisms of *IL-33*, which seem to be functional based on the analysis of the prediction software used. In our study, we do not analyze the expression and neither plasma levels of IL-33 in CAD patients and healthy controls. However, we consider that the evaluation of IL-33 production in monocyte cultures of individuals with different genotypes could be a more direct approach of the effect of these genotypes in the production of IL-33. Since this is the first work that documents the correlation of the *IL-33* polymorphisms with premature CAD and central obesity, further studies in an independent group of patients are required to validate the results. Indeed, a strength of our work is that the control group only included individuals without subclinical atherosclerosis (individuals without coronary artery calcification).

The *IL-33* polymorphisms were in strong linkage disequilibrium in the present work; and still, the haplotypes were not associated with premature CAD. Crawford et al. described that the haplotype architecture of candidate genes across the human genome is convoluted. Also, they mentioned that a considerable the amount of sequence variation has not been documented yet [32]. Consequently, the absence of association of *IL-33* haplotypes in our study is not definitive, owing to the incomplete knowledge of both the genetic variation within the *IL-33* gene and the structure of linkage disequilibrium in the analyzed region.

Conclusions

In conclusion, the association of the *IL-33* rs7044343 polymorphism with both premature CAD and central obesity is established here. This polymorphism had functional effects, based on an *in silico* prediction analysis. In this study, we demonstrate that the rs7044343 polymorphism has an effect in the production of IL-33 in monocytes stimulated by lipopolysaccharide. Notably, Mexican people form a population with a distinctive genetic background and important differences [33–36]. Thus, owing to these genetic characteristics, the associations of the *IL-33* polymorphisms shown here are not definitive and should be tested in other independent populations.

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Association of the I148M/PNPLA3 (rs738409) polymorphism with premature coronary artery disease, fatty liver, and insulin resistance in type 2 diabetic patients and healthy controls. The GEA study

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ABSTRACT

The aim of this study was to evaluate the potential use of the *I148M/PNPLA3* (rs738409) gene polymorphism as a susceptibility marker for premature coronary artery disease (pCAD) and/or cardiovascular risk factors in Mexican type 2 diabetes mellitus patients (T2DM). The polymorphism was genotyped by 5′ exonuclease TaqMan assays in a group of 2572 subjects (1103 with pCAD and 1469 healthy controls) belonging to the Genetics of Atherosclerotic Disease (GEA) Mexican Study. Anthropometric and biochemical measurements were performed in all individuals. The association between the *I148M/PNPLA3* (rs738409) gene polymorphism with pCAD and other metabolic and cardiovascular risk factors was evaluated using logistic regression analysis under different statistical approaches including dominant, recessive, heterozygous, additive, and co-dominant models. The polymorphism was not associated with pCAD in the whole group of participants, however, when patients and controls were divided into those with and without T2DM, under additive model, the polymorphism was associated with the presence of pCAD only in patients with T2DM (OR = 1.20, 95% CI: 1.01–1.42, P_{add} = 0.042). On the other hand, under several models adjusted for age, gender, body mass index and T2DM, the polymorphism was associated with increased risk of fatty liver and elevated levels of alanine transaminase (ALT) in the whole group of pCAD patients and controls. In the control group, the polymorphism was associated with insulin resistance and coronary artery calcification (CAC) score ≥ 10 under several models. The results suggest that the *I148M/PNPLA3* (rs738409) polymorphism is associated with the presence of pCAD in T2DM patients and with some cardiometabolic parameters. The association detected with CAC in the control group indicates that this polymorphism could be a marker for subclinical atherosclerosis.

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1. Introduction

Coronary artery disease (CAD) is a complex multifactorial and polygenic disorder resulting from an excessive inflammatory response to various forms of injurious stimuli to the arterial wall (Ross, 1999; Garcia-Moll, 2005; Lusis, 2000). Multiple genetic factors may work in conjunction with environmental factors to confer susceptibility to CAD. Genome wide association (GWA) studies have identified several genetic loci associated with CAD risk in different ethnic groups (CARDIoGRAMplusC4D Consortium et al., 2013; O'Donnell et al., 2011; Schunkert et al., 2011; Lee et al., 2013; Lu et al., 2012; The Coronary Artery Disease (C4D) Genetics Consortium et al., 2011). Results of association studies may vary between populations due to population genetic differences, including allele frequencies differences and linkage disequilibrium (LD) structures (Neale and Sham, 2004). Therefore, it is important to examine multiple ethnic populations for the identification of ethnicity-specific loci as well as common susceptibility loci.

Fatty liver has been considered as an independent risk factor for CAD (Assy et al., 2010). *PNPLA3* gene variant (*I148M*) has been strongly associated with fatty liver in several populations, includ-

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ing Hispanic (Zhang et al., 2014; Baclig et al., 2014; Lee et al., 2014; Goran et al., 2010). Coronary artery calcification (CAC) is a measure of subclinical atherosclerosis that predicts cardiovascular disease morbidity and mortality independently of traditional cardiovascular risk factors (Alexopoulos et al., 2003). However, the association of the *I148M/PNPLA3* polymorphism with pCAD plus T2DM, pCAD without T2DM, and in control subjects with subclinical atherosclerosis (SA), defined as CAC score $\geq$ 10, has not been yet described. The *PNPLA3* gene encodes the patatin-like phospholipase 3, known as adiponutrin. This molecule was originally identified as a member of the calcium-independent phospholipase A2 family and has both triacylglycerol hydrolase and acylglycerol transacetylase activity (Jenkins et al., 2004). In humans as well as animals, adiponutrin is primarily expressed in liver and white adipose tissue (Lake et al., 2005). It has been reported that adiponutrin expression is nutritionally regulated (Baulande et al., 2001), and its levels increase with obesity (Romeo et al., 2009). Moreover, it has been recently shown that adiponutrin may also have a role in adipogenesis, being up-regulated during the differentiation of white adipocytes (Calvo and Obregon, 2009).

Based on these findings and considering that fatty liver has been associated with an increased risk of coronary vascular disease, the aim of the present study was to establish if the *I148M/PNPLA3* (rs738409) polymorphism could be associated with risk of developing pCAD, the presence of fatty liver, cardiometabolic risk factors and CAC in the Mexican adult population. To test this hypothesis, we analyzed the distribution of the *I148M/PNPLA3* (rs738409) polymorphism in patients with pCAD with and without T2DM and healthy controls, using the CAC score $\geq$ 10 as a surrogate marker for SA.

2. Material and methods

2.1. Study subjects

All participants provided written informed consent. The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of the Instituto Nacional de Cardiología Ignacio Chávez (INCICH). The primary aim of the Genetics of Atherosclerotic Disease (GEA) Study is to investigate genetic factors associated with pCAD, and other coronary risk factors in the Mexican population. All GEA participants are unrelated and of self-reported Mexican-Mestizo ancestry (three generations). A total of 2572 individuals were included in the study, 1103 diagnosed with premature CAD and 1469 healthy controls. pCAD was defined as history of myocardial infarction, angioplasty, revascularization surgery or coronary stenosis >50% on angiography, diagnosed before age 55 in men and before age 65 in women. Controls were individuals apparently healthy without family history of pCAD, recruited from blood bank donors and through brochures posted in Social Services centers. Exclusion criteria for controls included congestive heart failure, liver, renal, thyroid or oncological disease. The selection of the patients and controls of the GEA study were described in a previous study (Villarreal-Molina et al., 2012). Demographic, clinical, anthropometric, biochemical parameters and cardiovascular risk factors were evaluated in both patients and controls. Anthropometric parameters were measured by trained personnel, and included waist circumference and body mass index (BMI) calculated as weight in kilograms divided by height in square meters. Blood pressure was measured on three different times by sphygmomannometry and the average of the last two measurements was used for analyses. Obesity was defined as BMI $\geq$30 kg/m^2. Hypoalphalipoproteinemia, hypertriglyceridemia and metabolic syndrome (MS), were defined using the criteria from the American Heart Association National Heart, Lung, and Blood Institute Scientific Statement on the MS (Grundy et al., 2005) except for central obesity that was considered when waist circumference was 90 cm in men and 80 cm in women (Sánchez-Castillo et al., 2003). Hypercholesterolemia was defined as total cholesterol (TC) levels $\geq$200 mg/dL. Hypertension was defined as systolic blood pressure $\geq$140 mmHg and/or diastolic blood pressure $\geq$90 mmHg or the use of oral antihypertensive therapy. T2DM was defined by the American Diabetes Association criteria (American Diabetes Association, 2009), with a fasting glucose $\geq$126 mg/dL and was also considered when participants reported glucose-lowering treatment or a physician diagnosis of diabetes. Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR). The presence of insulin resistance was considered when the HOMA-IR values were $\geq$75th percentile (3.66 in women and 3.38 in men). Hyperinsulinemia was defined when insulin concentration was $\geq$75th percentile (16.97 μU/mL in women and 15.20 μU/mL in men). Increase visceral abdominal tissue (VAT) was defined as VAT $\geq$ 75th percentile (122.0 cm^2 in women and 151.5 cm^2 in men). Elevated alanine transaminase (ALT) was defined as ALT activity $\geq$75th percentile (21 IU/L in women and 24.5 IU/L in men). These cutoff points were obtained from a GEA study sample of 131 men and 185 women without obesity and with normal values of blood pressure, fasting glucose and lipids.

2.2. Biochemical analyses

Venous blood samples were collected from subjects after a 12 h fast. Plasma glucose, total cholesterol, triglycerides, high density lipoprotein cholesterol, apolipoprotein A (apo A), apolipoprotein B (apo B), aspartate transaminase (AST), alanine transaminase (ALT), and gamma-glutamyl transpeptidase (GGT) were measured in fresh samples, using standardized enzymatic procedures in a Hitachi 902 analyzer (Hitachi Ltd,Tokio, Japan). Accuracy and precision of lipid measurements in our laboratory are under periodic surveillance by the Center for Disease Control and Prevention Service (Atlanta, GA, USA). Low density lipoprotein cholesterol was estimated using the De Long et al. formula (De Long et al., 1986). Total high-sensitivity C-reactive protein (hs-CRP) levels were determined by immunonephelometry on a BN Pro Spec nephelometer (Dade Behring Marburg GmbH, Germany). Interassay coefficient of variation (CV) values were <6% for all of these assays. Plasma insulin concentrations were determined by a RIA (Millipore; RIA Kit, Cat. No. HI–14 K, MO, USA), the intra- and interassay CV values were 2.1 and 6.8%, respectively.

2.3. Computed tomography study

Total, subcutaneous and visceral abdominal tissue (VAT), liver and spleen attenuation, and CAC were quantified by computed tomography in each participant. Computed tomography (CT) of the chest and abdomen was performed using a 64-channel multidetector helical computed tomography system (Somatom Cardiac Sensation, 64, Forcheim, Germany) and interpreted by experienced radiologists. Scans were read to assess and quantify the following: (a) total abdominal, subcutaneous, and VAT areas as described by Kvist et al. (1988); (b) liver to spleen attenuation ratio (L:SAR) as described by Longo et al. (1993); and (c) CAC score using the Agatston method (Mautner et al., 1994). SA was defined as the presence of a CAC score $\geq$10.0 and fatty liver as L:SAR $\leq$1.0 [McKimmie et al., 2008].

2.4. Genetic analysis

Genomic DNA from whole blood containing EDTA was isolated by standard techniques. The *I148M* (rs738409) single nucleotide polymorphism (SNP) was genotyped using 5′ exonuclease TaqMan

Table 1
Clinical characteristics of the study subjects.

	Control (n = 1469)	Premature CAD (N = 1103)	P
Age (years)	53 ± 9	54 ± 8	0.017
Sex (% male)	49.8	81.2	<0.001
Body mass index (kg/m^2)	28.0 [25.5–31.0]	28.2 [25.9–31.1]	0.048
Waist circumference (cm)	94.8 [87.0–102.0]	97.0 [91.0–104.5]	<0.001
Systolic blood pressure (mmHg)	115 [105–126]	116 [106–127]	0.030
Diastolic blood pressure (mmHg)	72 [66–78]	71 [65–78]	0.986
Total abdominal tissue (cm^2)	436 [351–540]	425 [339–522]	0.029
SAT (cm^2)	281 [212–359]	245 [193–312]	<0.001
VAT (cm^2)	150 [110–193]	168 [129–214]	<0.001
Total cholesterol >200 mg/dL (%)	39.8	20.5	<0.001
Hypoalphalipoproteinemia (%)	50.0	66.8	<0.001
LDL-C >130 mg/dL (%)	33.4	16.1	<0.001
Hypertriglyceridemia (%)	48.9	56.1	<0.001
Obesity (%)	30.7	34.5	0.044
Abdominal obesity (%)	82.0	83.0	0.286
Type 2 diabetes mellitus (%)	13.4	35.1	<0.001
Hyperinsulinemia (%)	54.7	71.1	<0.001
Insulin resistance (%)	57.2	76.6	<0.001
Increased VAT (>p75) (%)	58.6	64.4	0.001
Hypertension (%)	9.9	68.0	<0.001
Metabolic syndrome (%)	43.8	71.4	<0.001
CAC score ≥ 10	17.3	Nd	–

LDL-C: low density lipoprotein cholesterol, SAT: subcutaneous abdominal tissue VAT: visceral abdominal tissue, CAC: coronary artery calcification, Nd: not determined.
Data are expressed as means ± SD, median (interquartile range) or percentage.
Comparisons were made using the *t*-test or the Mann–Whitney *U* test for continuous variables and by Chi square analysis for categorical variables.

genotyping assays on an ABI Prism 7900HT Fast Real-Time PCR system, according to manufacturer's instructions (Applied Biosystems, Foster City, CA).

2.5. Statistical analysis

Data are expressed as mean ± S.D., median (interquartile range), or as frequencies for categorical variables. Comparisons were made using the *t*-test, the Mann–Whitney *U* test, ANOVA or Kruskal-Wallis test as appropriate, for continuous variables, and by Chi square test for categorical variables. Logistic regression analysis was used to test for associations of the *I148M/PNPLA3* polymorphism with pCAD, pCAD with and without T2DM, fatty liver, cardiometabolic parameters, cardiovascular risk factors and SA under the following inheritance models: additive (major allele homozygotes vs. heterozygotes vs. minor allele homozygotes), co-dominant (major allele homozygotes vs. heterozygotes and major allele homozygotes vs. minor allele homozygotes), dominant (major allele homozygotes vs. heterozygotes + minor allele homozygotes), heterozygous (heterozygotes vs. major allele homozygotes + minor allele homozygotes), and recessive (major allele homozygotes + heterozygotes vs. minor allele homozygotes). The models were adjusted for age, gender, BMI, and T2DM. In the pCAD group, we further adjusted for hypoglycemic and lipid lowering treatment. In the control group, in order to evaluate the association of the polymorphism with CAC the models were also adjusted for HOMA-IR. Genotype frequencies did not show deviation from Hardy–Weinberg equilibrium (P > 0.05). All statistical procedures were performed using the SPSS software (SPSS version 15.0, Inc.).

3. Results

3.1. Characteristics of the study sample

Clinical and demographic characteristics of the individuals included in the study are shown in Tables 1 and 2. In the pCAD group, age, proportion of males, BMI, waist circumference, systolic blood pressure and VAT were significantly higher compared with the control group (Table 1). Due to the statin treatment, the preva-

Table 2
Metabolic characteristics of the study subjects.

	Control (n = 1469)	Premature CAD (n = 1103)	P
Total cholesterol (mg/dL)	192 [167–214]	160 [132–193]	<0.001
HDL-C (mg/dL)	44 [36–54]	37 [32–44]	<0.001
LDL-C (mg/dL)	118 [97–138]	91 [69–116]	<0.001
Non-HDL-C (mg/dL)	144 [123–168]	120 [93–151]	<0.001
Triglycerides (mg/dL)	148 [112–204]	162 [118–219]	<0.001
Apo B (mg/dl)	95 [[77–115]	80 [63–102]	<0.001
Apo A1 (mg/dl)	134 [115–156]	120 [101–138]	<0.001
Apo B/Apo A1 ratio	0.72 [0.55–0.90]	0.67 [0.53–0.87]	0.001
ALT (IU/L)	24 [18–33]	26 [19–36]	0.009
AST(IU/L)	25 [21–30]	26 [22–31]	0.003
GGT (IU/L)	27 [18–42]	32 [22–49]	<0.001
Glucose (mg/dL)	91 [84–99]	94 [87–118]	<0.001
HOMA-IR	3.97 [2.70–5.77]	5.04 [3.49–7.50]	<0.001
hsCRP (mg/L)	1.59 [0.81–3.22]	1.18 [0.62–2.56]	<0.001

HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, Apo B: apolipoprotein B, Apo A: apolipoprotein A, ALT: alanine transaminase, AST: aspartate transaminase, GGT: gamma-glutamyl transpeptidase HOMA-IR: homeostasis model assessment–insulin resistance, hsCRP: high sensitivity C reactive protein.
Data are expressed as median (interquartile range). Comparisons were made using the Mann–Whitney *U* test.

Table 3
Association of PNPLA3 polymorphism with pCAD in T2DM patients.

Variable	Model	Odds Ratio (95%CI)	p*
pCAD (n = 386)	Co-dominant	1.41 (0.98–2.02)	0.062
	Dominant	1.26 (0.90–1.75)	0.177
	Recessive	1.28 (0.99–1.64)	0.055
	Heterozygous	0.90 (0.71–1.15)	0.406
	Additive	**1.20 (1.01–1.42)**	**0.042**

Bold numbers indicate significant associations.
In all models the reference genotype was *I148I* (*CC*).
The control group included only individuals without diabetes and normal values of glucose (<100 mg/dL) (n = 1093).
* Associations were tested using logistic regression adjusted for age, gender and BMI.

lence of total cholesterol >200 mg/dL and LDL-C >130 mg/dL, as well as total cholesterol, LDL cholesterol, non-HDL cholesterol, Apo B, and C reactive protein levels were significantly lower in the patient group than in controls. However, it is possible that before having the coronary event and statin treatment, these patients had total and LDL cholesterol levels similar or even higher than those observed in the control group. As expected, pCAD patients also had lower HDL cholesterol and Apo A1, and higher TG levels (Table 2). Fasting glucose and HOMA-IR were also significantly higher in pCAD patients compared to healthy subjects (Table 2).

3.2. Association of polymorphisms with pCAD

The distribution of the studied polymorphism was similar in pCAD patients and healthy controls under all inheritance models tested, and no association with pCAD was observed (data not shown). Considering the high frequency of T2DM in the pCAD group, we carried out an analysis in patients with and without this pathology. Under additive model, the polymorphism was associated with increased risk of pCAD only in T2DM patients (OR = 1.20, 95% CI: 1.01-1.42, P_{add} = 0.042) (Table 3).

3.3. Association of the polymorphism with metabolic cardiovascular risk factors and metabolic parameters

The effect of the polymorphism on various metabolic parameters and cardiovascular risk factors was analyzed separately in pCAD patients and healthy controls (Table 4). A different distribution of the genotypes was observed for HOMA-IR (P = 0.015), alanine transaminase (P < 0.001), aspartate transaminase (P < 0.001), liver to spleen attenuation ratio (P < 0.001), and the prevalence of fatty liver (P < 0.001) in the control group. Similar data were observed in pCAD patients with two differences, the whole group of pCAD patients presented different distribution of the genotypes for visceral abdominal tissue (P = 0.029) but no differences were observed for HOMA-IR.

The risk analysis of the *PNPLA3 I148M* polymorphism with cardiovascular risk factors and CAC is shown in Table 5. The polymorphism was associated with risk of fatty liver (co-dominant, dominant, recessive, and additive models P > 0.001 for all models), elevated ALT (co-dominant, dominant, recessive and additive models, P < 0.01 for all models), insulin resistance (dominant model, P = 0.030), and with CAC score ≥10 (dominant, P = 0.047, and heterozygote, P = 0.010) in the control group. The models were adjusted for age, gender, BMI, and T2DM, and for CAC score ≥10 we further adjusted the model for HOMA-IR values. In the whole group

Table 4
Metabolic parameters of the population according to *PNPLA3 I148 M* genotype.

Controls	*I148I* (267)	*I148M* (677)	*M148M* (525)	p
Sex (% male)	48.3	49.8	50.5	0.847
Age (years)	54 ± 10	53 ± 9	53 ± 9	0.747
Body mass index (kg/m^2)	27.9 [25.3–30.3]	28.2 [25.5–31.1]	27.8 [25.7–31.0]	0.597
Waist circumference (cm)	94.5 [86.0–101.9]	94.7 [87.0–102.3]	94.9 [87.5–101.5]	0.913
VAT (cm^2)	151 [114–201]	150 [110–197]	149 [110–189]	0.644
HOMA-IR	**3.59 [2.49–5.26]**	**4.03 [2.80–5.80]**	**4.11 [2.71–6.10]**	**0.015**
ALT (IU/L)	**21 [16–29]**	**24 [18–32]**	**26 [19–38]**	**<0.001**
AST (IU/L)	**24 [20–28]**	**24 [20–30]**	**26 [22–34]**	**<0.001**
L:SAR	**1.16 [1.02–1.26]**	**1.11 [0.92–1.22]**	**1.04 [0.83–1.18]**	**<0.001**
Prevalence of fatty liver (%)	**19.1**	**32.5**	**41.1**	**<0.001**
Premature CAD	*I148I* (205)	*I148M* (522)	*M148M* (376)	p
Sex (% male)	80.0	80.7	82.7	0.650
Age (years)	54 ± 8	54 ± 8	54 ± 8	0.791
Body mass index (kg/m^2)	28.7 [26.3–31.6]	28.1 [25.8–30.7]	28.1 [25.9–31.1]	0.141
Waist circumference (cm)	98.2 [92.4–106.0]	97.3 [90.8–104.0]	96.3 [90.9–104.0]	0.099
VAT (cm^2)	**177 [140–233]**	**162 [128–209]**	**169 [124–212]**	**0.029**
HOMA-IR	4.89 [3.43–7.27]	4.93 [3.52–7.41]	5.21 [3.50–7.92]	0.492
ALT (IU/L)	**24 [19–31]**	**25 [18–35]**	**28 [20–37]**	**0.013**
AST (IU/L)	**24 [21–29]**	**26 [21–31]**	**27 [22–32]**	**<0.001**
L:SAR	**1.13 [1.00–1.23]**	**1.11 [0.98–1.20]**	**1.07 [0.91–1.17]**	**<0.001**
Prevalence of fatty liver (%)	**22.0**	**25.7**	**34.6**	**0.001**

VAT: visceral abdominal tissue, HOMA-RI: homeostasis model assessment–insulin resistance, ALT: alanine transaminase, AST: aspartate transaminase, L:SAR: liver to spleen attenuation ratio.
Data are expressed as means ± SD, median (interquartile range) or percentage. Comparisons were made using ANOVA, Kruskal-Wallis test, as appropriate, for continuous variables, and by Chi square analysis for categorical variables.
Bold numbers indicate significant associations.

Table 5
Association of the *PNPLA3 I148M* polymorphism with fatty liver, elevated ALT levels, insulin resistance and SA.

		Controls		Premature CAD	
Variable	Model	Odds Ratio (95%CI)	p*	Odds Ratio (95%CI)	p*
Fatty liver	Co-dominant	**3.20 (2.21–4.64)**	**<0.001**	**2.09 (1.39–3.16)**	**<0.001**
	Dominant	**2.54 (1.80–3.59)**	**<0.001**	**1.66 (1.14–2.42)**	**0.008**
	Recessive	**1.83 (1.44–2.32)**	**<0.001**	**1.65 (1.24–2.19)**	**0.001**
	Heterozygous	0.93 (0.74–1.16)	0.506	0.84 (0.64–1.11)	0.226
	Additive	**1.71 (1.45–2.03)**	**<0.001**	**1.46 (1.20–1.78)**	**<0.001**
Elevated ALT	Co-dominant	**1.76 (1.29–2.39)**	**0.001**	**1.53 (1.08–2.15)**	**0.015**
	Dominant	**1.51 (1.14–1.99)**	**0.004**	1.28 (0.94–1.74)	0.111
	Recessive	**1.42 (1.14–1.78)**	**0.002**	**1.40 (1.09–1.80)**	**0.008**
	Heterozygous	0.93 (0.75–1.15)	0.474	0.86 (0.68–1.08)	0.199
	Additive	**1.32 (1.14–1.54)**	**<0.001**	**1.25 (1.06–1.48)**	**0.008**
Insulin resistance	Co-dominant	**1.43 (1.02–2.01)**	**0.032**	1.11 (0.69–1.80)	0.262
	Dominant	**1.40 (1.03–1.91)**	**0.030**	1.23 (0.80–1.87)	0.349
	Recessive	1.14 (0.89–1.46)	0.310	0.92 (0.64–1.32)	0.649
	Heterozygous	1.09 (0.86–1.38)	0.473	1.22 (0.87–1.72)	0.247
	Additive	1.17 (0.99–1.38)	0.063	1.03 (0.81–1.31)	0.829
CAC score ≥10	Co-dominant	1.28 (0.80–2.04)	0.308		
	Dominant	**1.54 (1.01–2.35)**	**0.047**		
	Recessive	0.084 (0.61–1.15)	0.227		
	Heterozygote	**1.50 (1.10–2.04)**	**0.010**		
	Additive	1.04 (0.84–1.29)	0.731		

ALT: alanine transaminase, CAC = Coronary artery calcification.
Bold numbers indicate significant associations.
In all models the reference genotype was *I148I* (*CC*).
* Associations were tested using logistic regression adjusted for age, gender, body mass index and type 2 diabetes mellitus. For CAC score we further adjusted for HOMA-IR. In premature CAD group, for fatty liver, elevated ALT, and insulin resistance we further adjusted for hypoglycemic, and lipid lowering treatment.

of pCAD patients, the polymorphism was associated with fatty liver (co-dominant, dominant, recessive and additive models, $P < 0.05$ for all models), and elevated ALT (co-dominant, recessive, and additive models, $P < 0.05$ for all models). The models were adjusted for age, gender, BMI, T2DM, and hypoglycemic, and lipid lowering treatment.

4. Discussion

The present study examined 2572 individuals, 1103 diagnosed with pCAD and 1469 healthy controls to investigate the relationship between *I148M/PNPLA3* (rs738409) polymorphism and pCAD susceptibility in Mexican population. The association of the polymorphism with cardiovascular risk factors and metabolic parameters was also evaluated. In this study no association with pCAD was observed, our result confirmed previous data in GWAS performed in other populations (CARDIoGRAMplusC4D Consortium et al., 2013; O'Donnell et al., 2011; Schunkert et al., 2011; Lee et al., 2013; Lu et al., 2012; The Coronary Artery Disease (C4D) Genetics Consortium et al., 2011). However, when the analysis included separately pCAD patients with and without T2DM, the polymorphism was associated with the presence of pCAD only in T2DM patients, suggesting a possible role of this polymorphism in the developing of T2DM. This finding should be taken with caution considering that the cross-sectional design, does not allow to establish causal or temporal relationships between the *I148M/PNPLA3* polymorphism and T2DM. Some associations of the polymorphism with cardiovascular risk factors were observed in both controls and pCAD patients. In both groups, under different models, the polymorphism was associated with fatty liver and elevated ALT. It was also associated with insulin resistance and CAC ≥ 10 in the control group. The association of the *I148M/PNPLA3* polymorphism with fatty liver has been reported previously in several populations (Zhang et al., 2014; Baclig et al., 2014; Lee et al., 2014) but not in Mexicans. The first report of association between *I148M/PNPLA3* polymorphism and fatty liver was made in a genome-wide association study in subjects with non-alcoholic fatty liver disease (NAFLD) (Romeo et al., 2008). This study was then replicated in several populations confirming that the *G* allele (*148M*) of this polymorphism is significantly associated with increased risk of fatty liver disease. Valenti et al. (2010) confirmed the association of *I148M/PNPLA3* polymorphism with the presence of nonalcoholic steatohepatitis, the severity of steatosis and fibrosis with a gene-dose effect, independently of age, BMI and diabetes in a large series of biopsied patients. The higher risk was observed in subjects who presented the M148 M genotype. In our study, CT was used to detect fatty liver, defined as L:SAR ≤ 1.0 (McKimmie et al., 2008). CT does not allow to estimate the severity of steatosis, therefore we were unable to evaluate if M148M genotype could be considered as a marker of severe steatosis in our population. The association of the *I148M/PNPLA3* polymorphism with high levels of ALT has been reported in normal weight and overweight children (Viitasalo et al., 2015). This finding has been corroborated in normal-weight and overweight/obese Mexican children and in Mexican Amerindians (Larrieta-Carrasco et al., 2013; Larrieta-Carrasco et al., 2014). Wang et al. reported the association of the *I148M/PNPLA3* polymorphism with insulin resistance in a normoglycaemic population (Wang et al., 2011). In our study, we corroborated the association of the *I148M/PNPLA3* polymorphism with fatty liver and high levels of ALT in Mexican pCAD patients and healthy controls, and with insulin resistance in the control group. The *I148M/PNPLA3* is a non-synonymous polymorphism that did not altered the subcellular distribution, and neither the expression of the protein. Structural modeling has indicated that the side chain of the methionine at residue 148 extends into the catalytic site, inhibits catalytic activity of the enzyme, and leads to triglyceride accumulation in the liver (He et al., 2010). This could be, at least in part, the mechanism that explains why the amino acid substitution in PNPLA3 confers susceptibility to increased hepatic fat content and elevated serum levels of ALT (a marker of liver inflammation). In addition, it has been reported that the PNPLA3*G* allele could be involved in the development of fatty liver by modulating adipocyte size (Santoro et al., 2010). Adipocyte size reflects the amount of lipid storage in subcutaneous tissue, and the inability of subcutaneous tissue to expand, has been considered as a possi-

ble key factor for fat accumulation in ectopic tissues and organs (Kim et al., 2000). The presence of small adipocytes in the subcutaneous tissue may contribute to the overflow of free fatty acids to the liver, which can accumulate as triglycerides and also can activate the protein kinase C resulting in inhibition of the hepatic insulin signaling (Samuel et al., 2007) and development of subsequent insulin resistance. Interestingly, in our study, the polymorphism was associated with CAC $\geq$10 (a surrogate marker for coronary atherosclerosis) in healthy controls, suggesting its association with SA. To the best of our knowledge, this is first study that establishes an association between CAC and the *I148M/PNPLA3* polymorphism. The relationship was maintained after adjustment for age, gender, BMI and T2DM. A recent study in two cohorts of Sicilian and Northern Italian non-alcoholic fatty liver disease (NAFLD) patients reported that the *PNPLA3 GG* (*MM*) genotype is associated with a higher risk of carotid atherosclerosis in patients younger than 50 (Petta et al., 2013). Considering this information, our study is the second one that detects an association of the *I148M/PNPLA3* polymorphism with SA, in our case, using a different surrogate marker for coronary atherosclerosis, the CAC score. Several studies have demonstrated the functional effect of the *I148M/PNPLA3* polymorphism. A recent study, Sookoian and Pirola (2012) using a silico analysis showed that the rs738409*C* or *G* alleles have the ability to modify miRNA binding sites. They detected two miRNAs (hsa-miR-769-3p and hsa-miR-516a-3p) that interacted in the 3′UTR region of the gene. Another study, using a multivariable regression model including cardiovascular risk factors and pharmacological treatment showed that hsa-miR-769-3p was associated with the presence of significant coronary atherosclerosis (Freedman et al., 2012).

The strength of this study is that we included a large cohort of Mexican individuals with and without pCAD well characterized from the clinical, biochemical and tomographic points of view. On the other hand, this study has some potential limitations: (1) As a cross-sectional analysis, we cannot infer causality from our observations. (2) The diagnosis of fatty liver was not performed by liver biopsies due to ethical considerations. Instead, fatty liver diagnosis was performed by CT scans with the exclusion of viral hepatitis B, C, HIV/AIDS, syphilis, and Chagas disease. However, a significant correlation has been demonstrated between the liver attenuation images on CT and the histological grade of steatosis (Limanond et al., 2004). (3) We estimated insulin resistance with HOMA-IR index instead of using the euglycemic clamp; however, the HOMA-IR index has proven to be a reliable measure of insulin sensitivity in non-diabetic individuals (Bonora et al., 2000). (4) CAC was not measured in pCAD patients because the presence of stents and previous coronary surgery results in artifacts that do not allow the correct interpretation of tomographic images, therefore we were unable to assess the association between the *I148M/PNPLA3* polymorphism and CAC in pCAD patients.

5. Conclusions

Our study suggests that the *I148M/PNPLA3* polymorphism is associated with the presence of pCAD only in patients with T2DM and confirms its association with fatty liver, high levels of ALT, and insulin resistance. It also demonstrates the association of the polymorphism with *SA,* using the CAC as a surrogate marker for coronary atherosclerosis, in an adult Mexican population, which is a population with characteristic genetic background and important differences with other populations (Lisker et al., 1986, 1988, 1990; Juárez-Cedillo et al., 2008). Due to these genetic characteristics of our population, we considered that the associations of the *I148M/PNPLA3* polymorphism with CAC detected in our study should be explored in other populations.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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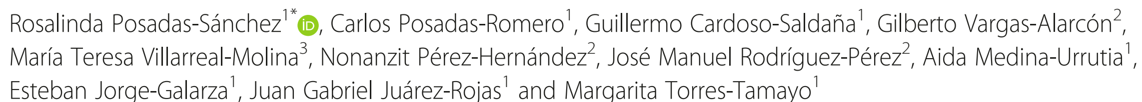
Serum magnesium is inversely associated with coronary artery calcification in the Genetics of Atherosclerotic Disease (GEA) study



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Abstract

Background: Serum magnesium is inversely associated to coronary artery calcification (CAC) in patients with chronic kidney disease. There is little information on this association in a general healthy population.

Objective: The aim of this study was to examine the cross-sectional association of serum magnesium levels with CAC.

Methods: We included 1276 Mexican-mestizo subjects (50 % women), aged 30–75 years, free of symptomatic cardiovascular disease. CAC was quantified by multidetector computed tomography using the method described by Agatston. Cross-sectional associations of serum magnesium with cardiometabolic factors and subclinical atherosclerosis defined as a CAC score > 0, were examined in logistic regression models adjusted for age, sex, education, smoking status, body mass index, systolic blood pressure, physical activity, elevated abdominal visceral tissue, fasting insulin and glucose, alcohol consumption, menopausal status (women only), low (LDL-C) and high density lipoprotein cholesterol (HDL-C), triglycerides, diuretic use, type 2 diabetes mellitus (DM2), and family history of DM2.

Results: After full adjustment, subjects in the highest quartile of serum magnesium had 48 % lower odds of hypertension (p = 0.028), 69 % lower odds of DM2 (p = 0.003), and 42 % lower odds of CAC score > 0 (p = 0.016) compared to those with the lowest serum magnesium. The analyses also showed that a 0.17 mg/dL (1SD) increment in serum magnesium was independently associated with 16 % lower CAC (OR 0.84, 95 % CI 0.724–0.986).

Conclusions: In a sample of Mexican-mestizo subjects, low serum magnesium was independently associated to higher prevalence not only of hypertension and DM2, but also to coronary artery calcification, which is a marker of atherosclerosis and a predictor of cardiovascular morbidity and mortality.

Keywords: Serum magnesium, Coronary artery calcification, Subclinical atherosclerosis

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Introduction

Magnesium plays an important role in many biochemical processes including all ATP transfer reactions. Magnesium is essential for insulin release by pancreatic β-cells, and is also and important second messenger for insulin action [1, 2]. Cross-sectional [3] and longitudinal studies [4, 5] have found a significant inverse association between serum magnesium and DM2, insulin resistance and inflammation. Moreover, magnesium is a natural calcium antagonist [6] and modulates vasomotor tone, blood pressure, and peripheral blood flow [7, 8]. In prospective studies, serum magnesium levels have been inversely associated with the incidence of hypertension [9] and coronary heart disease (CHD) [10]. Increased carotid intima-media thickness (CIMT) and coronary artery calcification (CAC) are both markers of subclinical atherosclerosis, and are also predictors of cardiovascular disease (CVD) morbidity and mortality independently of traditional CVD risk factors [11–13]. Inverse associations between serum magnesium and CIMT have been reported [3, 14]. However, conflicting results have been found in the two studies investigating the association of dietary magnesium and CAC [15, 16]. Moreover, a negative association between serum magnesium and vascular calcification has been observed in patients with chronic kidney disease [17]. The only observational study that has examined this association in a population-based study reported that low serum magnesium level is associated with CAC in Koreans subjects [18]. However, it is known that CAC prevalence and amount are heavily influenced by ethnicity [19, 20]. Therefore, in the present study we assessed the cross-sectional association of serum magnesium concentrations with coronary artery calcification in Mexican-mestizo subjects with no family history of premature CHD and asymptomatic for CVD.

Methods

Study population

The study population included participants of the Genetics of Atherosclerotic Disease (GEA) study. The GEA study was designed to examine the genomic bases of CHD and assess relationships between traditional and emerging risk factors with clinical and subclinical atherosclerotic vascular diseases in an adult Mexican-mestizo population [21]. Briefly, a convenience sample of 1200 CHD patients and 1500 control subjects aged 30–75 years was recruited from residents in Mexico City. Patients with established premature CHD were selected from the outpatient clinic of the National Institute of Cardiology. Volunteer control participants with a negative family history of premature CHD and no personal history of cardiovascular disease were recruited from blood bank donors, and through brochures posted in Social Services centers. Coronary patients and control subjects with a history of renal, liver, thyroid, or malignant disease, as well as those on treatment with corticosteroids, were excluded. The GEA study was approved by the Institutional Review Board of the National Institute of Cardiology and conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

This study is a cross-sectional analysis of the baseline measurements of 1500 control subjects included in the GEA study. The participants answered structured questionnaires that provide detailed information regarding family history, demographics, diet, physical activity, medications, smoking, and alcohol intake. We excluded all subjects with missing CAC (n = 25), missing physical activity data (n = 111), missing dietary data (n = 45), missing insulin (n = 1) and magnesium sampling (n = 42). The final study population included in the analysis comprised 1276 subjects. Systolic and diastolic blood pressures were measured after rest for at least 10 min, and the average of the second and third measurements was recorded for analyses. Hypertension was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg or the current use of antihypertensive medication. Height, weight, and waist circumference were measured and BMI was calculated as weight in kilograms divided by height in meters squared. The metabolic syndrome (MS) was defined using the criteria from the American Heart Association/National Heart, Lung, and Blood Institute scientific statement on the MS [22], except for central obesity that was considered when waist circumference was > 90 cm in men and > 80 cm in women [23]. Type 2 diabetes mellitus (DM2) was defined by the American Diabetes Association criteria [24], reported glucose-lowering treatment or previous DM2 diagnosis by a physician. Family history of DM2 was defined as either mother or father having DM2. Insulin resistance was estimated using the homeostasis model assessment (HOMA-IR). Insulin resistance was defined as HOMA-IR values ≥ 75th percentile (3.66 in women and 3.38 in men). These cutoff points were obtained from a GEA study sample of 131 men and 185 women without obesity and with normal of blood pressure, fasting glucose and lipids measurements.

Biochemical analyses

Venous blood samples were collected from subjects after a 12 h fast. Plasma glucose, total cholesterol, triglycerides, HDL-C, apolipoprotein A (apo A), apolipoprotein B (apo B), creatinine, uric acid, aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were measured in fresh samples, using standardized enzymatic procedures in a Hitachi 902 analyzer (Hitachi Ltd,Tokio, Japan).

Accuracy and precision of lipid measurements in our laboratory are under periodic surveillance by the Centers for Disease Control and Prevention service (Atlanta, GA, USA). LDL-C was estimated using the DeLong et al. formula [25]. Total high-sensitivity C-reactive protein (hs-CRP) levels were determined by immunonephelometry on a BN Pro Spec nephelometer (Dade Behring Marburg GmbH, Germany). Interassay coefficient of variation (CV) values were <6 % for all of these assays. Serum total adiponectin was determined by ELISA (R&D Systems, Minneapolis, USA) Quantine kit. The intra- and interassay CV were < 10 %. Plasma insulin concentrations were determined by a radioimmunoassay (Millipore; RIA Kit, Cat. No. HI-14 K, MO, USA) the intra- and interassay CV values were 2.1 and 6.8 %, respectively. Serum magnesium levels were measured by using the direct xylidyl blue complex method in a COBAS C311 automated chemistry analyzer (Roche diagnotics GmbH, Germany). The intra and interassay coefficient of variation were ≤1.0 %.

Computed tomography study

Visceral adipose tissue (VAT), subcutaneous fat, liver and spleen attenuation, and coronary artery calcium were quantified by computed tomography in each participant. Computed tomography of the chest and abdomen were performed using a 64-channel multidetector helical computed tomography system (Somatom Cardiac Sensation, 64, Forcheim, Germany) and interpreted by experienced radiologists. Scans were read to assess and quantify the following: i) total abdominal, subcutaneous, and VAT areas as described by Kvist et al. [26]; ii) liver to spleen attenuation ratio (L:SAR) as described by Longo et al. [27]; and iii) CAC score using the Agatston method [28]. Subclinical atherosclerosis was defined as the presence of a CAC score > 0.0 and hepatic steatosis as L:SAR ≤1.0 [29].

Data analysis

Data are expressed as mean ± S.D., median [interquartile range] for continuous variables, or as proportions for categorical variables. Serum magnesium concentrations were categorized by quartile distribution with the lowest quartile serving as the reference. Differences across quartiles were assessed with ANOVA or Kruskal-Wallis test for continuous variables, and Chi square test for categorical variables. Multivariate logistic regression analysis was used to evaluate the association between each S.D. increase in serum magnesium concentrations with coronary risk factors and CAC. For coronary risk factors, the forced-entry logistic regression analyses were adjusted for age, sex, education, smoking status, elevated abdominal visceral adipose tissue, body mass index, systolic blood pressure, low and high density lipoprotein cholesterol, triglycerides, fasting insulin and glucose, physical activity, alcohol consumption, menopausal status (women only) and diuretic use. For CAC, three forced-entry logistic regression models were constructed: an unadjusted model; a model adjusted for age, sex, education, smoking status, body mass index, systolic blood pressure, and physical activity (model 1); a regression model further adjusted for elevated abdominal visceral adipose tissue, fasting insulin and glucose, and alcohol consumption (model 2); and, finally, a regression model adjusted for the variables included in model 2 plus menopausal status (women only), low and high density lipoprotein cholesterol, triglycerides, diuretic use, DM2 and family history of DM2 (model 3). *P* values <0.05 were considered statistically significant. All statistical procedures were performed using the SPSS software (SPSS version 15.0, Inc.).

Results

This study included 1276 subjects (640 women, 636 men) with a mean age 54 ± 9 (SD) years, with no family history of premature CHD or CVD symptoms. Compared to women, men had a higher prevalence of high blood pressure (11.9 % vs 7.3 %, $p = 0.006$), insulin resistance (61.8 % vs 54.7 %, $p = 0.011$) and metabolic syndrome (49.2 % vs 40.9 %, $p = 0.003$). The prevalence of current smoking (25.1 % of men and 18.9 % of women) and type 2 diabetes mellitus (15 % of men and 13.4 % of women) were not significantly different between genders. Two thirds of women (66.4 %) were postmenopausal. The mean serum magnesium concentration in the whole population was 2.06 ± 0.17 (SD) mg/dl, and was slightly higher in men than in women (2.07 ± 0.16 (SD) mg/dl vs 2.06 ± 0.18 (SD) mg/dl, $p = 0.09$). The overall prevalence of CAC score > 0 was 27.2 %. (41.5 % of men and 13 % of women, $p < 0.001$).

Table 1 shows clinical, computed tomography and laboratory characteristics of participants according to serum magnesium quartiles. Systolic blood pressure, fasting glucose, HOMA-IR and C reactive protein were inversely associated with serum magnesium levels. High serum magnesium levels were also associated with lower prevalence of hypertension, DM2, insulin resistance, metabolic syndrome, and CAC score >0. A similar non-significant trend was observed for body mass index and fasting insulin levels. In contrast, total and LDL-cholesterol and apolipoprotein B were greater with increasing serum magnesium levels. Visceral adipose tissue, triglycerides, and adiponectin were not associated with serum magnesium levels.

Multivariate logistic regression analyses were performed to examine the associations of serum magnesium levels with cardiometabolic risk factors and CAC score >0 (Table 2). After adjustment for several confounding

Table 1 Characteristics of 1276 GEA study participants according to serum magnesium quartiles

	Q1<1.97 mg/dl	Q2≥1.97 to <2.07 mg/dl	Q3≥2.07 to <2.18 mg/dl	Q4≥2.18 mg/dl	P*
N	326	304	338	308	
Serum magnesium (mg/dl)	1.90 [1.83–1.94]	2.03 [2.00–2.05]	2.11 [2.09–2.14]	2.24 [2.20–2.29]	<0.001
Dietary magnesium (mg)	351 [290–410]	344 [292–421]	356 [289–424]	358 [293–422]	0.692
Male (%)	50	49.3	51.2	49	0.949
Age (years)	54 ± 10	53 ± 10	54 ± 9	55 ± 9	0.079
Body mass index (kg/cm^2)	28.8 ± 4.2	28.4 ± 4.2	28.0 ± 4.0	28.1 ± 3.8	0.056
Visceral adipose tissue (cm^2)	157 [112–211]	148 [112–190]	150 [109–194]	148 [115–193]	0.196
Systolic blood pressure (mmHg)	118 [108–130]	113 [103–125]	114 [106–123]	113 [103–123]	<0.001
Glucose (mg/dl)	95 [87–125]	91 [85–99]	89 [83–96]	89 [84–96]	<0.001
Insulin (μU/ml)	18.1 [13.2–24.9]	18.2 [12.6–24.7]	16.7 [12.4–22.8]	16.4 [12.3–22.4]	0.067
HOMA-IR	4.9 [3.0–7.5]	4.2 [2.8–5.7]	3.7 [2.6–5.4]	3.6 [2.6–5.1]	<0.001
Cholesterol					
Total (mg/dl)	190 ± 36	190 ± 37	193 ± 37	198 ± 37[a,b]	0.015
LDL (mg/dl)	116 ± 32	117 ± 33	117 ± 32	123 ± 33[a,b,c]	0.009
HDL (mg/dl)	45.7 ± 13.2	45.1 ± 13.3	47.8 ± 14.1	45.6 ± 13.0	0.065
Triglycerides (mg/dl)	148 [114–200]	152 [113–202]	145 [106–209]	153 [116–204]	0.844
Apolipoprotein B (mg/dl)	92 [74–112]	93 [75–111]	95 [79–115]	103 [85–122]	<0.001
Apolipoprotein A (mg/dl)	133 [114–154]	131 [111–152]	134 [117–158]	135 [117–155]	0.095
Adiponectin (μg/ml)	8.0 [4.8–11.8]	7.6 [5.0–12.1]	8.1 [5.2–13.1]	7.8 [5.0–12.2]	0.915
hsCRP (mg/l)	1.8 [0.9–3.6]	1.5 [0.8–3.3]	1.4 [0.7–2.9]	1.4 [0.8–3.0]	0.018
Current smoking (%)	21	23	23	21	0.765
Physical activity	7.75 [6.88–8.88]	7.88 [6.75–8.75]	7.88 [7.13–8.75]	7.75 [7.00–8.63]	0.256
Menopausal women (%)	62	62.3	64.8	76.6	0.018
Hypertension (%)	14	8	8	8	0.009
Type 2 diabetes mellitus (%)	34	10	8	5	<0.001
Insulin resistance (%)	66	63	53	51	<0.001
Metabolic syndrome (%)	54	44	41	41	0.003
CAC score >0 (%)	35	24	26	24	0.002

Values are expressed as mean ± SD, median [IR, interquartile range] or percentages. *HOMA-IR* Homeostasis model assessment insulin resistance, *LDL* Low density lipoprotein, *HDL* High density lipoprotein, *hsCRP* High sensitive C reactive protein
*for ANOVA, Kruskal-Wallis or Chi square test. P <0.05 [a]versus Q1, [b]versus Q2, [c]versus Q3

Table 2 Association of serum magnesium levels with cardiometabolic risk factors and subclinical atherosclerosis

	Q1	Q2	Q3	Q4
		Odds ratio [CI 95 %]	Odds ratio [CI 95 %]	Odds ratio [CI 95 %]
Hypertension	1	0.52 [0.295–0.917]	0.56 [0.323–0.961]	0.52 [0.294–0.931]
Insulin resistance	1	1.26 [0.891–1.940]	0.96 [0.632–1.467]	1.02 [0.694–1.506]
Metabolic syndrome	1	1.04 [0.715–1.512]	1.01 [0.696–1.467]	1.02 [0.964–1.506]
Type 2 diabetes mellitus	1	0.42 [0.199–0.886]	0.34 [0.161–0.705]	0.31 [0.143–0.672]
CAC score >0	1	0.68 [0.436–1.070]	0.78 [0.507–1.197]	0.58 [0.374–0.915]

Data are expressed as odds ratios and 95 % confidence interval (CI) as assessed by multivariate logistic regression analyses. Other covariates included in the multivariable regression model, along with serum magnesium were: age, sex, education, smoking status, elevated abdominal visceral adipose tissue, body mass index, systolic blood pressure, low and high density lipoprotein cholesterol, triglycerides, fasting insulin and glucose, physical activity, alcohol consumption, menopausal status (women only), family history of type 2 diabetes mellitus, type 2 diabetes mellitus and diuretic use

factors, individuals with the highest serum magnesium levels (fourth quartile) had 48 % lower odds lower odds of hypertension (p = 0.028), 69 % lower odds of DM2 (p = 0.003), and 42 % lower odds of CAC score > 0 (p = 0.016) as compared to those in the lowest quartile.

Associations of a 1SD (0.17 mg/dL) increment in serum magnesium levels with hypertension, insulin resistance, metabolic syndrome, and DM2 are described in Table 3. Variables included in the multivariable model were age, sex, education, smoking status, elevated abdominal visceral adipose tissue, body mass index, systolic blood pressure, LDL-C, HDL-C, triglycerides, fasting insulin and glucose, physical activity, alcohol consumption, menopausal status (women only) and diuretic use. Higher serum magnesium was associated with 19 % lower prevalence of hypertension and 38 % lower prevalence of DM2 per each 1 SD increment in magnesium concentration.

In addition, regression models were used to test the association between CAC and serum magnesium levels as a continuous variable. A 0.17 mg/dl (1SD) increment in serum magnesium concentration was associated with 16 % lower coronary artery calcification (odds ratio 0.84; 95 % confidence intervals (CI) 0.741–0.953, p = 0.007) in the unadjusted model. This association proved to be independent of age, sex, education, smoking status, body mass index, systolic blood pressure, and physical activity (model 1; odds ratio 0.84; CI 0.724–0.964, p = 0.014). A regression model further adjusted for elevated abdominal visceral adipose tissue, fasting insulin and glucose, and alcohol consumption (model 2; odds ratio 0.86; CI 0.737–0.995, p = 0.043) continued to show a significant independent association between serum magnesium concentrations and CAC. Finally, after a full adjustment (model 2 plus menopausal status -women only-, low and high density lipoprotein cholesterol, triglycerides, diuretic use, DM2 and family history of DM2), a 0.17 mg/dL (1SD) increment in serum magnesium concentration remained significantly associated with 16 % lower coronary artery calcification (odds ratio 0.84; 95 % confidence interval (CI) 0.724–0.986, p = 0.033).

Table 3 Associations of serum magnesium concentrations with coronary risk factors

	Odds ratio	P
Hypertension	0.81 [0.673–0.985]	0.034
Insulin resistance	0.89 [0.763–1.044]	0.155
Metabolic syndrome	1.03 [0.891–1.189]	0.694
Type 2 diabetes mellitus	0.62 [0.489–0.786]	<0.001

Odds ratios are expressed in terms of per SD 0.17 mg/dl increase in serum magnesium levels
Multivariate logistic regression analyses adjusted for age, sex, education, smoking status, elevated abdominal visceral adipose tissue, body mass index, systolic blood pressure, low and high density lipoprotein cholesterol, triglycerides, fasting insulin and glucose, physical activity, alcohol consumption, menopausal status (women only) and diuretic use

Discussion

We report here an inverse association between serum magnesium levels and the prevalence of CAC score >0 in a Mexican-mestizo population with no family history of premature CHD or clinical signs or symptoms of CVD. This association remained significant after adjustment for a range of confounding factors, including age, sex, education, smoking status, visceral adipose tissue, body mass index, systolic blood pressure, low and high density lipoprotein cholesterol, triglycerides, fasting insulin and glucose, physical activity, alcohol consumption, menopausal status (women only), diuretic use, DM2, and family history of DM2. Serum magnesium levels were also independently associated with a lower risk hypertension and DM2. The latter associations are consistent with previous reports showing that both conditions are frequently associated with a magnesium deficient state as assessed by decreased circulating concentrations of serum total or ionized magnesium [4, 9, 30, 31].

In regard to the association of magnesium with subclinical atherosclerosis, only two population-based epidemiologic studies have examined the relation of magnesium intake with coronary artery calcification. The first study was conducted in 5281 MESA (the Multi-Ethnic Study of Atherosclerosis) participants aged 45–84 years and free of clinically apparent CVD, reporting no association between magnesium intake and CAC [16]. The more recent study included 2695 offspring or third generation Framing Heart Study participants free of CVD and reported that higher magnesium intake was associated with lower levels of CAC [15].

Although an association between serum magnesium levels and CAC has been reported in patients with chronic kidney disease [20], to date, only one cross-sectional analysis has examined serum magnesium levels in relation to CAC, in large number of Korean individuals without cardiovascular disease participating in a health examination program [18]. After adjusting for potential confounders, a significant inverse association between CAC and serum magnesium was observed, which is consistent with the findings of the present study. In agreement with these investigations, two decades ago the Atherosclerosis Risk in Communities Study reported an inverse association between serum magnesium and carotid intima-media thickness (CIMT) [3]. More recently, in a population-based study, Hashimoto et. al. reported that low serum magnesium levels were not only significantly and independently associated with greater CIMT, but also with the presence of atherosclerotic plaques [14]. Increased CIMT is also a marker of atherosclerotic disease, and like CAC, is and

independent predictor of cardiovascular morbidity and mortality [11, 12]. Collectively, the results of this and other studies [3, 14, 15, 18] suggest independent associations between reduced magnesium dietary intake or low serum magnesium levels with subclinical atherosclerosis defined either as increased CIMT or as coronary artery calcification.

To date, the underlying mechanisms responsible for the associations between serum magnesium and CAC are incompletely understood. The adverse cardiovascular risk profile found in subjects with low levels of serum magnesium (higher prevalence of DM2, hypertension, insulin resistance, and metabolic syndrome) could explain the association. However, in our analysis and in the report of Lee et.al. [18], the associations between low magnesium and coronary artery calcification remained significant after adjustment for these potential confounders. This suggests that additional mechanisms, beyond classic cardiovascular and metabolic risk factors, could be partly responsible. Results from other experimental models support the role of these other mechanisms. Experiments in cultured endothelial cells have demonstrated that magnesium deficiency promotes a proatherogenic phenotype [32]. Moreover, dietary magnesium restriction in animal models resulted in reduced plasma and erythrocyte magnesium levels, which was accompanied by endothelial dysfunction and systemic inflammation, well known factors involved in the atherogenic process. Interestingly, these abnormalities were reverted by magnesium supplementation [33]. These findings are in line with the beneficial effects of magnesium on inflammation and endothelial dysfunction [34], and suggest that this mineral may prevent the onset and progression of atherosclerosis through mechanisms beyond known traditional pathways.

The present study has some limitations. First, the cross-sectional design does not allow to establish causal or temporal relationships between serum magnesium and coronary artery calcification. Second, despite the comprehensive nature of the data which allowed adjustment for multiple risk factors, the possibility of confounding from unknown or unmeasured factors cannot be completely ruled out. Third, our study population solely comprised Mexican-mestizo subjects, and thus our findings may not apply to populations of other ethnic backgrounds.

Conclusion

The results of this study strongly suggest that lower serum magnesium levels are associated with coronary artery calcification in Mexican subjects free of clinically apparent cardiovascular disease. Confirmation of these results in other populations is required. Additional prospective studies are also needed to determine if hypomagnesaemia predicts the development and progression of coronary atherosclerosis.

Abbreviations

ALT: Alanine aminotransferase; apo A: Apolipoprotein A; apo B: Apolipoprotein B; AST: Aspartate amonitransferase; CAC: Coronary artery calcification; CHD: Coronary heart disease; CIMT: Carotid intima-media thickness; CV: Coefficient of variation; CVD: Cardiovascular disease; DM2: Type 2 diabetes mellitus; GEA: Genetics of atherosclerotic disease; HDL-C: High density lipoprotein cholesterol; HOMA-IR: Homeostasis model assessment for insulin resistance; hs-CRP: High sensitive C-reactive protein; L:SAR: Liver to spleen attenuation ratio; LDL-C: Low density lipoprotein cholesterol; MS: Metabolic syndrome; VAT: Visceral adipose tissue.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RPS and CPR- designed the study, were responsible of the data analysis and extraction, and wrote the manuscript with final comment and approval by all the authors. TVM- has been involved in the critical revision of the manuscript for important intellectual content. GCS, GVA, MTT, NPH, JMRP, AMU, EJG and JGJR- provided data and advice regarding study design. All authors were involved in the drafting of the manuscript. All authors read and approved the final manuscript

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GACETA MÉDICA DE MÉXICO ARTÍCULO ORIGINAL

Deficiencia de vitamina D y su asociación con enfermedad arterial coronaria en población mexicana: estudio Genética de la Enfermedad Aterosclerosa (GEA)

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Resumen

Objetivo: *Investigar la asociación independiente de la deficiencia de vitamina D (DVD) con la enfermedad arterial coronaria (EAC) en población adulta.* **Método:** *Estudio de casos y controles pareado. Se obtuvieron datos de factores de riesgo cardiovascular, medicamentos, alcohol, tabaquismo, consumo de vitamina D y actividad física. Se midieron variables bioquímicas, antropométricas y de presión arterial. La 25(OH)D se cuantificó por quimioluminiscencia.* **Resultados:** *Se estudiaron 250 pacientes con EAC establecida y 250 sujetos control, pareados por edad, sexo e índice de masa corporal (IMC), de 53 ± 6.1 años e IMC de 28 ± 3.5 kg/m^2. La DVD fue significativamente mayor en el grupo control (21.2 vs. 16%). El análisis de regresión logística múltiple no mostró asociación entre la DVD y la EAC (OR: 1.37 [0.08-23.2]). El análisis de regresión lineal múltiple mostró que el uso de estatinas (b = 2.2; p = 0.004) y el no consumo de alcohol (b = –1.8; p = 0.03) incrementaron significativamente la concentración de 25(OH)D.* **Conclusiones:** *En adultos mexicanos no se encontró asociación independiente entre la DVD y la presencia de EAC. Los resultados sugieren que el tratamiento con estatinas y la abstinencia en el consumo de alcohol pueden ser la explicación para las concentraciones más altas de 25(OH)D encontradas en los pacientes con EAC.*

PALABRAS CLAVE: *Deficiencia de 25(OH)D. Enfermedad arterial coronaria. Estudio GEA.*

Association of vitamin D deficiency with coronary artery disease in Mexican population: Genetics of Atherosclerotic Disease (GEA) study

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Abstract

Objective: *To investigate the independent association between vitamin D deficiency (VDD) and coronary artery disease (CAD) in Mexican adult population.* **Method:** *Matched case-control study. Data cardiovascular on risk factors, medication use, physical activity, alcohol use, smoking and vitamin D consumption were obtained. Biochemical variables, anthropometric and blood pressure were measured. 25(OH)D was quantified by chemiluminescence.* **Results:** *We studied 250 patients with established*

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CAD and 250 age-gender-body mass index (BMI) matched control subjects, with a mean age of 53 ± 6.1 years and BMI of 28 ± 3.5 kg/m². Deficiency of 25(OH)D was significantly higher in the control group (21.2 vs. 16%). Multiple logistic regression analysis did not show association between VDD and CAD (OR: 1.37 [0.08-23.2]). Multiple linear regression analysis also showed that statin use (b = 2.2; p = 0.004) and no alcohol use (b = −1.8; p = 0.03) significantly increased 25(OH)D levels. **Conclusions:** *No independent association between VDD and the presence of coronary artery disease was found in Mexican adult population. The results suggest that treatment with statins and absence of alcohol consumption, might be the explanation for the higher concentrations of 25(OH)D observed in patients with CAD.*

KEY WORDS: *Vitamin D deficiency. Coronary artery disease. Genetics of atherosclerotic disease study.*

Introducción

La enfermedad arterial coronaria (EAC) es la primera causa de muerte en el mundo[1]. En México, por mucho tiempo fue considerada una enfermedad poco frecuente, pero en los últimos años su prevalencia ha tenido un incremento considerable y se ha situado como la principal causa de mortalidad[2]. Proyecciones para el año 2030 indican que continuará esta tendencia[3].

En los últimos años se ha incrementado el interés por el estudio del metabolismo de la vitamina D (VD). El consumo en la dieta aporta solo el 10% de la VD, por lo que la mayor fuente de obtención es la síntesis endógena, resultado de la transformación de 7-dehidrocolesterol presente en la piel por efecto de la exposición a los rayos solares ultravioleta B. Los estudios epidemiológicos realizados en sujetos aparentemente sanos han reportado prevalencias de deficiencia que varían del 2 al 90%, dependiendo del punto de corte utilizado y de la población estudiada[4]. Hace aproximadamente tres décadas, Robert Scragg postuló que el incremento en las concentraciones de 25(OH)D es un factor protector contra la enfermedad cardiovascular[5]. A partir de esa fecha, un número grande de estudios han informado que las concentraciones adecuadas de 25(OH)D se asocian con menor prevalencia de diabetes, hipertensión, dislipidemia y síndrome metabólico[6-9]. Por otra parte, se ha reportado asociación de la deficiencia de 25(OH)D con mayor prevalencia de EAC asintomática[10] y con eventos cardiovasculares como infarto del miocardio[11] y mortalidad de causa cardiovascular y no vascular[12]. Sin embargo, existen varios estudios que no han encontrado asociación entre la deficiencia de esta vitamina y la enfermedad cardiovascular[13,14].

Considerando la controversia existente sobre la asociación de la VD con la enfermedad cardiovascular, que en México la deficiencia de VD (< 30 ng/ml) tiene una prevalencia alta (30%)[15] y que la enfermedad cardiovascular es la primera causa de muerte, el objetivo del presente estudio fue investigar la independencia de la asociación entre la deficiencia de 25(OH)D y la EAC en población adulta mexicana.

Métodos

Se diseñó un estudio de casos y controles pareado, anidado al proyecto Genética de la Enfermedad Aterosclerosa (GEA), el cual fue diseñado para investigar las bases genéticas de la EAC y evaluar la relación entre los factores de riesgo tradicionales y emergentes con la enfermedad aterosclerosa clínica y subclínica en población adulta mexicana. La fase transversal del proyecto GEA se realizó de junio de 2008 a febrero de 2013, con especial cuidado en la amplia caracterización de los participantes con y sin EAC. El muestreo se realizó a conveniencia y se incluyeron 1000 pacientes con EAC y 1500 sujetos control con edad de 35-75 años, residentes de Ciudad de México. El grupo con EAC fue seleccionado de la consulta externa y del departamento de hemodinámica del Instituto Nacional de Cardiología Ignacio Chávez con EAC prematura definida como antecedente personal de infarto al miocardio, angioplastia, cirugía de revascularización o estenosis coronaria ≥ 50% determinada por angiografía, diagnosticada en hombres antes de los 55 años y en mujeres antes de los 65 años. Se excluyeron los pacientes con evento cardiovascular agudo en los 3 meses previos al estudio o con insuficiencia cardiaca congestiva. El grupo control se formó con voluntarios de la población abierta, sin manifestaciones clínicas de EAC y sin antecedentes familiares de EAC prematura, que acudieron por invitación a través de medios escritos. De ambos grupos se excluyeron los pacientes con enfermedad renal, hepática o tiroidea, con enfermedades malignas o bien que tuvieran tratamiento con corticosteroides[16]. El proyecto fue aprobado por el Comité de Ética

del Instituto Nacional de Cardiología Ignacio Chávez y realizado de acuerdo con los lineamientos de la Declaración de Helsinki. Todos los participantes firmaron el consentimiento informado.

De la muestra total se seleccionaron 250 pacientes con EAC y 250 sujetos control, de edad mayor de 40 años, pareados por edad ± 1 año, sexo e IMC ± 1 kg/m^2. El grupo de casos se definió por la presencia de EAC bien establecida, y el grupo control por la ausencia de aterosclerosis subclínica, para lo que se evaluó el calcio arterial coronario (CAC = 0 U.A.) a través de tomografía axial computada con multidetector. Para este estudio se excluyeron individuos con diabetes *mellitus,* antecedente o evidencia de enfermedad renal, hepática, tiroidea u oncológica, y aquellos con tratamiento corticosteroide o uso de suplementos de VD. A todos los participantes se les aplicaron cuestionarios estandarizados para obtener información demográfica, historia familiar y personal de factores de riesgo cardiovascular, patrones de alimentación[17], actividad física[18], uso de medicamentos y consumo de tabaco y alcohol.

El tamaño de muestra estimado a través de la fórmula de Freeman[19] fue de 250 sujetos por grupo, con una razón control: caso de 1:1.

Mediciones clínicas y antropométricas

El peso se midió en una báscula calibrada, y la talla utilizando un estadímetro de pared SECA 222 (Hamburgo, Alemania). El IMC se calculó dividiendo el peso en kilogramos entre el cuadrado de la talla en metros. Se consideró sobrepeso cuando el IMC fue de 25-29.9 kg/m^2, y obesidad cuando fue ≥ 30 kg/m^2. La circunferencia de cintura se midió con una cinta métrica de fibra de vidrio, en el punto medio de la distancia entre la parte inferior de la última costilla y la cresta iliaca. La presión arterial se midió en posición sedente, después de un periodo de reposo de 10 minutos, utilizando un esfigmomanómetro digital (Welch Allyn, series 52000), y el promedio de las dos últimas de tres mediciones consecutivas se utilizó en el análisis.

En ayuno de 12 horas, se obtuvieron muestras de sangre venosa. Las concentraciones de glucosa, colesterol total (CT), triglicéridos (Tg) y colesterol de las lipoproteínas de alta densidad (C-HDL) fueron medidas en muestras frescas, mediante procedimientos enzimático-colorimétricos estandarizados (Roche/Hitachi, Alemania) en un autoanalizador Hitachi 902 (Hitachi LTD, Tokio, Japón). El C-LDL fue calculado con la fórmula de Friedewald modificada por De Long, et al.[20]. En el laboratorio de endocrinología, la precisión y la exactitud de las determinaciones de lípidos son evaluadas periódicamente por el Centro para el Control y Prevención de Enfermedades Atlanta. La proteína C reactiva (PCR) y la apolipoproteína B se cuantificaron por inmunonefelometría (BN Pro Spec Nephelometer, Dade Behring Marburg GmbH, Alemania). El coeficiente de variación fue < 6% en todos los ensayos. La insulina en suero se determinó por radioinmunoanálisis (Millipore Cat. No. HI-14K, MO, EE.UU.) con coeficientes de variación intraensayo e interensayo de 2.1 y 6.8%, respectivamente. La hiperinsulinemia se definió como un valor ≥ 16.97 μU/ml en mujeres y ≥ 15.20 μU/ml en hombres. La resistencia a la insulina se estimó por medio del modelo homeostático de resistencia a la insulina (HOMA-RI)[21], y se consideró presente cuando los valores se encontraron por arriba de la percentila 75 (3.66 en mujeres y 3.38 en hombres). La concentración de adiponectina se determinó por inmunoensayo (Quantikine ELISA, R&D Minneapolis, EE.UU.). Para la adiponectina baja, se tomaron como referencia los valores por debajo del percentil 25 (8.67 μg/ml en mujeres y 5.3 μg/ml en hombres). Estos valores de HOMA-RI y de adiponectina fueron obtenidos de una submuestra del estudio GEA que incluyó 131 hombres y 185 mujeres sin obesidad, y con valores normales de presión arterial, glucosa y lípidos. La diabetes *mellitus* fue definida de acuerdo con los criterios de la Asociación Americana de Diabetes[22], o cuando los participantes manifestaron utilizar medicamentos para el control de la glucosa y en aquellos con diagnóstico previo de diabetes realizado por un médico. Las dislipidemias se definieron de acuerdo con los siguientes puntos de corte: hipercolesterolemia, CT > 200 mg/dl o C-LDL > 130 mg/dl; hipertrigliceridemia, Tg > 150 mg/dl; C-HDL bajo, < 40 mg/dl en hombres y < 50 mg/dl en mujeres; e índice aterogénico, relación CT/C-HDL > 4.5. La concentración de 25(OH)D se cuantificó por quimioluminiscencia (Architect plus CI8200), que tiene una buena correlación (r = 0.90)[23] con la cromatografía líquida por espectrometría de masas, considerada como el método de referencia. Se usaron calibradores bajo, medio y alto, que mostraron coeficientes de variación del 3.6, el 3.1 y el 4.05, respectivamente. El coeficiente de variación interensayo fue del 2.1%. La deficiencia de VD se consideró cuando las concentraciones de 25(OH)D fueron < 20 ng/ml[24]. Las estaciones del año se clasificaron de acuerdo con las fechas establecidas para el hemisferio norte. El

consumo de VD se obtuvo a través de una frecuencia de consumo de alimentos[17] diseñada y validada por el Instituto Nacional de Salud Pública. La cantidad consumida por día se calculó por medio del programa *Sistema de evaluación de hábitos nutricionales y consumo de nutrimentos* (SNUT)[25]. La actividad física se cuantificó mediante un cuestionario que proporciona información sobre la frecuencia, la intensidad y la duración[18].

La tomografía computada es un método validado para cuantificar el CAC[26]. Las mediciones se realizaron utilizando un tomógrafo multidetector de 64 cortes (Somaton Sensation, Siemens, Malvern, PA, EE.UU.) o 256 cortes (Somaton Definition Flash, Siemens, Erlangen, Alemania), antes y después de febrero de 2009, respectivamente. El estudio se obtuvo con sincronización cardiaca mediante protocolo prospectivo con los siguientes parámetros: 120 kV, 120 mA y grosor de corte de 3 mm. El CAC se cuantificó de acuerdo con el método de Agatston[27]. Las imágenes fueron interpretadas por un radiólogo experto, en una estación de trabajo (Leonardo Workstation, Siemens, Forcheim, Alemania) provista de un programa específico para el análisis del índice de calcio CaScoring (Siemens, Forcheim, Alemania). El test-retest para el puntaje de Agatston utilizado para evaluar la fiabilidad intraobservador mostró un coeficiente de correlación intraclase muy alto (0.99).

Los datos se presentan como media ± desviación estándar, mediana (rango intercuartílico) o porcentaje. Las comparaciones entre los grupos se realizaron con las pruebas estadísticas t de Student, U de Mann-Whitney y ji al cuadrado, según correspondiera. Se usó el análisis de regresión logística condicionada simple y múltiple para evaluar la relación independiente entre la deficiencia de 25(OH)D y la EAC con ajuste de acuerdo con cinco modelos que se construyeron con las variables que fueron significativamente diferentes en el análisis bivariado y que no tuvieran colinealidad. Se realizaron también análisis de regresión lineal y múltiple para conocer las variables modificadoras de la concentración de 25(OH)D. De manera adicional, se estimó la concentración de 25(OH)D eliminando el efecto del tratamiento con estatinas, con los valores obtenidos de la diferencia entre tener o no el tratamiento entre cada grupo (grupo de EAC –1.4 ng/ml y grupo control 0.33 ng/ml). El valor de $p < 0.05$ fue considerado significativo. El análisis estadístico se realizó con el *software* STATA/MP 13 (StataCorp, Inc., College Station, Texas, EE.UU.).

Resultados

Se estudiaron 250 pacientes con EAC y 250 sujetos control, pareados por edad, sexo e IMC, cuyas características se muestran en la tabla 1. En la población total, el 82% fueron de sexo masculino, la media de edad fue de 53 años y el IMC fue de 28 kg/m^2. En los pacientes con EAC, las concentraciones de CT, C-HDL, C-LDL y apolipoproteína B, el índice aterogénico y el consumo de VD con la dieta fueron significativamente menores en comparación con el grupo control ($p < 0.05$). Por el contrario, la concentración de insulina y el valor de HOMA-RI fueron significativamente mayores ($p < 0.005$). En los parámetros relacionados con el perfil de inflamación, los valores de PCR no fueron diferentes entre los dos grupos, pero se observó que la concentración de adiponectina fue más alta en el grupo control de manera estadísticamente significativa ($p = 0.007$). La proporción de participantes tratados con estatinas fue significativamente mayor en los pacientes con EAC (91.2 vs. 6.8%; $p < 0.001$). El tabaquismo y la frecuencia de consumo de bebidas alcohólicas fueron 10 puntos porcentuales más altos en el grupo control ($p < 0.01$).

La concentración de 25(OH)D en el grupo de EAC fue significativamente más alta en comparación con el grupo control (28 ng/ml *vs.* 25 ng/ml; $p <0.001$), aunque el consumo de VD fue significativamente más bajo en el grupo de EAC (129.6 vs. 158.9 UI; $p = 0.001$). La prevalencia de insuficiencia de 25(OH)D (20-29.9 ng/ml) fue mayor en los sujetos control (57.6 vs. 49.2%; $p = 0.13$), al igual que la prevalencia de deficiencia (< 20 ng/ml; 21.2 vs. 16%; $p = 0.001$). La toma de muestras de sangre en los dos grupos estudiados se realizó de manera proporcional en cada estación del año ($p = 0.20$), lo que permitió controlar el efecto producido por la exposición solar en las diferentes estaciones sobre las concentraciones de 25(OH)D circulante. El resto de las variables fueron similares en ambos grupos.

Las prevalencias de los factores de riesgo cardiovascular por grupo de estudio se muestran en la figura 1. La hipertensión arterial mostró una prevalencia significativamente más alta en los pacientes (63.2 vs. 13.2%; $p <0.0001$), y de manera similar, tanto la resistencia a la insulina elevada (66.4 vs. 54.4%; $p = 0.006$) como los valores bajos de adiponectina (57.6 vs. 45.3%, $p = 0.007$) se observaron con una frecuencia significativamente más alta en el grupo con EAC, mientras que el C-LDL elevado fue más prevalente entre los controles (30.4 vs. 19.2%; $p = 0.004$). Las otras anormalidades mostraron

Tabla 1. Características clínicas y bioquímicas de la población de estudio de acuerdo a la presencia de enfermedad arterial coronaria

	Grupo con EAC n = 250	Grupo control n = 250	p
Sexo (H), %*	82.4	82.4	0.99
Edad, años*	53.3 ± 6.0	53.5 ± 6.2	0.61
IMC, kg/m^2*	28.0 ± 3.5	27.9 ± 3.5	0.78
PAS, mmHg	114.5 [106-123.5]	113.2 [105.5-122]	0.47
PAD, mmHg	72 [65.5-77.5]	71 [66.5-76.5]	0.83
CT, mg/dl	163.7 [139.8-195.0]	187.0 [164.5-209.0]	< 0.001
Tg, mg/dl	159.5 [116.0-208.0]	160.6 [113-233.7]	0.77
C-HDL, mg/dl	38.1 [32.0-45.2]	40.3 [34.0-49.0]	0.01
C-LDL, mg/dl	97.8 [76.0-118.2]	114.2 [93.2-135.4]	< 0.001
Índice aterogénico	4.2 [3.4-5.1]	4.5 [3.6-5.5]	0.02
Glucosa, mg/dl	89.5 [84-95]	90 [85-95]	0.95
Insulina, µUI/ml	18.2 [14.5-26.4]	16.6 [12.3-23.1]	0.002
HOMA-RI	4.0 [3.1-6.0]	3.6 [2.6-5.2]	0.003
ApoB, mg/dl	80 [65-103]	96.5 [77-116]	<0.001
Adiponectina, µg/ml	5.1 [3.3-8.2]	6.1 [3.8-10.1]	0.007
PCR, mg/l	1.1 [0.6-2.2]	1.2 [0.6-2.4]	0.37
Tabaquismo, %	12	22.8	0.001
Uso de alcohol, %	72.1	82.6	0.006
Índice de actividad física	7.9 ± 1.2	7.9 ± 1.2	0.70
Vitamina D			
25(OH) D, ng/ml	28 ± 8.2	25 ± 6.8	< 0.001
Estado de 25(OH) D, %			
Óptimo (≥ 30 ng/ml)	34.8	21.2	0.001
Insuficiente (20-29.9 ng/ml)	49.2	57.6	0.13
Deficiente (< 20 ng/ml)	16	21.2	0.001
Consumo de vitamina D, UI/día	129.6 [88.1-219.0]	158.9 [102.3-249.1]	0.01
Estación de muestreo, %			
Primavera	22.4	20	
Verano	29.6	29.6	
Otoño	28.4	36	0.20
Invierno	19.6	14.4	
Uso de estatinas, %	91.2	6.8	<0.001

Los datos se muestran como media ± desviación estándar y mediana (rango intercuartílico).
*Variables de pareamiento. p < 0.05 significativa.
ApoB: apolipoproteína B; C-HDL: colesterol de las lipoproteínas de alta densidad; C-LDL: colesterol de las lipoproteínas de baja densidad; CT: colesterol total; EAC: enfermedad arterial coronaria; HOMA-RI: modelo homeostático de resistencia a la insulina; IMC: índice de masa corporal; PAD: presión arterial diastólica; PAS: presión arterial sistólica; PCR: proteína C reactiva; Tg: triglicéridos.

frecuencias similares en ambos grupos, pero se hace notar que sus prevalencias fueron altas: el C-HDL bajo tuvo una prevalencia del 62.4 vs. 55.6%, la hipertrigliceridemia del 54.4 vs. 56%, y la glucosa de ayuno alterada del 15.2 vs. 14% en el grupo de EAC y en el grupo control, respectivamente.

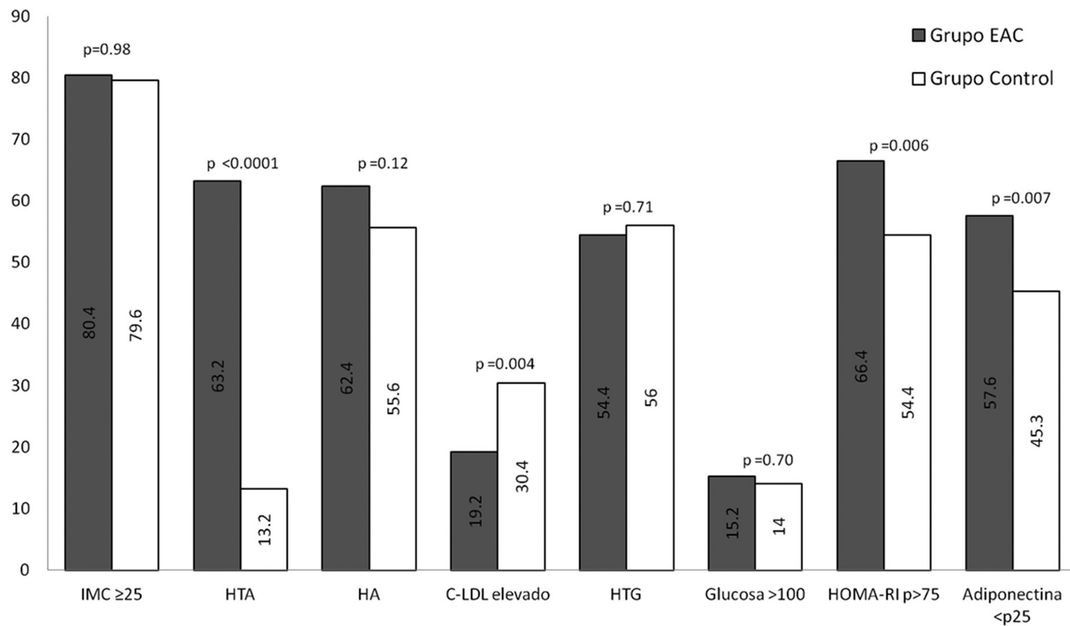

Figura 1. *Prevalencia de anormalidades metabólicas en los pacientes con enfermedad arterial coronaria (EAC) y en el grupo control. C-LDL: colesterol de las lipoproteínas de baja densidad; HA: hipoalfalipoproteinemia; HTA: hipertensión arterial; HOMA-RI: modelo homeostático de resistencia a la insulina; HTG: hipertrigliceridemia; IMC: índice de masa corporal.*

Para investigar la independencia de la asociación entre la deficiencia de 25(OH)D y la EAC se realizó un análisis de regresión logística condicionado ajustando por las variables que mostraron ser significativamente diferentes en el análisis bivariado. En la tabla 2 se muestra que aun en el modelo sin ajuste no se encontró asociación.

Con el fin de conocer los factores que pudieron haber influido en las concentraciones de 25(OH)D, también se utilizaron análisis de regresión lineal simple y múltiple ajustados por las variables que se han descrito en la literatura como modificadoras de la VD (Tabla 3). Los resultados del análisis simple y múltiple muestran que, en la población total, el uso de estatinas incrementa 2.2 ng/ml la concentración de 25(OH)D y explica el 14% de la variación (p = 0.004); en contraste, el uso de alcohol la disminuye 1.8 ng/ml y explica el 10% de las concentraciones bajas de 25(OH)D (p = 0.03). En los participantes en el estudio, la pravastatina fue la estatina usada con mayor frecuencia, seguida de la atorvastatina, la simvastatina y la rosuvastatina (Tabla 4). De manera adicional, se estimó la concentración de 25(OH)D eliminando el efecto del tratamiento con estatinas en el grupo de EAC (−1.4 ng/ml) y el grupo control (0.33 ng/ml). La concentración de 25(OH)D estimada en el grupo de EAC mantuvo valores significativamente más altos en comparación con el grupo control (26.7 *vs.* 25.04 ng/ml; p = 0.01), pero la prevalencia de deficiencia de VD aumentó 5.2 puntos porcentuales y se igualó con la del grupo control (21.2 *vs.* 20.8%; p = 0.50). Sin embargo, contra lo esperado, la concentración de 25(OH)D por tipo de estatina y dosis no mostró un incremento dosis-respuesta.

Tabla 2. Asociación de la deficiencia de 25(OH) D y la enfermedad arterial coronaria

	OR (IC 95%)	p
Deficiencia de 25(OH) D (< 20 ng/ml)	0.68 (0.42-1.10)	0.12
Modelo 1	0.85 (0.28-2.55)	0.78
Modelo 2	0.83 (0.27-2.51)	0.74
Modelo 3	0.97 (.29-3.2)	0.97
Modelo 4	1.48 (0.33-6.5)	0.60
Modelo 5	1.37 (0.08-23.2)	0.80

Valores expresados en ***odds ratio*** (OR) e intervalo de confianza del 95% (IC 95%).
Análisis multivariado de regresión logística condicionada.
Modelo 1: deficiencia de vitamina D + estatinas.
Modelo 2: modelo 1 + índice aterogénico > 4.5.
Modelo 3: modelo 2 + HOMA-RI y adiponectina.
Modelo 4: modelo 3 + tabaquismo y uso de alcohol.
Modelo 5: modelo 4 + ingesta de vitamina D.

Discusión

En el presente estudio, después de ajustar por el tratamiento con estatinas, el índice aterogénico > 4.5,

Tabla 3. Análisis de regresión lineal de los factores asociados con la concentración de 25(OH) D

	Simple			Multivariado*		
	b	β	p	b	β	p
Uso de estatinas	2.73	0.17	< 0.001	2.2	0.14	0.004
Uso de alcohol	–2.10	–0.11	0.01	–1.8	–0.10	0.03

Valores expresados como coeficientes b y β. Análisis de regresión simple y multivariado (ajustado por sexo, edad, tabaquismo positivo, IMC, actividad física total, estación de muestreo e ingesta de vitamina D)
*R^2= 4%.

Tabla 4. Uso de estatinas por grupos de estudio

	Grupo con EAC n = 228	Grupo control n = 16	p
Pravastatina, n (%)	83 (36.4)	7 (43.7)	0.59
Atorvastatina, n (%)	50 (21.9)	5 (31.2)	0.36
Simvastatina, n (%)	50 (21.9)	2 (12.5)	0.53
Rosuvastatina, n (%)	43 (18.8)	2 (12.5)	0.74
Simvastatina + ezetimiba, n (%)	2 (0.88)	0	0.99

Prueba de Fisher: $p < 0.05$ significativa.
EAC: enfermedad arterial coronaria.

HOMA-RI, la adiponectina, el tabaquismo, el uso de alcohol y la ingesta de VD, el análisis de regresión multivariado no mostró asociación de la deficiencia de 25(OH)D con la presencia de EAC ni con factores de riesgo cardiovascular. Estos resultados contrastan con estudios que han mostrado que los valores bajos de VD se asocian a un estado inflamatorio sistémico, favorecen la resistencia a la insulina y activan el sistema renina-angiotensina-aldosterona, con la subsecuente elevación de la presión arterial y la hipertrofia del músculo cardiaco y de las células musculares lisas[28-30]. En concordancia con estos hallazgos, varios estudios observacionales han reportado una asociación entre la deficiencia de 25(OH)D y la presencia de EAC clínica[13,31-33] y subclínica[34-38]. En un estudio de casos y controles en el que se incluyeron tanto pacientes con enfermedad coronaria como pacientes con enfermedad vascular cerebral, la deficiencia de 25(OH)D, definida por concentraciones inferiores a 15 ng/ml, mostró una alta prevalencia en pacientes (68%) y en controles (54%), y se encontró asociada a la presencia de enfermedad cardiovascular (OR: 2.9 [1.67-5.12]; $p < 0.001$)[33]. De manera similar, en 1370 sujetos de 45 a 84 años, de los cuales 394 cursaban con insuficiencia renal crónica, De Boer et al.[14] reportaron deficiencia de 25(OH)D (< 15 ng/ml) en el 73.2% de la población, así como su relación modesta, pero independiente, con la prevalencia de CAC (RR: 1.06 [1.00-1.13]; p = 0.06). En otro estudio, Lim et al.[38] informaron de que, en 921 sujetos mayores de 65 años, la deficiencia de 25(OH)D (< 30 ng/ml), observada en el 94% de la población estudiada, se asoció con estenosis coronaria ≥ 50% (OR: 2.08 [1.16-4.68]). Sin embargo, otras investigaciones no han observado esta asociación. En un estudio realizado en 387 sobrevivientes del primer infarto al miocardio, con sus respectivos 387 controles, pareados por edad y sexo, la deficiencia de 25(OH)D (< 30 ng/ml) encontrada en el 80% de los pacientes y el 75% del grupo control no mostró asociación con la cardiopatía coronaria (OR: 1.01 [0.82-1.25])[13]. Sin embargo, el apoyo más importante a los resultados obtenidos en nuestro estudio está dado por dos revisiones sistemáticas y metaanálisis recientes[39,40]. En una se incluyeron 82 estudios prospectivos de cohorte, 84 intervenciones aleatorizadas controladas, 20 metaanálisis de 208 estudios prospectivos y 8 metaanálisis de 88 estudios de intervención aleatorizados controlados[39]. En la otra, los autores analizaron 76 revisiones sistemáticas de estudios observacionales, 48 metaanálisis de estudios observacionales y 57 metaanálisis de estudios de intervención aleatorizados controlados[40]. Estas dos revisiones de la amplia literatura sobre el tema identificaron una discrepancia entre los estudios de tipo observacional y los ensayos clínicos controlados, en la que la mayoría de los estudios de suplementación no mostraron un efecto de la VD sobre la enfermedad cardiovascular. Por tanto, los autores concluyeron que la deficiencia de VD es muy probablemente un marcador de estado de salud deficiente más que la causa de la enfermedad.

Entre los factores que influyen en las concentraciones de 25(OH)D circulante[41], en la población de nuestro estudio no se observó influencia del sexo, la edad, el consumo de tabaco, el IMC, la actividad física, la estación del año en que se realizó el muestreo ni la ingesta de VD. Sin embargo, el no consumo de alcohol y el tratamiento con estatinas se asociaron con valores más altos de 25(OH)D.

Tanto en la población total como en el grupo con EAC, el no consumo de alcohol se asoció con concentraciones significativamente más altas de 25(OH) D, lo que está de acuerdo con la observación de que los sujetos con consumo mayor de 20 gramos de alcohol al día tienen concentraciones menores de 25(OH)D[42].

La terapia con estatinas es otro factor que modifica los valores de la 25(OH)D en suero. La asociación del uso de estatinas con las concentraciones de 25(OH)D se observó en un estudio de 208 mujeres con suplementación de VD durante 3 años[43]. Los valores de esta forma de la VD fueron más altos en las 51 mujeres usuarias de estatinas, tanto en la muestra basal como durante el tratamiento, independientemente de si recibían placebo o VD. En otro estudio que investigó el efecto de la atorvastatina en dosis de 10-80 mg diarios sobre las concentraciones de VD en pacientes con cardiopatía isquémica, Pérez-Castrillón et al.[44] encontraron un aumento de la 25(OH)D, de 17.08 ± 7 a 19.6 ± 7.9 ng/ml ($p = 0.003$), y una disminución del 75 al 57% en la proporción de pacientes con deficiencia de VD después de 1 año de terapia con la estatina. Estos resultados son notablemente similares a los del presente estudio, en el cual los valores de 25(OH)D en el 91.2% de los pacientes tratados con alguna estatina (28 ± 8.1 ng/ml) fueron significativamente más altos que los observados en el 8.8% de los no usuarios de estatinas (26.6 ± 9.5 ng/ml) y en el grupo control (25.03 ± 6.7 ng/ml). Aunque al momento actual no se tiene certeza del mecanismo mediante el cual las estatinas incrementan las concentraciones circulantes de esta vitamina, se ha especulado que debido a que el 7-dehidrocolesterol es precursor tanto del colesterol como de la 25(OH)D, al inhibirse la hidroximetilglutaril-coenzima A reductasa por acción de la estatina se incrementaría el sustrato para la síntesis de 25(OH)D[44].

En las fortalezas de nuestro estudio se incluye que los participantes sin datos clínicos de enfermedad vascular, con y sin CAC, y aquellos con cardiopatía coronaria bien definida, han sido ampliamente caracterizados desde los puntos de vista clínico, bioquímico y radiológico. La amplia caracterización de los grupos de estudio permitió ajustar por un número grande de factores confusores, y además se excluyeron a los sujetos con diabetes, eliminando así un factor altamente confusor para la EAC y modificador de las concentraciones de 25(OH)D; además, a diferencia de estudios previos, se tuvo la certeza de que el grupo control no presentaba aterosclerosis subclínica.

Entre las limitaciones de este trabajo se encuentran, primero, debido al carácter transversal del estudio, que no se puede establecer relación causal entre la DVD y la EAC. Segundo, la medición de la 25(OH)D se realizó en una sola ocasión, pero se ha mostrado que las concentraciones de 25(OH)D parecen ser muy constantes en el tiempo[45]. Tercero, la mayor parte de los pacientes estaban tratados con estatinas, por lo que no podían ser excluidos; sin embargo, al corregir por el efecto de la estatina las concentraciones de 25(OH)D y la prevalencia en la deficiencia de VD fueron similares entre el grupo de pacientes y el grupo control.

En conclusión, los resultados de este estudio en población mestiza mexicana no mostraron asociación entre la deficiencia de 25(OH)D y la presencia de EAC. El tratamiento con estatinas y el menor consumo de alcohol son factores importantes con influencia en las concentraciones de 25(OH)D circulantes. En los pacientes con EAC, la concentración de 25(OH)D fue mayor y la prevalencia de un estado de deficiencia de esta vitamina fue menor que en sus controles pareados por sexo, edad e IMC. Los resultados sugieren que el tratamiento con estatinas y el menor consumo de alcohol pudieran ser las explicaciones para las concentraciones más altas de 25(OH)D encontradas en los pacientes con EAC.

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Archives of Medical Research 48 (2017) 73−78

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ORIGINAL ARTICLE

Association of Adiponectin with Subclinical Atherosclerosis in a Mexican-Mestizo Population

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Background and Aims. Adiponectin (ADPN) is a cardioprotective adipocytokine, and its association with atherosclerosis development is controversial. The aim of the present study was to assess the association of low ADPN plasma levels with the presence of subclinical atherosclerosis in a Mexican-Mestizo population without history of diabetes or coronary artery disease (CAD).

Methods. In 818 subjects (53.4 ± 9 years; 49.9% women) anthropometry, subcutaneous and visceral adipose tissue, lipids, glucose, C-reactive protein (CRP), insulin, and ADPN levels were determined. Carotid artery intima-media thickness (CIMT) was measured with ultrasound in B mode and the sex-age specific value higher than 75th percentile defined the presence of subclinical atherosclerosis. Low ADPN was considered when plasma concentrations were lower than 25th percentile (8.67 μg/mL in women, 5.30 μg/mL in men).

Results. Prevalence of low ADPN was 43.6% (42.9% in women and 44.4% in men; $p = 0.66$) and elevated CIMT (eCIMT) was 23.8% (25.8% in women and 21.9% in men; $p = 0.184$). In addition to their higher prevalence of low ADPN, subjects with eCIMT had higher values of body mass index, blood pressure, total cholesterol, triglycerides, glucose, insulin, and CRP. Multivariate analysis revealed that independent of these factors, low ADPN was associated with eCIMT (OR [95% CI]: 1.505 [1.051−2.153]).

Conclusions. In the studied population, low adiponectin concentrations are associated with a higher prevalence of subclinical atherosclerosis, independent of traditional cardiovascular risk factors. © 2017 IMSS. Published by Elsevier Inc.

Key Words: Adiponectin, Atherosclerosis, Intima-media thickness, Cardiovascular risk factors, Mexican-Mestizo population.

Introduction

Coronary artery disease (CAD) is the main cause of morbidity and mortality in Mexico (1). Although clinical, epidemiological, and experimental studies have shown that CAD development is related to multiple traditional risk factors (2), effective treatment of lipids, high blood pressure, hyperglycemia, and unhealthy lifestyles only reduces cardiovascular risk between 35 and 50% in high-risk populations (3−5). As a result, there has been an increased interest in identifying novel biomarkers that might improve the risk prediction of CAD (6,7). Indeed, several investigators have focused attention on adipose tissue excess that may favor CAD through different pathways (8−10).

Adiponectin (ADPN) is an anti-inflammatory cytokine mainly produced in adipose tissue, and owing to its endocrine, paracrine, and autocrine effects, it could participate in CAD through regulation of multiple metabolic processes (11−15). Moreover, the decreased ADPN plasma levels

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observed in obesity, particularly visceral adipose tissue excess, has been associated with the presence of cardiovascular risk factors such as insulin resistance, hypertension, dyslipidemia, and inflammation (11–15). In addition, low ADPN levels have been associated with subclinical atherosclerosis only in patients with diabetes (16) or in men with high cardiovascular risk but not in women (17). In three studies conducted in subjects free of cardiovascular disease, one study found an inverse association (18), whereas the others showed no independent association (8,19).

Considering the increasing prevalence of obesity and atherosclerotic cardiovascular disease (20–23) as well as the reduced ADPN plasma levels (23–25) found in the Mexican population, the aim of the present study was to investigate whether low ADPN is associated with increased common carotid intima-media thickness (CIMT), a measure of subclinical atherosclerosis (26,27) that has been shown to be a predictor of future cardiovascular events (28–30). To test this hypothesis, ADPN and CIMT were studied in Mexican-Mestizo subjects without diabetes mellitus or personal history of premature CAD.

Materials and Methods

Study Population

This investigation is part of the ‘‘Genetics of Atherosclerosis Disease’’ (GEA, in Spanish) study, designed at the National Institute of Cardiology Ignacio Chavez to examine the genomic basis of coronary artery disease and to evaluate its relationship with traditional and emerging cardiovascular risk factors in the adult Mexican population. The study included a group of 1,000 patients with CAD and a control group of 1,500 individuals without CAD, who were all between the ages of 35 and 70 years and residents of Mexico City. Subjects from the control group were volunteers with no clinical or family history of CAD who attended the blood bank of the National Institute of Cardiology Ignacio Chavez or were invited via written messages placed in social service centers. The GEA study was approved by the Ethics and Research Committee of the National Institute of Cardiology Ignacio Chavez based on the guidelines from the Declaration of Helsinki. All subjects who participated in the study signed informed consent.

This is a cross-sectional analysis of the baseline measurements of 1,500 control subjects included in the GEA study. Because diabetes is associated with higher CAD risk (31), we excluded all subjects with previously diagnosed type 2 diabetes (n = 202). We also excluded 480 subjects with unavailable ADPN (n = 86) or CIMT (n = 394) measurements. Initially, CIMT measurement was not considered in the GEA study. The final population for this analysis comprised 818 subjects. When compared with the included participants, those excluded were similar in age, frequency of both genders, and cardiovascular risk factors (data not shown).

Anthropometry and Arterial Tension

All subjects were interviewed by trained research staff and completed questionnaires to collect information pertaining to demographic characteristics, CAD history, medication, alcohol and tobacco use. All participants had a complete clinical examination. Height was measured to the nearest 0.1 cm using a rigid stadiometer, and weight was measured to the nearest 0.1 kg with the use of a balance scale. Body mass index (BMI) was calculated as weight in kilograms divided by height in square meters. After a 10-min rest, blood pressure was measured three times; the average of the second and third blood pressure measurements was used for the analysis. Hypertension was defined as self-reported treatment with antihypertensive medications or a systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mm Hg.

Laboratory Analyses

Venous blood samples were collected from subjects after a 12-h fast and 20 min in a sitting position. Plasma glucose, total cholesterol, triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) were measured using standardized enzymatic procedures (Roche Diagnostics GmbH, Mannheim, Germany). Accuracy and precision of lipid measurements in our laboratory are under periodic surveillance by the Center for Disease Control and Prevention service (Atlanta, GA). Inter-assay coefficients of variation were <6% for all assays. Low-density lipoprotein cholesterol (LDL-C) was estimated using the method of De Long et al. (32). Hypercholesterolemia was defined when total cholesterol was ≥200 mg/dL and hypertriglyceridemia when TG concentration was ≥150 mg/dL. High-sensitivity C-reactive protein (hsCRP) was determined by immunonephelometry on a BN Pro Spec nephelometer (Dade Behring, Marburg, Hesse, Germany) according to the manufacturer’s method. Plasma insulin concentrations were determined by a radioimmunometric assay (Millipore, St. Charles, MO) with intra- and inter-assay variation coefficients of 2.1 and 6.8%, respectively. The cut-off point to define hyperinsulinemia was calculated in a subsample of subjects without cardiovascular risk factors of the GEA control group (127 men and 169 women). Hyperinsulinemia was considered when insulin concentration in serum was > 75th percentile (17.2 μU/mL in women and 14.3 μU/mL in men). Type 2 diabetes mellitus was defined with fasting glucose values ≥126 mg/dL, if there was a previous diagnosis, or when the participant reported the use of glucose-lowering therapy.

Human total ADPN levels were determined with a Quantikine ELISA kit (R&D Systems, Minneapolis, MN) with intra- and inter-assays variation coefficients <10%. The cut-off point to define low ADPN values was also calculated in the subsample of subjects without cardiovascular risk factors. Low ADPN was considered when its

concentration in serum was < 25th percentile (8.67 μg/mL in women and 5.30 μg/mL in men).

Computed Tomography

Computed tomography (CT) is a validated method for measuring visceral adipose tissue (33). In the present study, CT of the abdomen and chest were performed using a 64 channel multidetector helical system (Somatom Cardiac Sensation 64, Forcheim, Bavaria, Germany) and interpreted by experienced radiologists. Scans were read to assess and quantify total, subcutaneous, and visceral abdominal adipose tissue as described by Kvist et al. (34) Twenty different scans were randomly selected to evaluate consistency of interpretation. Intra-observer coefficient correlation was 0.99 (p <0.001).

Carotid Intima-Media Thickness

To analyze subclinical atherosclerosis, the CIMT was measured using high-resolution ultrasound equipment in B mode (Sonosite MicroMaxx) with a 13−6 MHz transducer. The study was performed in supine with extended neck position. The measurements of the intima-media of the carotid artery were performed in the horizontal plane in the distal wall of the carotid artery at 2 cm from the bifurcation of the carotid sinus. Thickness of the intima-media was calculated as the distance between the arterial intima-lumen interface and the interface of the media-adventitia of the distal wall. Five measurements were performed each in the right and left carotid arteries, and the average of all measurements was used to determine the CIMT. Subclinical atherosclerosis was defined as a CIMT higher than the 75th percentile (eCIMT), specifically established for the Hispanic population by gender and age group and/or presence of atherosclerotic plaque (35). Procedures were performed by only one trained observer. Reproducibility of measurements was calculated with 5% of the cohort, obtaining an intra-observer correlation coefficient of 0.96.

Statistical Analysis

Data are presented as mean ± standard deviation (SD), median (interquartile range) or simple frequencies and percentages. Comparison of means was accomplished with Student t test for two groups. Median comparison was performed with Mann-Whitney U test for two groups. Prevalence was compared with chi square test. To determine the association of subclinical atherosclerosis with low ADPN, the studied population was stratified in subjects with normal CIMT (CIMT < 75th percentile) and subjects with elevated CIMT (CIMT > 75th percentile). To identify independence of the association between eCIMT and low ADPN, a logistic regression analysis was performed including age, gender, BMI, visceral adipose tissue, smoking, blood pressure, total cholesterol, HDL-C, TG, CRP, glucose, and insulin as adjusting variables. Statistical significance was set at p < 0.05. SPSS v15.0 software (SPSS, Chicago, II) was used for statistical analyses.

Results

The studied population was comprised of 818 subjects (49.9% women) with a mean age of 53.4 ± 9 years, median ADPN levels of 7.55 (4.7−12.3) μg/mL and CIMT of 0.62 (0.55−0.72) mm. General prevalence of overweight (BMI 25−29.9 kg/m^2) was 46.5%; obesity (BMI >29.9 kg/m^2) 32.4%; hypertension 23.3%; tobacco smoking 22.4%; hypercholesterolemia 40.2%; hypertriglyceridemia 48.3%; hyperinsulinemia 54.2%; eCIMT 23.8% and low ADPN levels 43.6%. Stratification of subjects according to CIMT revealed that compared to those with normal CIMT, those with eCIMT had significantly higher values in BMI, visceral adipose tissue, blood pressure, total cholesterol, LDL cholesterol, glucose, and CRP (Table 1). As shown in Figure 1, prevalence of low ADPN was also significantly higher in subjects with eCIMT (40.8 vs. 49.2%; p = 0.037).

To evaluate the independence of the eCIMT association with low ADPN values, a logistic regression analysis was performed adjusted by the variables that were found different between the subjects with and without eCIMT and by factors traditionally associated with CAD. As shown in Figure 2, when compared to subjects with normal ADPN values, those with low values of this cytokine had a significantly higher probability of presenting eCIMT (OR: 1.409 [95% CI: 1.020−1.946]). This association showed a moderate increase after adjusting by clinical variables (OR: 1.425 [95% CI: 1.012−2.007]) and persisted with statistical significance after including the biochemical variables in the model (OR: 1.505 [95% CI: 1.051−2.153]). A similar trend was observed when men and women were separately analyzed (data not shown). The aforementioned suggests an independent association between low ADPN plasma levels and subclinical atherosclerosis (eCIMT).

Discussion

The results of the present study showed a 43.6% prevalence of low ADPN and 23.8% of elevated CIMT in this Mexican-Mestizo population without diabetes and without history of CAD. More importantly, our data demonstrated that low ADPN plasma levels are independently associated with the presence of subclinical atherosclerosis. These data suggest that beyond cardiovascular risk factors, ADPN could be considered an important modifiable atherosclerotic risk factor.

Previous studies have reported ethnic differences in ADPN plasma levels, with lower values found in Hispanics

Table 1. General characteristics of the study population

	Total (n = 818)	Normal CIMT (n = 623)	Elevated CIMT (n = 195)	K[a]
Age (years)	53.4 ± 9	53 ± 9	54 ± 8	*NS*
Women (%)	49.9	48.6	53.8	*NS*
BMI (kg/m^2)	28.1 (25.4−31.3)	27.9 (25.2−31.0)	28.6 (26.3−31.9)	0.013
Visceral adipose tissue (cm^2)	150 (110−191)	146 (108−190)	160 (119−199)	0.026
Subcutaneous adipose tissue (cm^2)	286 (215−366)	284 (211−357)	299 (226−377)	0.088
SBP (mmHg)	112 (104−123)	111 (103−121)	116 (107−130)	<0.001
DBP (mmHg)	71 (65−76)	70 (65−76)	73 (66−79)	0.002
Total cholesterol (mg/dL)	193 ± 37	191 ± 37	200 ± 39	0.003
LDL cholesterol (mg/dL)	118 ± 33	116 ± 32	125 ± 34	0.001
HDL cholesterol (mg/dL)	46.3 ± 13.7	45.9 ± 13.6	47.6 ± 13.9	*NS*
Triglycerides (mg/dL)	147 (113−203)	148 (113−203)	143 (111−202)	*NS*
Glucose (mg/dL)	89 (84−95)	89 (83−95)	91 (86−96)	0.008
Insulin (μU/mL)	17.1 (12.4−23.4)	16.9 (12.2−23.1)	18.0 (13.2−24.2)	*NS*
CRP (mg/L)	1.41 (0.77−3.08)	1.28 (0.73−2.39)	1.76 (1.00−3.47)	0.001

CIMT, carotid intima-media thickness; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; CRP, C-reactive protein.
Values are provided as mean ± SD, median (inter-quartile range) or percentage.
[a]*p* values based on normal vs. elevated CIMT groups comparison.

(23−25,36). However, investigations on the association between adiponectin and CIMT are limited and have been mainly performed in non-Hispanic populations. In fact, to the best of our knowledge, the relationship between ADPN and cardiovascular disease has not been previously analyzed in the Mexican population. Therefore, the present study is the first to show that low ADPN plasma levels are associated with high prevalence of eCIMT. Furthermore, in multivariate modeling we found that independent of biochemical parameters, body composition data, and significantly related carotid CIMT factors, low ADPN plasma levels were associated with 50% higher odds of eCIMT.

The independent association between ADPN and CIMT is controversial. In a high cardiovascular risk European population, Persson et al. (17) observed a relationship between ADPN and elevated CIMT among men, but not among women. In another study of subjects with high cardiovascular risk, Gardener et al. (16) showed that in black, white and Hispanics, ADPN was independently associated with eCIMT in diabetic, but not in non-diabetic, subjects. Investigations conducted in Caucasian subjects without personal history of cardiovascular disease or diabetes reported that subjects with low ADPN values had higher CIMT (8,19). However, this association was lost after adjusting for multiple cardiovascular risk factors. Interestingly, in 1033 healthy Korean subjects without diabetes and no personal history of cardiovascular disease, Yoon et al. (18) reported that low ADPN values were independently associated with CIMT >0.9 mm in both genders. In agreement with these results, our findings in the GEA population with similar characteristics showed that, in men and women, this association was independent of confounding factors. Possible explanations for the discrepant results between studies include differences in ethnic origins (23−25,36) and in the characteristics of the population studied such as presence of diabetes or cardiovascular disease.

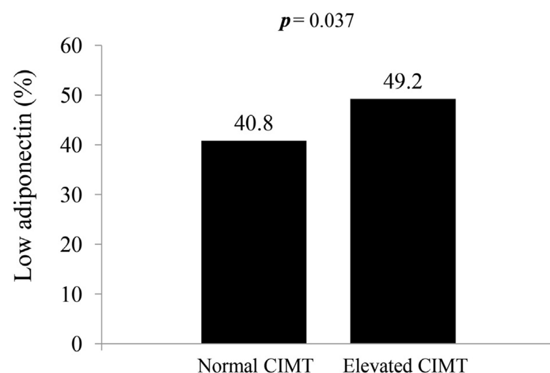

Figure 1. Low adiponectin prevalence by presence (elevated CIMT) or absence (normal CIMT) of subclinical atherosclerosis.

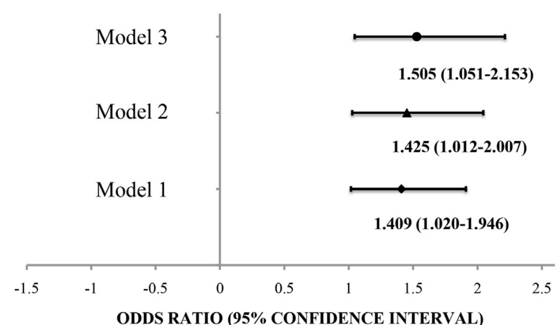

Figure 2. Odds ratio of subclinical atherosclerosis (elevated CIMT) in subjects with low adiponectin levels (<8.67 μg/mL in women and <5.30 μg/mL in men) relative to those with normal adiponectin levels.

Given its multiple metabolic functions that include anti-diabetic, anti-inflammatory and anti-atherogenic properties (11), adiponectin has been proposed as the link between obesity and increased cardiovascular risk (8). A notable finding of our study was that adiposity measurements such as body mass index and visceral adipose tissue did not modify the association between low ADPN and eCIMT (Figure 2). This result suggests that ADPN could have a direct effect on atherosclerosis development. This hypothesis is supported by previous reports demonstrating that ADPN is able to regulate nitric oxide synthesis, adhesion molecules expression, differentiation of monocytes to macrophages and foam cells as well as proliferation and migration of smooth muscle cells to the endothelial space (12−15). Collectively, these data suggest that this adipo-cytokine could be considered not only a marker, but also a risk factor, for cardiovascular disease.

This study has some limitations. First, due to the cross-sectional nature of the GEA study, the results of the present analysis do not enable us to identify causality on the association of ADPN with CIMT. Nonetheless, earlier studies that have analyzed possible mechanisms about this association do support the hypothesis that ADPN may favor atherosclerosis development and CAD appearance (12−15). Second, the GEA study included only subjects who were free of personal or family history of premature CAD; thus, the results may not be applicable to the general population. Third, because there are no well-established cut-off points to define low ADPN in our population, percentile cut-off points were used in the present analysis. However, this has been recommended for the study of other metabolic parameters (37,38). In addition, to calculate the cut-off points we chose subjects without cardiovascular risk factors as these could modify the ADPN plasma concentration (11−15).

In conclusion, results of the present study show that low ADPN levels are associated with subclinical atherosclerosis defined as elevated CIMT. We demonstrated that in a Mexican-Mestizo population without history of diabetes and premature cardiovascular disease, ADPN provides additional information for the assessment of atherosclerotic risk, beyond that provided by conventional cardiovascular risk factors including adiposity excess. These findings support the evidence about a novel and modifiable atherosclerotic risk factor with potentially substantial clinical benefits for CAD reduction.

Acknowledgments

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No potential conflicts of interest relevant to this article were reported.

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Diabetology & Metabolic Syndrome

RESEARCH

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Adipose tissue dysfunction increases fatty liver association with pre diabetes and newly diagnosed type 2 diabetes mellitus

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Abstract

Background: To evaluate the role of adipose tissue function on the association of fatty liver (FL) with impaired fasting glucose (IFG) or newly diagnosed type 2 diabetes mellitus (nT2D).

Methods: In 1264 subjects, computed tomography was used to evaluate FL and elevated visceral adipose tissue (VAT). Fasting plasma glucose, <5.6, 5.6–6.9 and ≥7 mmol/l, were used to defined normoglycemic (NG), IFG or nT2D, respectively. Elevated free fatty acids, low serum adiponectin levels and adipose tissue insulin resistance (Adipo-IR), were used as markers of adipose tissue dysfunction.

Results: Compared to NG subjects, those with IFG or nT2D had higher prevalence of FL and elevated VAT. FL was found to be independently associated with IFG and nT2D. Adipo-IR increased the association between FL and IFG [OR: 2.46 (95% I.C.: 1.73–3.49) to 5.42 (3.11–9.41)], whereas low adiponectin levels had a higher effect on the FL and nT2D association [OR: 4.26 (2.18–8.34) to 8.53 (2.96–24.55)].

Conclusion: Fatty liver was independently associated with IFG and nT2D. Our results indicate for the first time, that adipose tissue dysfunction increases these associations.

Keywords: Impaired fasting glucose, Type 2 diabetes mellitus, Liver fat, Insulin resistance, Visceral fat

Background

For many years adipose tissue was considered an organ of energy deposit and thermal insulation. However, this concept has changed during the last decades, and it is now clear that adipose tissue is a complex endocrine organ with high metabolic activity [1]. It has been postulated that dysfunction of adipose tissue begins when fat storage capacity of the subcutaneous compartment is diminished, which leads to fat accumulation in other organs and tissues [2]. Intrabdominal visceral adipose tissue (VAT) is one of the most important ectopic depots. Under insulin resistance conditions, VAT is a source of excessive release of free fatty acids (FFA) and inflammatory adipokines to the portal vein leading to hepatic fat accumulation, which in turn affects glucose and lipoprotein metabolism and contributes to the inflammatory process [3]. The total adipose tissue insulin resistance (Adipo-IR) may participate in this process by increasing triglycerides lipolysis [4, 5]. Dysfunction of adipose tissue is also characterized by low levels of adiponectin [6.]. In humans, adiponectin which is mainly synthesized by adipocytes, has been directly correlated with insulin sensitivity but inversely related with cardiovascular risk factors [7] and with hepatic fat content [8]. Because Adipo-IR, elevated FFA, and low adiponectin are abnormalities associated with adipose tissue excess, liver injury and related comorbidities, their presence could be considered as a marker of dysfunctional adipose tissue [4, 7].

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Impaired fasting glucose (IFG) and type 2 diabetes mellitus have been associated with high total mortality risk [9]. VAT and hepatic fat are depots commonly associated with these two conditions [3, 10, 11] and it has been previously reported that VAT and fatty liver (FL) share similar effects on lipid and glucose metabolism [3, 12, 13]. Recent studies have proposed the hypothesis that compared to VAT, FL could have a greater impact on the development of metabolic derangements [14, 15]. Kantarzis et al. found that liver fat predicted glucose tolerance categories more strongly than VAT [15]. However, it is currently unknown whether functional features of adipose tissue could have a greater impact than its quantity on the association of liver fat with the risk of pre diabetes and type 2 diabetes mellitus. Therefore, the aim of the present study was to test the hypothesis that adipose tissue dysfunction (measured through FFA, adiponectin and Adipo-IR) participates on the association of liver fat with either IFG or newly diagnosed type 2 diabetes mellitus (nT2D), independently of the amount of VAT.

Methods

Study population

The study population included participants in the Genetics of Atherosclerotic Disease (GEA) study. The GEA study was designed to examine the genomic bases of coronary heart disease (CHD), and to assess relationships between traditional and emerging risk factors with clinical and subclinical atherosclerotic vascular disease in an adult Mexican population [16]. Briefly, a convenience sample from residents in Mexico City was recruited; this sample included non randomized, consecutive volunteers to form a control group of 1500 subjects aged 35 to 70 years. Patients with established premature CHD were consecutively selected from the outpatient clinic of the National Institute of Cardiology. Control participants without family history of premature CHD and no personal history of cardiovascular disease were recruited from Blood Bank donors, and through brochures posted in social service centers. Coronary patients and control subjects with history of renal, liver, thyroid or malignant disease, as well as those on treatment with corticosteroids, were excluded. Subjects with positive serology for viral hepatitis B and C, HIV, syphilis, and Chagas disease were also excluded.

In the present study, we included 1264 participants from the original GEA control group (n = 1500). Subjects without FFA determination (n = 23) and with type 2 diabetes mellitus previously diagnosed (n = 147) were not eligible; whereas those with high plasma triglycerides (TG ≥ 6.78 mmol/l, n = 11) or low glomerular filtration rate (GFR ≤ 60 ml/min, n = 55) were excluded (Fig. 1). Participants were stratified as: (1) normoglycemic when fasting plasma glucose was <5.6 mmol/l (NG); (2) IFG when glucose levels were ≥5.6 mmol/l but <7.0 mmol/l; and (3) nT2D when glucose values were ≥70.0 mmol/l, using the cutoff points of the American Diabetes Association [17].

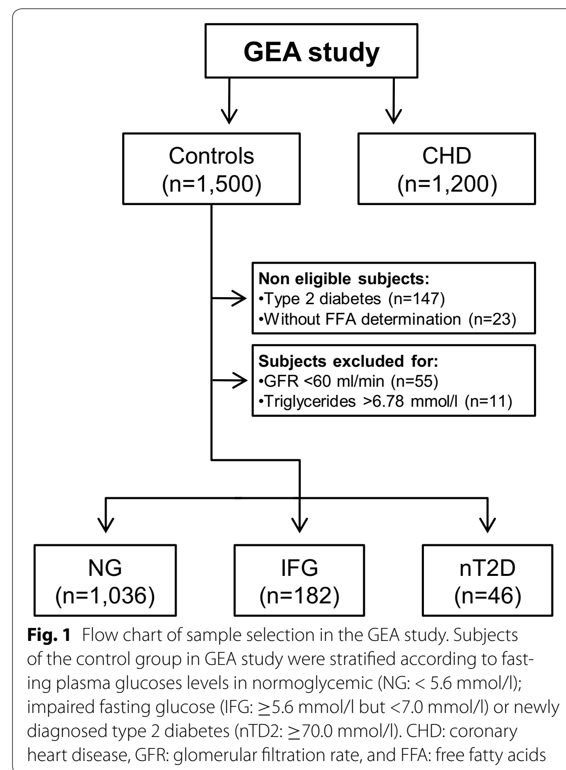

Fig. 1 Flow chart of sample selection in the GEA study. Subjects of the control group in GEA study were stratified according to fasting plasma glucoses levels in normoglycemic (NG: < 5.6 mmol/l); impaired fasting glucose (IFG: ≥5.6 mmol/l but <7.0 mmol/l) or newly diagnosed type 2 diabetes (nTD2: ≥70.0 mmol/l). CHD: coronary heart disease, GFR: glomerular filtration rate, and FFA: free fatty acids

Clinical assessment

All subjects were interviewed by a trained research staff and completed questionnaires to collect information pertaining to demographic characteristics, CHD history, medication, alcohol and tobacco use. All participants had a complete clinical examination. Height was measured to the nearest 0.1 cm using a rigid stadiometer, and weight was measured to the nearest 0.1 kg with the use of a balance scale. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. After a 10-min rest, blood pressure was measured 3 times; the average of the second and third blood pressure measurements was used for the analysis. Hypertension was defined as self-reported treatment with antihypertensive medications or a systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg. Low adiponectin levels was defined as adiponectin values below 4 μg/ml [6]. Elevated VAT, elevated FFA

and the presence of Adipo-IR were considered when their values were ≥75th percentile (VAT: 121 cm^2 for women; 153 cm^2 for men; FFA: 0.75 mmol/l for women, 0.61 mmol/l for men; Adipo-IR: 11.09 mmol/l·μU/l for women, 8.24 mmol/l μU/l for men). These cutoff points were obtained from a GEA study subsample of 101 men and 180 women without obesity, history of CHD and normal values of blood pressure, fasting glucose and lipids.

Biochemical analysis

Venous blood samples were collected from subjects after a 12 h fasting and 20 min in a sitting position. Plasma glucose, TG, high density lipoprotein cholesterol (HDL-C) and FFA were measured using standardized enzymatic procedures (Roche Diagnostics GmbH, Mannheim, Germany). Accuracy and precision of lipid measurements in our laboratory are under periodic surveillance by the Center for Disease Control and Prevention service (Atlanta, GA, USA). Inter assay coefficients of variation were less than 6% for all of these assays. Low-density lipoprotein cholesterol (LDL-C) was estimated by using the De Long et al. method [18]; and GFR was estimated using the Cockroft-Gault formula [19]. High-sensitivity C-reactive protein (hsCRP) was determined by immunonephelometry on a BN Pro Spec nephelometer (Dade Behring, Marburg, Hesse, Germany), according to the manufacturer method. Plasma insulin concentrations were determined by a radioimmunometric assay (Millipore, St. Charles, Missouri, USA) and human total adiponectin was determined with a Quantikine ELISA kit (R&D Systems, Boston, Massachusetts, USA). Insulin resistance (IR) was estimated with the use of the homeostasis model assessment (HOMA-IR) [20] and the Adipo-IR was computed with a validated adipose tissue insulin resistance index (Adipo-IR = FFA [mmol/l] X insulin concentration [μU/l]) [4].

Computed tomography study

CT is a validated method for measuring VAT [21] and FL [22]. In the present study, these measurements were obtained using a 64-slice scanner (Somatom Cardiac Sensation 64, Forcheim, Bavaria, Germany). To determine the liver and spleen attenuation, a single slice CT scan was obtained at the level of T11–T12 or T12–L1. Fatty liver was defined as a liver/spleen attenuation ratio lower than 1.0 [22]. To calculate the amount of total abdominal tissue (TAT) and VAT, a single slice scan was done at the level of L4–L5 and the area was expressed in cm^2. Subcutaneous abdominal tissue (SAT) was calculated by subtracting the VAT from the TAT area.

Statistical analysis

All variables were analyzed for normal distribution, using skewness and kurtosis. Data are expressed as mean ± standard deviation for variables with normal distribution, median (interquartile range) for skewed variables, and number of subjects (%) for categorical variables. Comparisons of means, medians and frequencies were made with ANOVA, Kruskal–Wallis and Chi squared tests, respectively. Bonferroni post hoc test was used for multiple pairwise comparisons. The relative contribution of fat depots or markers of adipose tissue function to IFG and nT2D was analyzed with the use of multinomial logistic regression analyses. To evaluate the role of adipose tissue function markers on the association of FL or elevated VAT, with IFG or nT2D, subjects with NG, IFG or nT2D were stratified by the presence of FL or elevated VAT alone, or its combination with each of the abnormal markers in a full adjusted multivariate model. All analyses were carried out with the software program STATA 12 (STATA CORP Texas, USA.). All p values <0.05 or confidence intervals 95% that excluded the unity, were considered statistically significant.

Results

Metabolic characteristics of the studied groups are summarized in Table 1. Compared with the NG group, IFG and nT2D groups were older (51.7 ± 9, 54.2 ± 8 and 54.9 ± 9 years; respectively) and had a higer proportion of male subjects (51.8, 56 and 63%; respectively), as well as higher values of BMI, TG, fasting glucose, HOMA-IR, Adipo-IR, hsCRP, SAT, TAT and VAT, and lower values of HDL-C, adiponectin and liver/spleen attenuation ratio. Compared with IFG, nT2D group was higher in fasting plasma glucose and HOMA-IR, but lower in SAT and Adipo-IR. No differences were observed in physical activity, current smoking, and statin use among different groups (data not shown). Compared with NG, subjects with IFG and nT2D had higher prevalences of FL, elevated VAT, Adipo-IR, elevated FFA and low adiponectin. No differences were found between subjects with glucose abnormalities (IFG and nT2D) (Fig. 2).

Logistic regression analysis was used to evaluate the individual associations of fat depots and markers of adipose tissue function with IFG and nT2D (Table 2). Results showed that independent of age and gender, IFG and nT2D subjects were more likely to have FL, elevated VAT, Adipo-IR, and low adiponectin levels (model 1). In a fully adjusted model (model 2), FL but not VAT, remained associated with IFG and with nT2D. Among the adipose tissue function markers, Adipo-IR was associated with both glucose abnormalities, whereas low adiponectin levels were associated only with nT2D. To confirm the results, subjects from IFG or nT2D groups were matched by age and gender with control subjects that did not have obesity and

Table 1 Metabolic characteristics of the studied groups

	Normoglycemic n = 1036	Impaired fasting glucose n = 182	Newly diagnosed type 2 diabetes mellitus n = 46	p trend
Age (years)	51.7 ± 9	54.2 ± 8*	54.9 ± 9	<0.001
Gender (male)	499 (51.8%)	102 (56%)	29 (63%)*	0.017
BMI (kg/m^2)	28.2 ± 4	30.2 ± 4*	30.4 ± 5*	<0.001
Alcohol consumption (>30 gr/day)	18 (1.7%)	3 (1.7%)	2 (4.3%)	0.610
Hypertension (%)	209 (20)	55 (30)*	12 (26)	<0.001
LDL-C (mmol/l)	3.08 ± 0.8	3.21 ± 0.9	3.10 ± 0.8	0.195
HDL-C (mmol/l)	1.16 (0.96–1.40)	1.05 (0.88–1.24)*	1.04 (0.85–1.16)*	<0.001
Triglycerides (mmol/l)	1.60 (1.21–2.18)	2.01 (1.45–2.73)*	2.29 (1.67–3.13)*	<0.001
Fasting glucose (mmol/l)	4.89 (4.6–5.2)	5.77 (5.7–6.1)*	8.49 (7.7–12.2)*†	<0.001
HOMA-IR	3.4 (2.49–4.89)	6.16 (4.6–8.1)*	8.98 (6.4–12)*†	<0.001
hsCRP (nmol/l)	14.0 (7.52–28.6)	21.8 (9.5–38.0)*	21.8 (10.5–36.2)*	<0.001
Adipo-IR (mmol/l·μU/l)	8.8 (5.7–13.1)	13.3 (9.2–20)*	12.3 (8.9–17.9)*†	<0.001
Free fatty acids (mmol/l)	0.55 (0.43–0.69)	0.57 (0.43–0.69)	0.59 (0.52–0.76)*	0.0312
Adiponectin (μg/ml)[a]	8.3 (5.1–13)	6.7 (3.9-10–2)*	6.4 (2.8–9.2)*	<0.001
Subcutaneous adipose tissue (cm^2)	282 (212–360)	307 (250–384)*	297 (2–405)†	0.005
Total adipose tissue (cm^2)	428 (3–531)	491 (424–587)*	502 (403–607)*	<0.001
Visceral adipose tissue (cm^2)	143 (105–184)	176 (139–229)*	177 (139–229)*	<0.001
Liver to spleen attenuation ratio	1.12 (0.96–1.22)	0.96 (0.75–1.11)*	0.89 (0.68–1.01)*	<0.001

Values are expressed as mean ± standard deviation, median (interquartile range) or number of subjects (%)

HDL-C high density lipoprotein cholesterol, *LDL-C* low density lipoprotein cholesterol, *HOMA-IR* homeostasis model assessment of insulin resistance

* p < 0.05 vs normoglycemic

† p < 0.05 vs impaired fasting glucose

[a] n = 985 for normoglycemic group, n = 167 for impaired fasting glucose group and n = 41 for newly diagnosed type 2 diabetes mellitus group

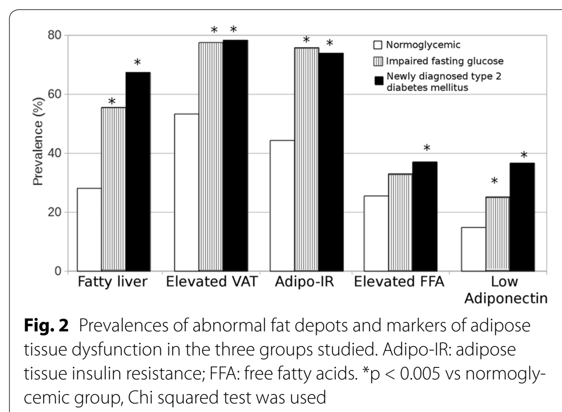

Fig. 2 Prevalences of abnormal fat depots and markers of adipose tissue dysfunction in the three groups studied. Adipo-IR: adipose tissue insulin resistance; FFA: free fatty acids. *p < 0.005 vs normoglycemic group, Chi squared test was used

metabolic syndrome. The fully adjusted model showed that compared with control subjects without obesity and metabolic syndrome, those with IFG or nT2D had higher risk of fatty liver disease [OR: 2.35 (C.I. 95%: 1.32–4.16) and 3.78 (1.67–8.53); respectively].

To evaluate the potential effect of adipose tissue dysfunction on the association of FL with IFG or nT2D, we assessed the effect of FL alone and also the effect of FL plus each one of the adipose tissue function markers (Table 3). These analyses showed that the presence of Adipo-IR had the strongest effect on the association between FL and IFG, followed by low adiponectin levels and elevated FFA. Conversely, the presence of low adiponectin had a significantly higher effect on the FL and nT2D association, followed by Adipo-IR and elevated FFA. In the paired-matched sub-analysis, the effect of low adiponectin on the association of FL with glucose categories was attenuated, and the effect of the other adipose tissue markers on the association of FL with glucoses abnormalities did not change (Additional file 1: Table S1). Although elevated VAT was not independently associated with IFG or nT2D, a similar analysis was performed to evaluate the effect of adipose tissue dysfunction on the association between elevated VAT and glucose abnormalities. The results showed that Adipo-IR was the only marker that increased the association of elevated VAT with IFG [2.99 (1.62–5.55), p < 0.05].

Discussion

Previous studies have shown that both, visceral and hepatic fat depots are associated with increased risk of IFG and type 2 diabetes mellitus [11, 15, 23]. Recently, it has been

Table 2 Association of fat depots and markers of adipose tissue dysfunction with impaired fasting glucose and newly diagnosed type 2 diabetes mellitus

Abnormalities	Normoglycemic	Impaired fasting glucose	Newly diagnosed type 2 diabetes mellitus
	Odd ratio (95% I.C)	Odd ratio (95% I.C)	Odd ratio (95% I.C)
Model 1	Reference values		
Fatty liver	1	3.36 (2.43–4.65)	5.68 (3.01–10.69)
Elevated VAT	1	2.79 (1.91–4.05)	2.84 (1.38–5.82)
Adipo-IR	1	3.80 (2.65–5.47)	2.15 (1.54–3.02)
Low adiponectin	1	1.84 (1.22–2.77)	3.29 (1.64–6.58)
Elevated FFA	1	1.37 (0.97–1.94)	1.62 (0.87–3.01)
Model 2			
Fatty liver[a]	1	2.46 (1.73–3.49)	4.26 (2.18–8.25)
Elevated VAT[b]	1	1.37 (0.89–2.11)	1.09 (0.49–2.44)
Adipo-IR	1	2.69 (1.82–3.96)	2.15 (1.06–4.37)
Low adiponectin	1	1.46 (0.96-2.22)	2.54 (1.26–5.12)
Elevated FFA	1	1.29 (0.91-1.85)	1.51 (0.80–2.85)

Model 1 adjusted for age and gender

Model 2 adjusted for age, gender, body mass index, high density lipoprotein cholesterol and triglycerides

VAT visceral abdominal tissue, *Adipo-IR* adipose tissue insulin resistance, *FFA* free fatty acids

[a] Additional adjustment for elevated VAT

[b] Additional adjustment for fatty liver

Table 3 Combined association of fatty liver and markers of adipose tissue dysfunction with impaired fasting glucose and newly diagnosed type 2 diabetes mellitus

Abnormalities	Normoglycemic	Impaired fasting glucose	Newly diagnosed type 2 diabetes mellitus
	Reference values	Odds ratio (95% I.C)	Odds ratio (95% I.C)
Fatty liver	1	2.46 (1.73–3.49)	4.26 (2.18–8.34)
Fatty liver + Adipo-IR	1	5.42 (3.11–9.41)	6.81 (2.29–20.23)
Fatty liver + low adiponectin	1	3.89 (2.11–7.17)	8.53 (2.96–24-55)
Fatty liver + elevated FFA	1	2.66 (1.56–4.57)	4.99 (2.04–12.19)

Model adjusted for age, gender, body mass index, high density lipoprotein cholesterol, triglycerides and elevated VAT

Adipo-IR adipose tissue insulin resistance, *VAT* visceral adipose tissue, *FFA* free fatty acids

proposed that compared to VAT, FL could have a greater impact on the development of metabolic derangements. Moreover, hepatic fat accumulation has been associated to dysfunctional adipose tissue, which is characterized by Adipo-IR, elevated FFA, and low adiponectin plasma levels [4, 24]. The results of the present study confirm these associations and show that Adipo-IR and low adiponectin could have an important role in the association of FL with IFG or nT2D. Our data also show that the combined effect of FL plus dysfunctional adipose tissue on IFG and nT2D is independent of VAT. These findings extend the knowledge about adipose tissue influence on the association of fat depots and glucose metabolic abnormalities.

Pre diabetes is a condition where early abnormalities in glucose metabolism are present but elevation in blood glucose is below cutoff point for establishing the diagnosis of type 2 diabetes mellitus [9]. IFG is a state of pre diabetes, closely associated with type 2 diabetes mellitus and is originated by multiple risk factors. The present and previous reports [15, 25, 26] have shown that NAFLD is independently associated with pre diabetes. However, in a very recent study, Ming et al. found no association between fatty liver and pre diabetes [27]. The contrasting results could be explained by differences in study design, sample size, ethnicity, studied population and pre diabetes definition. It is important to consider that the correlation between visceral and liver fat makes it difficult to discern the relative contribution of each fat depot on the risk of glucose abnormalities. Recent data has shown an independent association of VAT with the presence of

type 2 diabetes mellitus [10, 11], but there are also previous reports indicating that liver fat content was associated with type 2 diabetes mellitus independently of VAT [28, 29]. Fabbrini et al. compared subjects with different VAT volume paired by liver fat content and found no differences regarding metabolic abnormalities of insulin resistance. On the other hand, when comparing subjects with different liver fat content but similar VAT, they found that metabolic alterations and insulin resistance were explained by intra hepatic fat content [14]. Consistent with these findings, the results of the present study showed that FL is associated with a higher probability of having nT2D, independent of traditional risk factors and elevated VAT (Table 2). Together these results are in line with the proposed hypothesis that in some cases, the reported association between VAT and derangements in glucose metabolism may be explained through the close relationship between VAT and liver fat content [14]. However, there are also data suggesting that obesity and FL may act through different mechanisms to increase the risk of type 2 diabetes mellitus [30].

Several studies have postulated that dysfunctional adipose tissue, favors the release of FFA to the portal circulation, and then to the liver where they accumulate and induce hepatic steatosis, inflammation, insulin resistance and 2 diabetes mellitus [3, 4, 31]. Dysfunctional adipocytes also show an abnormal anti-inflammatory response, characterized by lower synthesis and secretion of adiponectin. Low levels of adiponectin have been associated with insulin resistance, type 2 diabetes mellitus and FL [2, 6, 7]. Lomonaco et al. showed that a modest increase in Adipo-IR is associated with low adiponectin plasma levels, dyslipidemia, hepatic and muscle insulin resistance and hepatic steatosis. Similarly, the results of the present study showed that subjects with IFG and nT2D have higher visceral and hepatic fat content, as well as lower levels of adiponectin and higher levels of Adipo-IR. Moreover, these results indicate that in subjects with FL, both Adipo-IR and low adiponectin, respectively increase 110 and 50%, the probability of having IFG. The risk of having nT2D was higher in subjects with FL plus low adiponectin (84%) or FL plus Adipo-IR (48%). Adipo-IR was the only variable that significantly increased the association of elevated VAT with IFG (117%). The finding that Adipo-IR increased the risk of IFG in subjects with FL or elevated VAT, suggests that lipolysis induced by insulin resistance may be a key mediator in the early stages of metabolic derangements in subjects with ectopic fat excess. Our findings are further supported by the recent findings showing that liver fat accumulation is associated with decreased branched-chain amino acids catabolism, which suggest that adipose tissue dysfunction may play a key role in the systemic nature of NAFLD pathogenesis (32). On the other hand, the association of low adiponectin with nT2D found in the present study could reflect more advanced stages of metabolic alterations where inflammation plays a more definitive role [33, 34]. Furthermore, it has been previously reported that adiponectin expression is decreased by 20–40% in the presence of NAFLD, and plasma adiponectin concentrations are inversely related to hepatic fat content in patients with type 2 diabetes mellitus [8]. All these data suggest that adiponectin may also play an important pathophysiological role in the metabolic abnormalities associated with liver injury. Although the cause of total adipose tissue dysfunction, is not fully understood, hypoxia [35], PPAR gamma activation [2], defects in fatty acids oxidation [36], down-regulation of branched-chain amino acids catabolism [32], and genetic predisposition [37] could be involved.

The present study has some limitations. First, causality cannot be determined due to the cross-sectional nature of the analyses. Second, the presence of subjects with glucose intolerance could not be ruled out in the population studied, however, similar to our observations, previous studies have found that IFG is mainly associated with derangements in hepatic insulin sensitivity [38]. Third, the diagnosis of FL was not confirmed with hepatic biopsy specimens; however, significant correlations have been reported between imaging attenuation and the histology grade of steatosis [39]. Although subjects with viral hepatitis B and C, human immunodeficiency virus, syphilis and Chagas disease were excluded from the analyses, other causes of fatty liver such as viral hepatitis A, D, E and G, autoimmune hepatitis, metabolic liver disease or genetic factors were not excluded. We only analyzed the impact of PNPLA3 genotypes, and found no association between PNPLA3 and glucose metabolism abnormalities (data not shown). However, other fatty liver associated genotypes such as TM6SF2 variants were not explored. Therefore, their influence on the results cannot be ruled out. Fourth, our study included a Mexican-mestizo population; therefore, our findings may not be generalized to other ethnic groups. Finally, due to the small number of subjects with nT2D, these findings should be interpreted with caution and considered as hypotheses generating. These results should be confirmed by studies with a larger number of subjects.

Conclusion

Our results show that FL is independently associated with IFG and nT2D. Furthermore, this study suggests that Adipo-IR and low levels of adiponectin may increase the association of FL with IFG and nT2D. Even though the volume of VAT was not independently associated with higher type 2 diabetes mellitus risk in this population, the presence of Adipo-IR significantly increased the risk of IFG, in subjects with elevated VAT.

Additional file

Additional file 1: Table S1. Combined association of fatty liver and markers of adipose tissue dysfunction with the risk of IFG and nT2D in the paired-matched subpopulation.

Abbreviations

FL: fatty liver; IFG: impaired fasting glucose; nT2D: newly diagnosed type 2 diabetes mellitus; VAT: visceral adipose tissue; Adipo-IR: adipose tissue insulin resistance; FFA: free fatty acids.

Authors' contributions

EJG, JGJR and AMU participated in conception, design, analysis, interpretation of data, and final approval of the manuscript submitted. RPS, CPR, GCS, GVA, NCP, CGS, and MTT contributed in data collection, drafting of the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The GEA study was approved by the Institutional Review Board of the National Institute of Cardiology and conducted according to the Declaration of Helsinki. Written informed consent was obtained from participants.

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PLA2G2A polymorphisms are associated with metabolic syndrome and type 2 diabetes mellitus. Results from the genetics of atherosclerotic disease Mexican study

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ABSTRACT

The secretory phospholipase A_2 II A ($sPLA_2$-IIA) encoded by *PLA2G2A* gene hydrolyzes phospholipids liberating free fatty acids (FFAs) and lysophospholipids. If lipolysis exceeds lipogenesis, the free fatty acids undergo a continuous release into circulation. A sustained excessive increase in this release contributes to metabolic disease. The aim of the present study was to evaluate the role of *PLA2G2A* gene polymorphisms as susceptibility markers for metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM) in Mexican population. Three *PLA2G2A* gene polymorphisms (rs876018, rs3753827 and rs11573156) were genotyped by 5′ exonuclease TaqMan assays in a group of 338 patients with T2DM, 460 individuals with MetS and 366 healthy controls. Under codominant 1 (codom1), dominant (dom) and additive (add) models adjusted by age, gender, body mass index (BMI), smoking habit, and hypertension, the rs876018 *T* allele was associated with increased risk of MetS [Odds Ratio (OR) = 1.66, $P_{codom1} = 0.005$; OR = 1.67, $P_{dom} = 0.003$; OR = 1.49, $P_{add} = 0.005$] as compared to controls. On the other hand, under several models adjusted by the same variables, the rs3753827 *A* (OR = 1.52, $P_{codom1} = 0.039$ and OR = 1.49, $P_{dom} = 0.039$) and rs11573156*C* alleles (OR = 6.46, $P_{codom1} = 0.013$; OR = 6.70, $P_{codom2} = 0.009$; OR = 6.65, $P_{dom} = 0.009$) were associated with increased risk of T2DM when compared with controls. In addition, the rs876018 *T* allele was associated with hypercholesterolemia ($P_{dom} = 0.017$, $P_{add} = 0.009$) and risk of subclinical atherosclerosis (SA) ($P_{dom} = 0.041$) in MetS when compared with controls. Also, this allele was associated with SA in T2DM patients ($P_{dom} = 0.007$). The *TAG* haplotype was significantly associated with increased risk of MetS (OR = 1.54, P = 0.006). Results suggest that *PLA2G2A* polymorphisms are involved in the risk of developing MetS and T2D and are associated with SA in this group of patients.

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1. Introduction

The prevalence of metabolic syndrome (MetS) is increasing in epidemic proportions worldwide, not only in the urbanized world, but also in developing countries (Cornier et al., 2008; Cruz et al., 2010). Although the etiology of MetS is not fully understood and it remains unclear whether there is a unifying pathophysiological mechanism explaining this syndrome, it is evident that central obesity and insulin resistance leading to alterations in the lipid profile and glucose metabolism are involved in developing of MetS (Cruz et al., 2010). The risk of developing type 2 diabetes mellitus (T2DM) is 5-fold greater in people with MetS (Alberti et al., 2005), and MetS prevalence ranges from 13-30% and 70-80% among Caucasian non-diabetic (Reaven, 1988; Kaplan, 1989) and diabetic (The Metabolic Syndrome, 2009; Zimmet et al., 2005) populations, respectively,

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also, it is estimated the risk is near 70% among African-Americans (Alberti et al., 2006).

Adipose tissue plays a crucial role in MetS. Approximately 80% of adipose tissue weight is lipid, and over 90% of lipid is stored as triglycerides. Adipose tissue is capable of secreting free fatty acids derived from lipolysis of stored triglycerides, a process regulated by hormones and enzymes (Iyer et al., 2011; Varastehpour et al., 2006). The secretory phospholipase A_2 ($sPLA_2$) family of enzymes hydrolyzes the sn-2 ester bond of phospholipids and cell membranes, generating non-esterified free fatty acids and lyso-phospholipids, which may promote diverse pro-inflammatory processes (Six and Dennis, 2000). Several lines of evidence suggest that $sPLA_2$ has a role in MetS, atherosclerosis and coronary artery disease (CAD). $sPLA_2$-IIA and $sPLA_2$-V expression was found to be higher in placenta of obese neonates as compared to control ones, and was also associated with increased levels of omega-3 polyunsaturated fatty acids (Birts et al., 2009). On the other hand, Iyer *et. al.*, demonstrated that the inhibition of $sPLA_2$-IIA might reverse and protect against diet-induced adiposity and metabolic dysfunction in rats (Iyer et al., 2011). Moreover, increased $sPLA_2$ enzyme activity has been considered atherogenic (Mallat et al., 2007); increased levels of plasma $sPLA_2$-IIA were found to be a significant predictor of coronary events in patients with CAD (Kugiyama et al., 1999); increased $sPLA_2$-IIA expression levels were found in epicardial adipose tissue of patients with CAD (Dutour et al., 2010), and $sPLA_2$ inhibitors have been considered as potential therapeutic for treatment of metabolic disorder (Magrioti and Kokotos, 2010).

$sPLA_2$-IIA belongs to the subfamily of group II $sPLA_2$s, comprising PLA_2 group IIA, IIA-IIF, and V. All these enzymes are encoded by a cluster of highly homologous genes located within a ~250 kb genomic segment on human chromosome 1p35 (Exeter et al., 2012). Single nucleotide polymorphisms (SNPs) located in the coding region of *PLA2G2A* (gene that encode $sPLA_2$-IIA) have been previously associated with CAD and T2DM (Exeter et al., 2012; Wootton et al., 2006). Moreover, *PLA2G2A* SNPs rs11573156 (in the 5′UTR region) and rs876018 (nearby the SNP 3′UTR) were found to be independently associated with $sPLA_2$-IIA plasma levels (Exeter et al., 2012). In addition, the *PLA2G2A* rs876018 and rs3753827 polymorphisms have been previously associated with T2DM (Wootton et al., 2006). Because young adults with MetS present increased sPLA2 serum levels (Mattsson et al., 2010), and because the role of PLA2G2A gene variation in MetS and T2D remains inconclusive, the aim of the present study was to determine the effect of three PLA2G2A polymorphisms in the risk of develop MetS and T2DM.

2. Materials and methods

All participants provided written informed consent, and the study complies with the Declaration of Helsinki. It was approved by the Ethics Committee of the Instituto Nacional de Cardiología Ignacio Chávez.

2.1. Subjects

The study included 343 patients with T2DM, 454 individuals with MetS and 379 healthy controls. The controls were apparently healthy, asymptomatic individuals, without family history of premature CAD, voluntarily recruited from blood donors. All Genetics of Atherosclerotic Disease Study participants are unrelated and of self-reported Mexican-mestizo ancestry (3 generations) (Villarreal-Molina et al., 2012). Participants with MetS were selected according to NCEP – ATP III criteria that included 3 or more of the following traits: waist circumference ≥ 90 cm for men, ≥ 80 cm for women; fasting glucose ≥ 100 mg/dl; triglycerides ≥ 150 mg/dl; high density lipoprotein (HDL) cholesterol < 40 g/dl for men, <50 mg/dl for women; systolic blood pressure ≥ 130 mm Hg or diastolic ≥ 85 mmHg (Ma et al., 2009). T2DM was defined by the American Diabetes Association criteria (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2000) considering fasting blood glucose levels ≥ 126 mg/dl and was also considered when participants reported glucose-lowering treatment or a physician diagnosis of diabetes. Demographic, clinical, anthropometric and biochemical parameters and cardiovascular risk factors were evaluated in all participants.

2.2. Computed tomography of the chest and abdomen

Total subcutaneous and visceral abdominal fat and coronary artery calcification (CAC) were quantified by computed tomography in each participant. Computed tomography (CT) of the chest and abdomen was performed using a 64-channel multidetector helical computed tomography system (Somatom Cardiac Sensation, 64, Forcheim, Germany) and interpreted by experienced radiologists. Scans were read to assess and quantify the following: a) total abdominal, subcutaneous, and visceral abdominal fat areas as described by (Kvist et al., 1988); b) CAC score using the Agatston method (Mautner et al., 1994). Subclinical atherosclerosis (SA) was defined as the presence of a CAC score ≥ 1.0 (McKimmie et al., 2008).

2.3. Genetic analysis

Genomic DNA from whole blood containing EDTA was isolated by standard techniques. The rs876018, rs3753827 and rs11573156 SNPs were genotyped using 5′ exonuclease TaqMan genotyping assays in ABI Prism 7900HT Fast Real-Time PCR system, according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Samples previously sequenced of the different genotypes of the polymorphisms studied were included as positive controls.

2.4. Statistical analysis

All calculations were performed using SPSS version 18.0 (SPSS, Chicago, Il) statistical package. The data are shown as means ± S.D., median (interquartile range) or as frequencies for categorical variables. Comparisons were made using Mann-Whitney *U* test or *t*-Student, as appropriate, for continuous variables, and by Chi-square tests for categorical variables. Logistic regression analysis was used to test for associations of polymorphisms with MetS and T2DM under different inheritance models. Inheritance patterns were defined as follows: co-dominant (major allele homozygotes vs. heterozygotes and major allele homozygotes vs. minor allele homozygotes), dominant (major allele homozygotes vs. heterozygotes + minor allele homozygotes), recessive (major allele homozygotes + heterozygotes vs. minor allele homozygotes) and additive (major allele homozygotes vs. heterozygotes vs. minor allele homozygotes). The models were adjusted by age, gender, BMI, smoking habit and hypertension. Statistical power to detect associations of polymorphisms with MetS exceeded 0.80 as estimated with QUANTO software (http://hydra.usc.edu/GxE/). Genotype frequencies did not show deviation from Hardy-Weinberg equilibrium ($P > 0.05$). Pairwise linkage disequilibrium (LD, D′) estimations between polymorphisms and haplotype reconstruction were performed with Haploview version 4.1 (Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA).

Table 1
Comparison of anthropometric parameters in individuals with metabolic syndrome, type 2 diabetes mellitus and controls.

	Controls (n = 366)	MetS (n = 460)	p	T2DM (n = 338)	p	p*
Age (years)	53 [48–60]	53.5 [49–59]	0.883	58 [52–62]	<0.0001	<0.0001
Gender n (% Male)	181 (49.5)	312 (67.8)	<0.0001	226 (66.9)	<0.0001	0.416
Body Mass Index (kg/m2̂)	26.73 [24.28–29.43]	30.17 [28.06–32.74]	<0.0001	29.23 [26.18–32.14]	<0.0001	<0.0001
Obesity n (%)	80 (21.9)	240 (52.2)	<0.0001	144 (42.6)	<0.0001	0.005
Waist circumference (cm)	90.56 ± 10.92	102 ± 10.24	<0.0001	99.58 ± 11.73	<0.0001	0.007
Central obesity n (%)	146 (39.9)	395 (85.9)	<0.0001	227 (67.2)	<0.0001	<0.0001
Total Abdominal Fat (cm^2)	402.85 ± 143.84	513.52 ± 132.77	<0.0001	476.79 ± 160.58	<0.0001	0.002
Subcutaneous Abdominal Fat (cm^2)	252 [183.50-325.25]	299 [244–370]	<0.0001	254 [202.50–352.75]	0.073	<0.0001
Visceral Abdominal Fat (cm^2)	132 [98–172]	191 [153–239]	<0.0001	188 [145.50–238.25]	<0.0001	0.265
Visceral/Subcutaneous adipose tissue ratio	0.528 [0.367–0.763]	0.644 [0.471–0.871]	<0.0001	0.724 [0.502–0.980]	<0.0001	0.002
Current smokers n (%)	67 (18.3)	92 (20.0)	0.301	43 (12.7)	0.026	0.004
Former smokers n (%)	134 (45.0)	230 (62.3)	<0.0001	186 (63.1)	<0.0001	0.457
Hypertension n (%)	48 (13.1)	256 (55.7)	<0.0001	228 (67.5)	<0.0001	<0.0001
Diastolic Blood Pressure (mmHg)	72.05 + 9.36	77.80 + 10.62	<0.0001	75.50 + 10.52	<0.0001	0.006
Systolic Blood Pressure (mmHg)	115.50 [106.33–142]	123.66 [111.75–136.66]	<0.0001	123.66 [113.16–139.33]	<0.0001	0.464
Heart rate (bpm)	64.06 ± 8.77	65.16 ± 9.81	0.384	69 ± 10.77	<0.0001	<0.0001
SA n (%)	101 (27.6)	82 (17.8)	<0.0001	47 (13.9)	<0.0001	<0.0001
CAD n (%)	0	236 (51.3)	<0.0001	245 (72.5)	<0.0001	<0.0001

Data are expressed as means ± SD, median (interquartile range) or percentage. log-transformed values were used for statistical analysis. P values were estimated using Mann-Whitney *U* test or *t*-Student for continuous variables and Pearsonís Chi-square test for categorical values. MetS: Metabolic Syndrome; T2DM: Type 2 Diabetes Mellitus; SA: Subclinical Atherosclerosis; CAD: Coronary Artery Disease. p*: T2DM vs MetS.

Table 2
Comparison of biochemical parameters in individuals with metabolic syndrome, type 2 diabetes mellitus and controls.

	Controls (n = 366)	MetS (n = 460)	P	T2DM (n = 338)	p	p*
Total Cholesterol (mg/dL)	192.67 ± 37.08	192.62 ± 46.47	1.000	176.65 ± 51.18	<0.0001	<0.0001
Hypercholesterolemia > 200 mg/dL n (%)	154 (42.1)	345 (75)	<0.0001	268 (79.3)	<0.0001	0.091
HDL-C (mg/dl)	50.10 [42.90–60.00]	37 [32.00–44.38]	<0.0001	40 [34–47.10]	<0.0001	<0.0001
Hipo-α-lipoproteinemia n (%)	113 (30.9)	417 (90.7)	<0.0001	291 (86.4)	<0.0001	0.037
LDL-C (mg/dL)	117.46 [99.25–136.22]	112.42 [91.12–138.38]	0.189	97.60 [71.60–125.32]	<0.0001	<0.0001
Triglycerides (mg/dL)	121.75 [93–148.88]	197.60 [162.70–255.75]	<0.0001	170.40 [126.45–236.75]	<0.0001	<0.0001
Hypertriglyceridemia n (%)	89 (24.5)	404 (87.8)	<0.0001	221 (65.6)	<0.0001	<0.0001
Statin and/or Fibrate treatment n (%)	13 (3.6)	228 (49.6)	<0.0001	123 (68.0)	<0.0001	<0.0001
apoA (mg/dL)	139.15 [121.80–168.40]	119.60 [103.40–138.90]	<0.0001	124 [103.60–144.85]	<0.0001	0.087
apoB (mg/dL)	85 [68–106]	94 [73–115]	<0.0001	79 [61.50–103.50]	0.030	<0.0001
apoB/apoA	0.587 [0.457–0.781]	0.768 [0.60–0.97]	<0.0001	0.646 [0.491–0.850]	0.012	<0.0001
Glucose (mg/dL)	89 [83–94]	94 [87–103]	<0.0001	141 [113–189]	<0.0001	<0.0001
Insulin (μU/mL)	15.40 [11.21–20.65]	23.16 [17.2–31.21]	<0.0001	20.63 [15.32–29.59]	<0.0001	0.011
HOMA-IR	3.30 [2.37–4.60]	5.38 [3.91–7.46]	<0.0001	7.54 [5.10–11.10]	<0.0001	<0.0001
HOMA- β	222.59 [168.87–302.74]	277.91 [207.22–377.94]	<0.0001	104.87 [57.97–202.84]	<0.0001	<0.0001
Uric Acid (mg/dL)	5.45 ± 1.33	6.52 ± 1.54	<0.0001	5.91 ± 1.60	<0.0001	<0.0001
Creatinine (mg/dL)	0.87 [0.73–1.00]	0.93 [0.80–1.08]	<0.0001	0.91 [0.78–1.08]	0.002	0.564
Alanine transaminase (IU/L)	25 [20–30]	27 [23–34]	<0.0001	25 [20–32]	0.008	<0.0001
Aspartate transaminase (IU/L)	21 [15–29]	26 [19–38]	<0.0001	23 [17–32]	0.477	<0.0001
Alkaline phosphatase (IU/L)	79 [66–93]	77 [65–96]	0.973	80 [65–100]	0.251	0.256
Gamma-glutamyl transpeptidase (IU/L)	23 [16–37]	33 [22.49]	<0.0001	32 [22–50]	<0.0001	0.804
Adiponectin (μg/mL)	8.50 [5.02–13.67]	6.20 [3.60–9.60]	<0.0001	6.00 [3.40–9.10]	<0.0001	0.388
Free fatty acids (mq/L)	0.52 [0.40–0.67]	0.545 [0.43–0.69]	0.101	0.590 [0.46–0.71]	0.005	0.103

Data are expressed as means ± SD, median (interquartile range) or percentage; log-transformed values were used for statistical analysis. P values were estimated using Mann-Whitney U-test or t-Student for continuous variables and Pearsonís Chi-square test for categorical values. HDL: High density lipoprotein; LDL: Long density lipoprotein; HOMA: Homeostasis model assessment. p*: T2DM vs MetS.

3. Results

General characteristics of the population are shown in Tables 1 and 2. Three independent groups were considered for the analysis: controls, MetS (diagnosed according to NECP—ATP III), and T2DM (defined according to the ADA criteria). Some differences among the groups were observed. Compared with the healthy group, individuals with MetS and T2DM had higher levels of total abdominal fat, visceral abdominal fat, visceral/subcutaneous adipose tissue ratio, triglycerides, glucose, insulin, HOMA-IR, uric acid, creatinine, alanin trasaminase, gamma-glutamyl transpeptidase and free fatty acids as well as high prevalence of hypertension, obesity, central obesity, hypercholesterolemia, hipo-α-lipoproteinemia, and hipertriglyceridemia. However, levels of HDL-C, ApoA, and adiponectin were lower in the MetS and T2DM groups when compared with the control group.

3.1. Association of polymorphisms with MetS and T2D

Genotype and allele frequencies of the three polymorphisms are presented in Table 3. The rs876018 *T* allele was more frequent in the MetS group when compared with controls, and was associated with an increased risk of MetS under the co-dominant1, dominant and additive models (OR = 1.66, 95% CI: 1.16–2.37, $P_{co\text{-}dom1}$ = 0.005; OR = 1.67, 95% CI: 1.18–2.36, P_{dom} = 0.003 and OR = 1.49, 95% CI: 1.12–1.98, P_{add} = 0.005). On the other hand, under co-dominante1, dominant and additive models, the rs876018 *T* allele was associated with decreased risk of developing T2DM (OR = 0.62, 95% CI: 0.45–0.85, $P_{co\text{-}dom1}$ = 0.003; OR = 0.62, 95%

Table 3
Association of the rs876018 (*A > T*), rs3753827 (*C > A*) and rs11573156 (*G > C*) *PLA2G2A* polymorphisms with metabolic syndrome and type 2 diabetes mellitus.

	Genotype frequency (%)			MAF	MODEL	OR (95% CI)	P
rs876018	*A/A*	*A/T*	*T/T*				
Control (n = 366)	237 (0.648)	112 (0.306)	17 (0.046)	0.192			
MetS (n = 460)	254 (0.552)	175 (0.380)	31 (0.067)	0.233	Co-dominant1*	**1.66 (1.16–2.37)**	**0.005**
					Co-dominant2*	1.77 (0.84–3.73)	0.133
					Dominant*	**1.67 (1.18–2.36)**	**0.003**
					Recessive*	1.45 (0.69–3.01)	0.318
					Additive*	**1.49 (1.12–1.98)**	**0.005**
T2DM (n = 338)	219 (64.8)	102 (0.302)	17 (0.050)	0.199	Co-dominant1†	0.85 (0.56–1.28)	0.452
					Co-dominant2†	1.12 (0.47–2.66)	0.783
					Dominant†	0.88 (0.60–1.31)	0.555
					Recessive†	1.18 (0.50–2.76)	0.695
					Additive†	0.94 (0.68–1.30)	0.737
					Co-dominant1‡	**0.62 (0.45–0.85)**	**0.003**
					Co-dominant2‡	0.62 (0.33–1.21)	0.171
					Dominant‡	**0.62 (0.46–0.84)**	**0.002**
					Recessive‡	0.76 (0.40–1.43)	0.395
					Additive‡	**0.70 (0.54–0.89)**	**0.005**
	Genotype frequency (%)			MAF	MODEL	OR (95% CI)	P
rs3753827	*C/C*	*C/A*	*A/A*				
Control (n = 366)	161 (0.440)	155 (0.423)	50 (0.137)	0.356			
MetS (n = 460)	180 (0.391)	213 (0.463)	67 (0.146)	0.368	Co-dominant1*	1.11 (0.77–1.60)	0.543
					Co-dominant2*	1.10 (0.65–1.86)	0.702
					Dominant*	1.11 (0.79–1.56)	0.528
					Recessive*	1.04 (0.64–1.69)	0.862
					Additive*	1.06 (0.83–1.236)	0.591
T2DM (N = 338)	123 (0.364)	170 (0.503)	45 (0.133)	0.384	Co-dominant1†	**1.52 (1.02–2.28)**	**0.039**
					Co-dominant2†	1.38 (0.77–2.45)	0.273
					Dominant†	**1.49 (1.02–2.18)**	**0.039**
					Recessive†	1.09 (0.64–1.87)	0.727
					Additive†	1.25 (0.95–1.64)	0.099
					Co-dominant1‡	1.24 (0.91–1.71)	0.173
					Co-dominant2‡	0.97 (0.61–1.54)	0.918
					Dominant‡	1.17 (0.87–1.59)	0.283
					Recessive‡	0.86 (0.56–1.31)	0.499
					Additive‡	1.04 (0.84–1.29)	0.674
	Genotype frequency (%)			MAF	MODEL	OR (95% CI)	P
rs11573156	*C/C*	*C/G*	*G/G*				
Control (n = 366)	9 (0.025)	68 (0.186)	289 (0.790)	0.116			
MetS (n = 460)	6 (0.013)	71 (0.154)	383 (0.833)	0.081	Co-dominant1*	2.05 (0.57–7.36)	0.269
					Co-dominant2*	2.81 (0.82–9.60)	0.098
					Dominant*	2.65 (0.78–9.02)	0.118
					Recessive*	1.47 (0.95–2.27)	0.078
					Additive*	**1.47 (1.01–2.13)**	**0.044**
T2DM (N = 338)	4 (0.012)	61 (0.180)	273 (0.808)	0.096	Co-dominant1†	**6.46 (1.48–28.13)**	**0.013**
					Co-dominant2†	**6.70 (1.61–27.84)**	**0.009**
					Dominant†	**6.65 (1.60–27.55)**	**0.009**
					Recessive†	1.20 (0.76–1.91)	0.421
					Additive†	1.35 (0.89–2.05)	0.146
					Co-dominant1‡	1.60 (0.42–6.07)	0.484
					Co-dominant2‡	1.30 (0.35–4.72)	0.689
					Dominant‡	1.34 (0.37–4.88)	0.651
					Recessive‡	0.83 (0.57–1.22)	0.360
					Additive‡	0.88 (0.63–1.24)	0.484

Data are presented as (*n*(%)) number and proportion. All association were tested using logistic regression adjusted by age, gender, IBM, smoking and Hypertension. MAF: Minor Allele Frequency; MetS: Metabolic Syndrome; T2DM: Type 2 Diabetes. * MetS vs Controls. † T2DM vs Controls. ‡ T2DM vs MetS.

CI: 0.46–0.84, P_{dom} = 0.002 and OR = 0.70, 95% CI: 0.54–0.89, P_{add} = 0.005) as compared to MetS group. In addition, under co-dominant1 and dominant models, the rs3753827 *A* allele was associated with an increased risk of developing T2DM (OR = 1.52, 95%CI: 1.02–2.28, $P_{co\text{-}dom1}$ = 0.039; OR = 1.49, 95% CI: 1.02–2.18, P_{dom} = 0.039) when compared to healthy controls. Furthermore, under codominant 1, codominant 2 and dominant models, the rs11573156*C* allele was associated with an increased risk of developing T2DM (OR = 6.46, 95% CI: 1.48–28.13, P_{codom1} = 0.013; OR = 6.70, 95% CI: 1.61–27.84, P_{codom2} = 0.009; OR = 6.65, 95% CI: 1.60–27.55, P_{dom} = 0.009) when compared to healthy controls. In addition, under additive model, the rs11573156*C* allele was associated with increased risk of MetS (OR = 1.47, 95% CI: 1.01–2.13, P_{add} = 0.044) when compared to controls. Models were adjusted for age, gender, IBM, smoking habit, and hypertension.

Table 4
PLA2G2A haplotypes frequencies in the study groups.

Haplotype	Control	MetS	T2DM	OR (95% CI)*	P*
H1(A-C-G)	0.442	0.424	0.416	0.92 (0.77–1.10)	0.445
H2(A-A-G)	0.251	0.235	0.289	0.91 (0.74–1.12)	0.450
H3(T-A-G)	0.097	0.142	0.095	**1.54 (1.17–2.02)**	**0.006**
H4(T-C-G)	0.092	0.109	0.098	1.20 (0.90–1.61)	0.250
H5(A-C-C)	0.107	0.083	0.096	0.75 (0.55–1.02)	0.105

MetS, Metabolic syndrome; OR, odds ratio; CI, confidence interval; T2DM, Type 2 diabetes mellitus. The order of the polymorphisms in the haplotypes is according to the positions in the chromosome (rs876018, rs3753827, rs1157315).*P-value Compared to individuals with Metabolic Syndrome vs control.

3.2. Association of the polymorphisms with metabolic traits

The association of the *PLA2G2A* polymorphisms with quantitative metabolic parameters and cardiovascular risk factors was evaluated in the three groups (controls, MetS and T2DM) (data not shown). No associations were detected with quantitative metabolic parameters. However, some associations were observed with cardiovascular risk factors. Under dominant and additive models, the rs876018 polymorphism was associated with hypercholesterolemia in MetS patients when compared to healthy controls (OR = 1.80, 95% CI: 1.11–2.91, P_{dom} = 0.017 and OR = 1.72, 95% CI: 1.14–2.58, P_{add} = 0.009). In addition, under dominant model, the rs3753827 polymorphism was associated with SA in MetS (OR = 2.01, 95% CI: 1.02–3.94, P_{dom} = 0.041) and T2DM patients (OR = 3.03, 95% CI: 1.35–6.8, P_{dom} = 0.007)when compared to healthy controls. Models were adjusted by age, gender, BMI, smoking habit and hypertension.

3.3. Haplotype analysis

Polymorphisms showed a moderate linkage disequilibrium (D' > 0.63, r^2 = 0.014) and five haplotypes were observed: *AGC, AAG, TAG, TCG, ACC*. The *TAG* haplotype was significantly associated with an increased risk of MetS (OR = 1.54, P = 0.006) (Table 4). Also, this haplotype was associated with higher total-cholesterol levels in MetS patients (*TAG* 199.64 ± 47.4 *vs* others haplotypes 189.26 ± 45.7, P = 0.025).

4. Discussion

PLA2G2A genetic variation was analyzed to investigate possible associations with MetS, T2DM and metabolic traits. We observed a significant association of *PLA2G2A* polymorphisms with MetS, T2DM, hypercholesterolemia and SA in individuals participating in the GEA study of the Mexican population. sPla2 is usually associated with increased lipolysis, however, in 2012, Iyer et al., reported a study that included rats fed with a high carbohydrates and saturated fats diet, where immune cells overexpressed $sPLA_2$-IIA, generating metabolites of phospholipids, such as PGE2, to act on secondary target cells (adipocytes) and inhibiting lipolysis through Prostaglandin E_2-Prostaglandin EP_3 receptor-$G\alpha_1$-cAMP signaling (PE_2-EP_3-$G\alpha_1$-cAMP), promoting adipocyte and metabolic dysfunction together with cardiovascular symptoms of MetS (Iyer et al., 2011)**.** Our results provide evidence supporting that *PLA2G2A* plays a role in the development of MetS and T2DM. We found that the rs876018 *T* allele was significantly associated with an increased risk of MetS and rs3753827 *A* allele was significantly associated with an increased risk of developing T2DM. These polymorphisms have been previously associated with T2DM (Wootton et al., 2006), but not with MetS. The dbSNP database was used to compare allelic frequencies of the polymorphisms in our study with those reported in other populations (European Caucasian, Africans, and Native Americans). In our study, rs876018 *T* (19.2%), rs3753827 *A* (35.6%) and rs11573156*C* (11.6%) were the less frequent alleles. These also are the less frequent alleles in European Caucasians (15%, 47%, 21%, respectively), in Africans (27%, 31%, 13%) and in Native Americans (24%, 38%, 11%).

The association of rs876018 polymorphism with hypercholesterolemia and the *TAG* haplotype with total cholesterol levels could be the result of a higher activity of $sPLA_2$-IIA. If intracellular lipolysis exceeds lipogenesis, then free fatty acids undergo a net release into circulation. Increases in circulating free fatty acids contribute to the typical dyslipidemia found in MetS (Bays et al., 2008).

Most importantly, adipose tissue lipolysis is especially sensitive to insulin. Under conditions of insulin resistance, increased lipolysis of stored triacylglycerol molecules in adipose tissue produces more fatty acids, which in turn inhibits insulin antilipolytic action, thereby initiating a cycle of additional lipolysis and further fatty acid production. In the liver, increased uptake of free fatty acids (FFAs) stimulates secretion of apolipoprotein (apo) B-100, the structural protein of atherogenic lipoproteins.

Although central obesity as determined by waist circumference measurement has recently been identified as a chief predictor of MetS in certain patients, not all obese subjects develop MetS. During positive caloric balance, the development of metabolic disease is more closely related to how fat is stored (through adipocyte hypertrophy versus hyperplasia) than simply the amount of fat stored (Vinik, 2005; Morange and Alessi, 2013). It has also been demonstrated that the accumulation of fat around abdominal viscera and inside intraabdominal solid organs is strongly associated with obesity-related complications like T2DM and CAD. The visceral adipose tissue and the adipose-tissue resident macrophages produce more pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) and less adiponectin. These changes in the production of cytokines induce insulin resistance and play a major role in the pathogenesis of endothelial dysfunction and subsequent atherosclerosis (Hamdy et al., 2006)**.** This could be the reason why we found the association of the rs3753827 polymorphism with risk of SA in MetS and T2DM.

Previous studies have linked $sPLA_2$ with insulin resistance (Leinonen et al., 2003) and T2DM (Leinonen et al., 2004). In atherosclerotic lesions, $sPLA_2$ is present in smooth muscle cells and macrophages and in close association with lipid deposits. $sPLA_2$ may play a role in remodeling of HDL to proatherogenic particles (Leinonen et al., 2004), the release of proinflammatory lipid mediators, and modification apoB-containing particles to a more atherogenic form, leading to lipoprotein retention and foam cell formation (Tietge et al., 2000). In addition to *PLA2G2A* genetic variation, these potentially proatherogenic mechanisms could modify the risk associated to MetS. Seo et al., reported that the components of MetS are strongly associated with the presence of SA and this risk increases gradually with the number of components having an individual diagnosed with MetS (Seo et al., 2015; Al Rifai et al., 2015). Nevertheless, the data reported for Seo and the participation of $sPLA_2$-IIA in mechanisms involved in the remodeling of HDL to proatherogenic particles, in the release of proinflammatory lipid mediators, and in the modification apoB-containing particles to a more atherogenic form that could lead to lipoprotein retention and foam cell formation justify the role of this molecule in the developing of cardiovascular disease in MetS individuals. In this way, and considering our results, *PLA2G2A* polymorphisms could be considered as susceptibility markers for cardiovascular disease in MetS individuals**.**

Study limitations need to be addressed. This study only included the analysis of three polymorphisms of the *PLA2G2A* gene. Considering that this is the first report of an association of the *PLA2G2A* polymorphisms with MetS, replication in another group of patients is necessary. The models were adjusted for diverse variables, however they were not adjusted for workout habits or caloric intake.

The results suggest that *PLA2G2A* gene variation has a role in the development of MetS and SA, although $sPLA_2$-IIA serum levels were not measured in our study populations. Our study provides the groundwork for future investigations in large prospective or case-control studies that pretend to determine the relationship among *PLA2G2A* genotypes, serum sPLA2-IIA levels and MetS risk.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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RESEARCH Open Access

Dietary fat and carbohydrate modulate the effect of the ATP-binding cassette A1 (*ABCA1*) R230C variant on metabolic risk parameters in premenopausal women from the Genetics of Atherosclerotic Disease (GEA) Study

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Abstract

Background: Although the R230C-ATP-binding cassette A1 (*ABCA1)* variant has been consistently associated with HDL-C levels, its association with diabetes and other metabolic parameters is unclear. Estrogen and dietary factors are known to regulate *ABCA1* expression in different tissues. Thus, we aimed to explore whether gender, menopausal status and macronutrient proportions of diet modulate the effect of this variant on various metabolic parameters.

Methods: One thousand five hundred ninety-eight controls from the GEA study were included (787 men, 363 premenopausal women and 448 menopausal women), previously assessed for anthropometric and biochemical measurements and visceral to subcutaneous abdominal fat (VAT/SAT) ratio on computed tomography. Taqman assays were performed for genotyping. Diet macronutrient proportions were assessed using a food frequency questionnaire validated for the Mexican population. Multivariate regression models were constructed to assess the interaction between the proportion of dietary macronutrients and the R230C polymorphism on metabolic parameters.

Results: All significant interactions were observed in premenopausal women. Those carrying the risk allele and consuming higher carbohydrate/lower fat diets showed an unfavorable metabolic pattern [lower HDL-C and adiponectin levels, higher VAT/SAT ratio, homeostasis model assessment for insulin resistance (HOMA-IR) and higher gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) levels]. Conversely, premenopausal women carrying the risk allele and consuming lower carbohydrate/higher fat diets showed a more favorable metabolic pattern (higher HDL-C and adiponectin levels, and lower VAT/SAT ratio, HOMA-IR, GGT and ALP levels).
(Continued on next page)

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Conclusion: This is the first study reporting a gender-specific interaction between *ABCA1*/R230C variant and dietary carbohydrate and fat percentages affecting VAT/SAT ratio, GGT, ALP, adiponectin levels and HOMA index. Our study confirmed the previously reported gender-specific *ABCA1*-diet interaction affecting HDL-C levels observed in an independent study. Our results show how gene-environment interactions may help further understand how certain gene variants confer metabolic risk, and may provide information useful to design diet intervention studies.

Keywords: *ABCA1*, R230C, Gene-diet interaction, Insulin resistance, Adiponectin, Visceral to subcutaneous abdominal fat ratio, GGT, HDL-C

Background

The ATP-binding cassette A-1 protein (ABCA1) is essential for the transport of lipids across plasma membranes and cholesterol homeostasis, is known to have various functions in distinct tissues and plays an essential role in metabolism [1]. ABCA1 is crucial for high-density lipoprotein cholesterol (HDL-C) biogenesis in the liver and intestine [2–4] and is known to regulate insulin secretion in pancreatic β–cells through the modulation of insulin granule exocytosis [5, 6]. Moreover, hepatic ABCA1 was found to improve glucose tolerance by enhancing β-cell function through both HDL production and interaction with β–cell ABCA1 [7], while adipocyte-specific ABCA1 was found to prevent fat storage, and to play a role in adipocyte lipid metabolism, body weight and whole-body glucose homeostasis [8, 9].

The *ABCA1* gene is located at 9q31.1, spans 149 kb, comprises 50 exons and is highly polymorphic. Observations from genetic association studies are in agreement with the role of ABCA1 in HDL-C formation, as several single nucleotide variants (SNVs) have been associated with HDL-C plasma levels in many studies, although their overall contribution to HDL-C levels is low [10–15]. In comparison, few studies have reported associations of *ABCA1* gene variants with other metabolic traits such as type 2 diabetes (T2D) [16–20], body mass index (BMI) [15], or body fat distribution in women [21, 22]. Particularly in the case of T2D, associations with *ABCA1* variants have been inconsistent [23, 24]. Among the many possible explanations for these inconsistencies are the possibility of spurious associations due to population stratification, differences in study design, gender bias and dietary factors known to affect *ABCA1* expression.

Gender-related metabolic differences are well known. *ABCA1* is one of the 1249 sex-biased genes found in liver, expressed at significantly higher levels in females [25]. Some of these gender-related metabolic differences, such as higher HDL-C levels in premenopausal women, can be explained by the effect of estrogen [26, 27]. Both in the murine model and in humans, estrogen has been found to increase *ABCA1* expression in different tissues [28, 29]. Moreover, certain dietary components have been found to regulate *ABCA1* expression: different *ABCA1* transcripts were upregulated by a high fat diet in mice [30]; high glucose, linoleic acid and omega-6 unsaturated fatty acid suppressed *ABCA1* expression while saturated fatty palmitic acid increased *ABCA1* expression in primary human monocyte-derived macrophages [31]; unsaturated fatty acids decreased ABCA1 protein levels by promoting its degradation in HepG2 and human small intestine epithelial cells [32, 33]; ABCA1 hepatic levels were increased in mice fed a high fat diet, while mulberry leaf and fruit extract treatment counteracted this high fat-induced ABCA1 expression [34]; and black soybean supplementation significantly increased hepatic *ABCA1* mRNA levels in mice with diet-induced non-alcoholic fatty liver disease (NAFLD) [35]. Although evidence of dietary factors affecting *ABCA1* expression is abundant, there are relatively few studies reporting of *ABCA1* genetic variants with dietary components. Significant interactions of *ABCA1* gene variants with dietary macronutrients affecting plasma lipid levels have been reported in the Inuit population [36], in healthy young Chinese individuals [37] and in Mexican premenopausal women [38], and two diet intervention studies reported significantly different responses in BMI, HDL-C and serum adiponectin levels in individuals bearing the *ABCA1/*R230C variant [39, 40].

The *ABCA1/*R230C variant (rs9282541) was found to be private to the Americas and strongly associated with low HDL-C in Mexican mestizos and Native American populations [15, 41]. This allele is of particular interest, because it is frequent in the Mexican mestizo population (10 %) and the sole presence of the C risk allele explains almost 4 % of plasma HDL-C concentration variation, which is higher than all HDL-C variation associated with a single nucleotide polymorphism identified through genome-wide association studies in European and Asian populations [42]. Although the *ABCA1*/R230C variant has been consistently associated with HDL-C levels, its association with diabetes and other metabolic parameters is unclear. Thus, we aimed to explore whether gender, menopausal status and dietary macronutrient composition modulate the effect of the *ABCA1/*R230C variant on plasma lipid levels and other cardiometabolic risk parameters.

Methods

The study included a total of 1598 controls recruited from the GEA Study, which was designed to investigate genetic factors associated with premature coronary artery disease, subclinical atherosclerosis and other coronary risk factors in the Mexican population [21]. All participants provided written informed consent, and the study was approved by the Ethics Committee of the Instituto Nacional de Cardiología "Ignacio Chávez" (INCICH) and the Ethics Committee of the Instituto Nacional de Medicina Genómica (INMEGEN). All participants answered standardized and validated questionnaires to obtain information on family and medical history, menopausal status, alcohol and tobacco consumption, dietary habits and physical activity [43, 44]. Menopausal status was defined as absence of menses for more than 12 months on interrogation. Anthropometric and metabolic measurements included BMI, HDL-C, low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglyceride (TG), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), adiponectin serum levels, homeostasis model for assessment of insulin resistance (HOMA-IR)], and visceral abdominal fat to subcutaneous abdominal fat (VAT/SAT) ratio on computed tomography scans were measured as previously described [21, 45].

Dietary assessment

Dietary assessment was performed using a Food Frequency Questionnaire (FFQ) previously validated for the Mexican population by the Instituto Nacional de Salud Pública, using a 116-item semiquantitative FFQ designed to estimate the usual dietary intake over the previous 12-month period [44]. Specifically, this questionnaire was designed to classify individuals by the relative intake of 20 nutrients from 82 foods of the Mexican diet. The FFQ collects specific information on the consumption of fats, carbohydrates, proteins and other nutrients. The validity and reproducibility of this questionnaire to assess dietary intake of a number of nutrients has been assessed previously in this population [44]. Energy intake, and the proportion of macronutrients consumed daily were estimated using the system evaluation of nutritional habits and food intake (SNUT) [46].

Genetic analysis

Genomic DNA was extracted and purified from white blood cells using the salting-out procedure [47]. The *ABCA1*/R230C variant (rs9282541) was genotyped using TaqMan assay number C_11720861_10, following the manufacturer's recommendations in an ABI Prism 7900HT sequence detection system (Applied Biosystems). Genotyping call exceeded 95 % and no discordant genotypes were observed in 184 duplicate samples. Previously sequenced samples of all genotypes (RR, RC and CC) were used as positive controls.

Statistical analyses

Statistical calculations and graphs were made using R [48]. For all calculations, anthropometric and metabolic variables (except age, waist circumference and LDL-C) were log-transformed due to skewed distribution. Genotype frequencies did not deviate from Hardy-Weinberg equilibrium ($P = 0.57$). Because of the reduced number of CC homozygous individuals, RC and CC genotypes were analyzed as a single group using a dominant model. Pearson's correlations were calculated to study the linear relationship between macronutrient proportions and anthropometric and metabolic variables, stratified by gender, menopausal status and *ABCA1*/R230C genotype. To address multiple testing for the correlation analysis, we calculated Bonferroni's correction using Simple Interactive Statistical Analyses Software [49] assuming 12 measurements and an average pairwise correlation of 0.21; *P*-value below 0.007 was considered significant. All variables that showed no significant correlation with macronutrients for RR genotypes, but a significant correlation with at least one macronutrient for RC/CC genotypes, were tested for gene-diet interactions. Multivariate regression models were used to assess the interaction between the proportion of dietary components and the R230C variant on the dependent variables and to assess their predictive effect, adjusting for age, BMI and alcohol consumption as appropriate. The study power to detect interactions between the *ABCA1*/R230C variant and dietary fat percentage affecting HDL-C, HOMA-IR, VAT/SAT ratio, adiponectin, ALP and GGT levels in premenopausal women was calculated using QUANTO software version 1.2.4 [50], assuming a dominant model with a minor allele (T) frequency of 0.11 and parameters estimated from the study population. Statistical power ranged between 60 % and 90 % for all variables except for HOMA-IR, which only reached 36 %.

Results

Anthropometric and biochemical characteristics of the population stratified according to gender and menopausal status are shown in Additional file 1: Tables S1 and S2. All correlations between macronutrient percentages and metabolic parameters stratified by gender, menopausal status and *ABCA1*/R230C genotypes are depicted as a correlation matrix in Fig. 1. Overall, correlations between macronutrient percentages and various metabolic parameters were of greater magnitude in women bearing RC and CC genotypes, particularly in premenopausal women. Interestingly, in the latter group, five metabolic parameters (HDL-C, HOMA-IR, VAT/

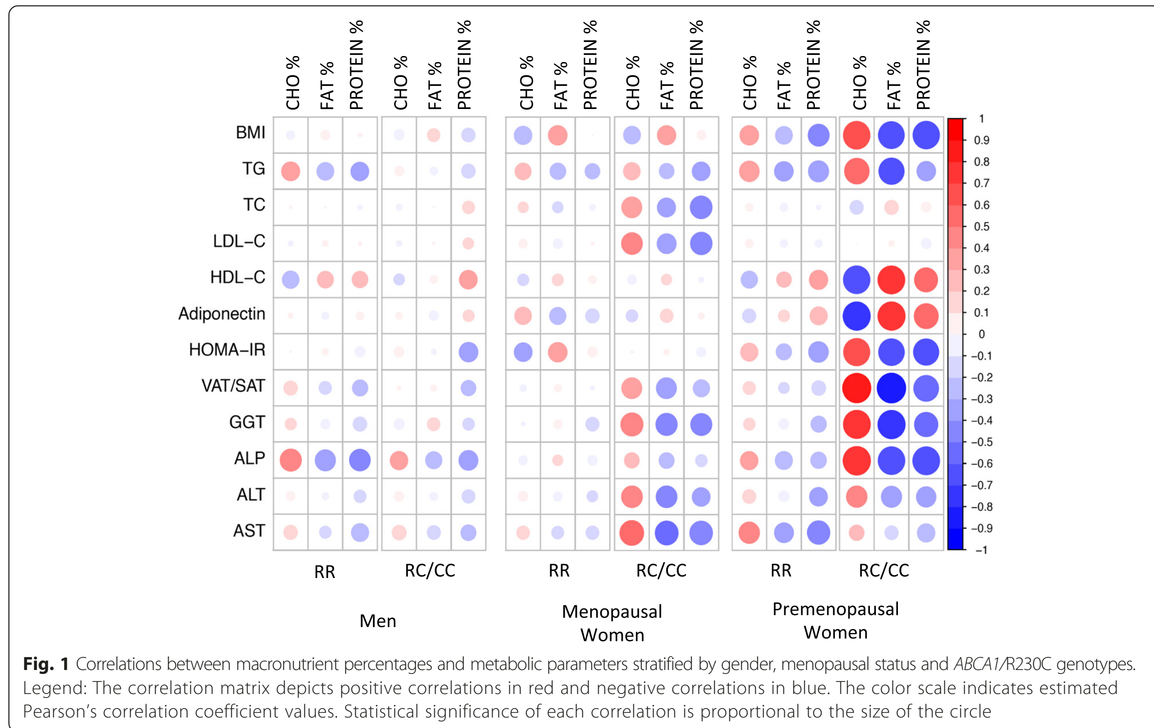

Fig. 1 Correlations between macronutrient percentages and metabolic parameters stratified by gender, menopausal status and *ABCA1*/R230C genotypes. Legend: The correlation matrix depicts positive correlations in red and negative correlations in blue. The color scale indicates estimated Pearson's correlation coefficient values. Statistical significance of each correlation is proportional to the size of the circle

SAT ratio, Adiponectin, and ALP) showed no significant correlation with macronutrients for RR genotypes, but significant correlations with at least one macronutrient for RC/CC genotypes after Bonferroni's correction (Additional file 1: Table S3). The correlation between dietary fat percentage and GGT levels in RC/CC premenopausal women showed borderline significance ($P = 0.008$) and was also tested for interactions. Median values for these six metabolic parameters according to *ABCA1*/R230C genotypes in the entire study population and in premenopausal women are described in Additional file 1: Table S4. Additional file 1: Tables S5 and S6 show median values for these metabolic parameters according to genotype and stratified by dietary carbohydrate and fat percentage tertiles.

HDL-C and adiponectin serum levels showed negative and significant correlations with dietary carbohydrate percentage and positive significant correlations with fat percentage in premenopausal women with RC and CC genotypes, but not in women with wild-type (RR) genotypes (Additional file 1: Table S3). Both carbohydrate and fat *ABCA1*/R230C interactions affecting HDL-C and adiponectin serum levels showed statistical significance ($P_{interaction} < 0.04$) (Figs. 2a and 3a).

Correlations between dietary macronutrients and HOMA-IR, VAT/SAT ratio, ALP and GGT showed similar patterns in premenopausal women. All these parameters showed positive and significant correlations with dietary carbohydrate percentage and negative significant correlations with fat percentage in premenopausal women with RC/CC genotypes, but not in women with wild-type (RR) genotypes. Interactions between *ABCA1*/R230C and dietary carbohydrate and fat affecting VAT/SAT ratio, ALP and GGT levels were statistically significant ($P < 0.03$) (Figs. 2c, 3b and 3c); these interactions showed a tendency to significance for HOMA-IR ($P = 0.075$ and $P = 0.058$, respectively) (Fig. 2b). Interestingly, TG levels showed a positive and significant correlation with dietary carbohydrate percentage ($P < 0.002$), and a negative significant correlation with dietary fat percentage ($P < 0.001$) in premenopausal women with RC/CC genotypes, however gene-diet interactions lacked statistical significance.

Dietary protein percentage correlated negatively with ALP levels in premenopausal women with RC/CC genotypes, with borderline significance ($P = 0.008$). This interaction was statistically significant ($P_{interaction} = 0.016$).

Discussion

We present here evidence suggesting that dietary macronutrient proportions modulate the effect of the functional *ABCA1*/R230C gene variant on several metabolic parameters in premenopausal women. Although the total number of premenopausal women was small (n = 363),

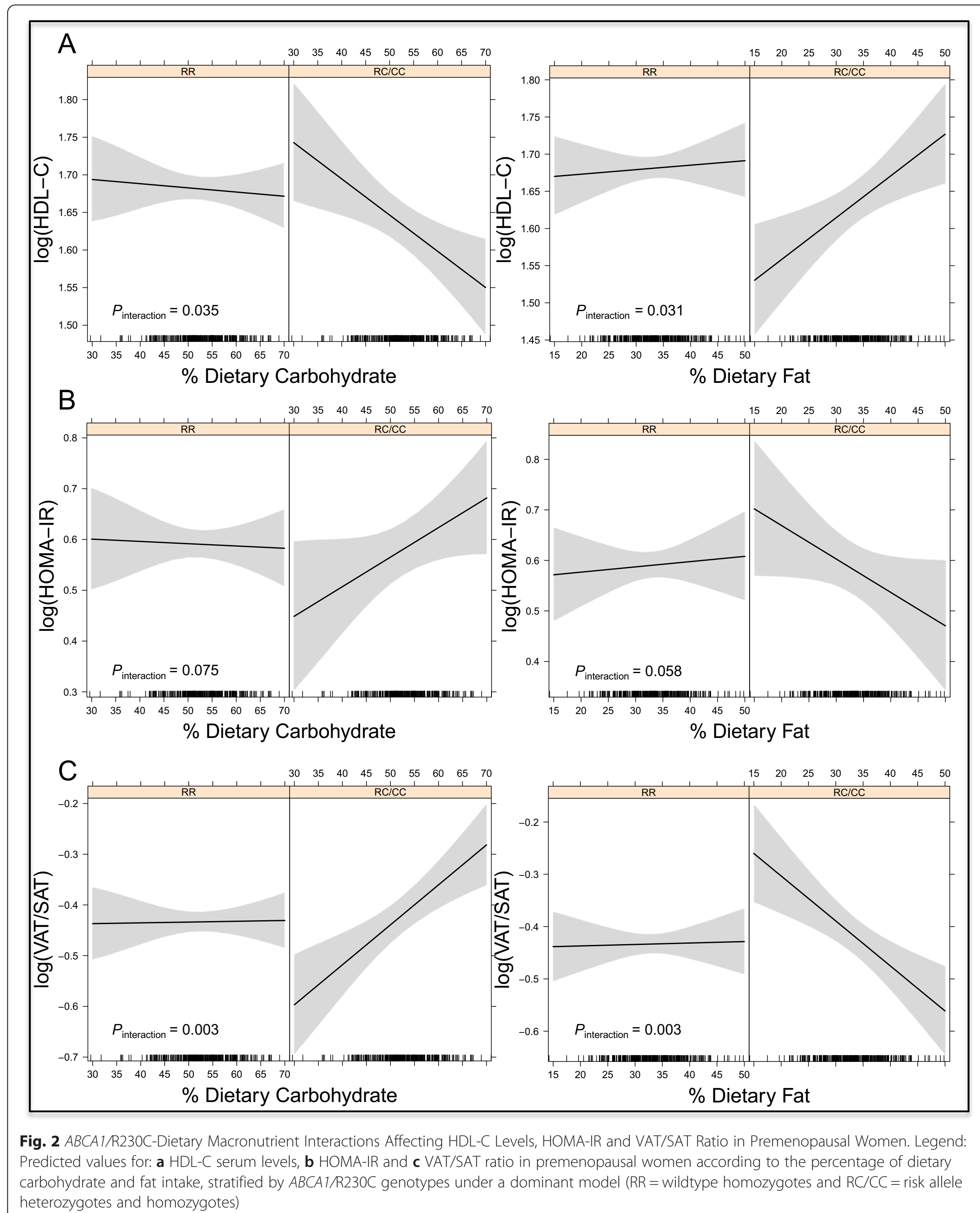

Fig. 2 *ABCA1*/R230C-Dietary Macronutrient Interactions Affecting HDL-C Levels, HOMA-IR and VAT/SAT Ratio in Premenopausal Women. Legend: Predicted values for: **a** HDL-C serum levels, **b** HOMA-IR and **c** VAT/SAT ratio in premenopausal women according to the percentage of dietary carbohydrate and fat intake, stratified by *ABCA1*/R230C genotypes under a dominant model (RR = wildtype homozygotes and RC/CC = risk allele heterozygotes and homozygotes)

several gene-diet interactions became evident and significant only in this group. All interactions indicate a metabolically unfavorable pattern in premenopausal women carrying the risk allele consuming higher carbohydrate and lower fat diets (lower HDL-C and adiponectin levels, higher VAT/SAT ratio, HOMA-IR and higher GGT and

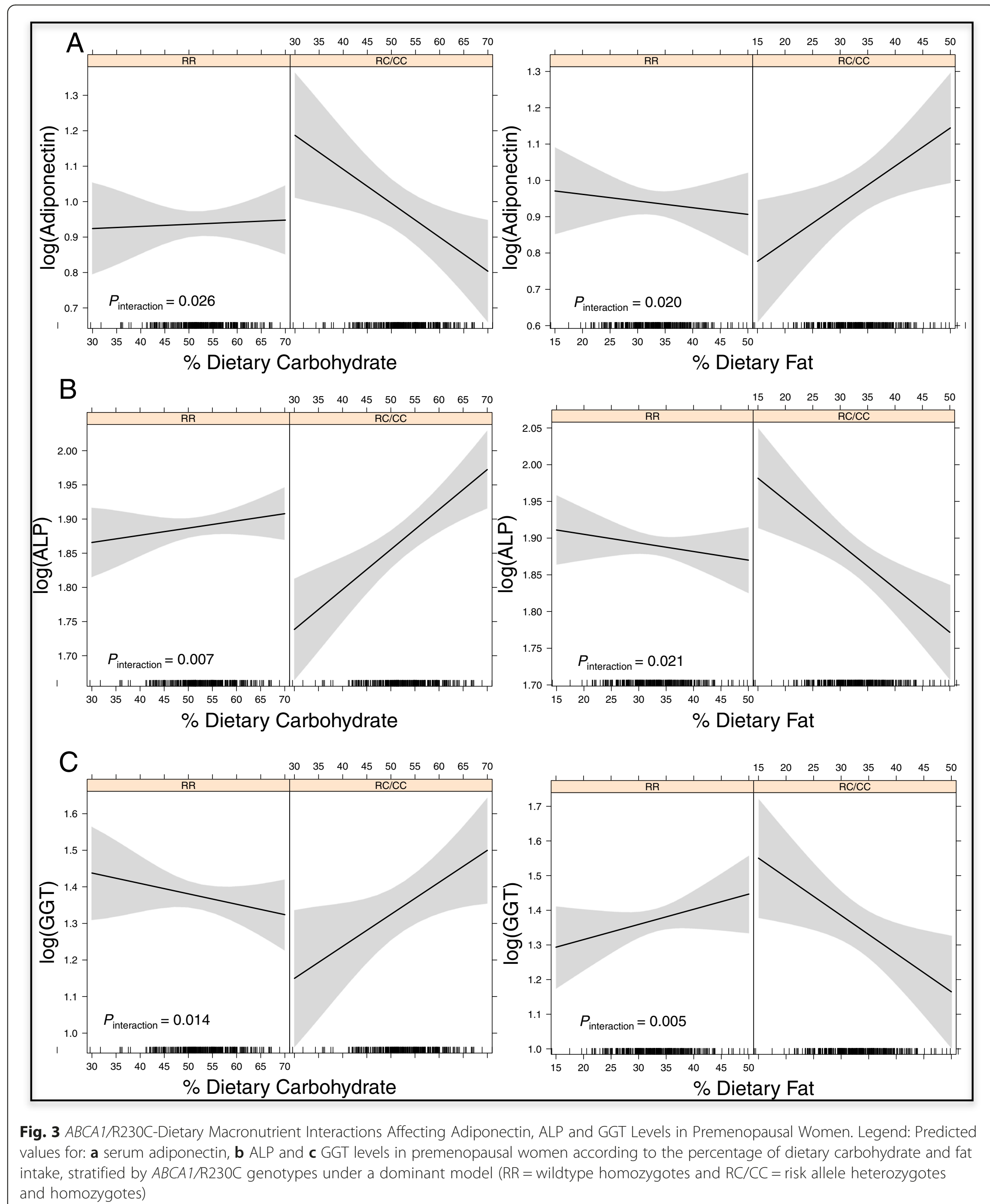

Fig. 3 *ABCA1*/R230C-Dietary Macronutrient Interactions Affecting Adiponectin, ALP and GGT Levels in Premenopausal Women. Legend: Predicted values for: **a** serum adiponectin, **b** ALP and **c** GGT levels in premenopausal women according to the percentage of dietary carbohydrate and fat intake, stratified by *ABCA1*/R230C genotypes under a dominant model (RR = wildtype homozygotes and RC/CC = risk allele heterozygotes and homozygotes)

ALP levels); and a more favorable metabolic pattern in premenopausal women carrying the risk allele with higher fat and lower carbohydrate diets (higher HDL-C and adiponectin levels, and lower VAT/SAT ratio, HOMA-IR, GGT and ALP levels). Gender differences in carbohydrate and fat metabolism are well known [51, 52] and gender

differences in metabolic changes in response to diet have been previously published [53–57], mostly evident in women of reproductive age. Although the molecular mechanisms for these differences are not fully understood, evidence both in humans and animal models has shown that the effects of estrogen on genes involved in lipid metabolism play an important role [25–29]. In the GEA control group, *ABCA1*/R230C was significantly associated with HDL-C levels [21], but not with any other metabolic variable. Apparently, the presence of other factors (estrogen levels, low dietary fat, and high dietary carbohydrate) is required for this variant to exert an effect on the other metabolic parameters.

ABCA1 and serum lipid levels

Many association studies support the role of *ABCA1* gene variation in plasma lipid levels. The vast majority of these studies analyze the role of *ABCA1* polymorphisms on plasma HDL-C levels [10–15]. ABCA1-mediated cholesterol efflux being the first step in HDL-C particle formation likely explains the consistency of the associations of *ABCA1* gene variation with HDL-C levels among different populations. The well-known inverse correlation between dietary carbohydrate intake and HDL-C levels [58, 59] was also observed in control individuals from the GEA study regardless of gender or genotype. However, in agreement with the report of Romero-Hidalgo et al. in an independent Mexican population [38], this inverse correlation was significantly higher in premenopausal women bearing the *ABCA1/*R230C allele ($P_{interaction}$ = 0.04). Also in agreement with this observation, hyperlipidemic R230C allele carriers showed a better HDL-C serum concentration response when fed a low-saturated fat diet supplemented with 25 g soy protein and 15 g soluble fiber, and this response was more evident in women [39], although the total proportion of macronutrients of this diet was not indicated. Thus, three independent studies, two based on food frequency questionnaires and one diet intervention study, confirm a gender-specific *ABCA1/*R230C-diet interaction affecting HDL-C serum levels.

ABCA1 gene variants have failed to show significant associations with triglyceride levels in almost all genetic association studies analyzing serum lipid levels. To our knowledge, the R230C variant was significantly associated with lower TG and total cholesterol levels in Mayans and Pimas from the United States in only one study [41], and the authors suggested that other genetic or environmental factors may play a role on the effect of ABCA1 on these and other metabolic traits. Interestingly, in the present study although the positive correlation between dietary carbohydrate intake and TG in premenopausal women was higher for RC/CC genotypes ($r = 0.366$; $P = 0.002$) than for RR genotypes ($r = 0.132$; $P = 0.031$), and the inverse correlation between dietary fat percentage and TG levels was greater in premenopausal women with RC/CC genotypes ($r = -0.384$; $P = 0.001$) than in those with RR genotypes ($r = -0.122$; $P = 0.046$), these interactions lacked statistical significance. A larger study using food frequency questionnaires reported that high saturated fat intake was associated with higher triacylglycerol serum levels in Inuit individuals bearing the CC genotype of *ABCA1/*R219K (rs2230806), with no gender effect reported [36]. Because *ABCA1/*230C allele is less frequent than the *ABCA1/*219K allele, a greater sample size may be necessary to uncover a possible R230C-macronutrient interaction affecting TG levels.

ABCA1, insulin resistance, VAT/SAT ratio and adiponectin levels

Although several lines of evidence both in human studies and animal models indicate that ABCA1 plays a role in insulin secretion and insulin resistance, the role of *ABCA1* gene variation in T2D susceptibility and insulin resistance is not fully understood. T2D is not a feature of Tangier disease, which is caused by recessive loss of function *ABCA1* mutations. However, while *ABCA1* knockout mice do not develop T2D, pancreatic beta-cell specific *ABCA1* knockout mice ($Abca1^{-P/-P}$) showed a gene-dose dependent and age-related accumulation of cellular cholesterol in β-cells, marked reduction in insulin secretion *in vivo* and progressive impairment in glucose tolerance that was independent of islet development or β-cell mass [5], and disruption of insulin granule exocytosis [6]. Adipocyte specific knockout mice ($Abca1^{-ad/-ad}$) also showed impaired glucose tolerance, lower insulin sensitivity and decreased insulin secretion [9].

There are few studies analyzing β-cell function and insulin sensitivity in human heterozygotes for loss-of-function *ABCA1* mutations, with inconsistent results. In agreement with the mouse model, a small study showed that 15 individuals with loss-of function *ABCA1* mutations showed decreased insulin secretion, mild hyperglycemia but no difference in insulin response to an oral glucose challenge measured by hyperglycemic clamp [60]. Because none of these *ABCA1* mutation carriers had diabetes, the authors suggested that carriership is a relatively mild islet susceptibility factor for diabetes by itself. In contrast, in a recent study including only young adults, 3 homozygotes and nine heterozygotes for *ABCA1* mutations exhibited enhanced oral glucose tolerance and increased β-cell secretory capacity as compared to control subjects suggesting a gene dose effect, with no differences in insulin sensitivity [61]. To explain discrepancies with previous studies, the authors suggested that age and systemic cholesterol availability might influence the effect of *ABCA1* mutations on insulin secretion. The largest study of loss-of-function mutations included

94 *ABCA1* heterozygotes from the Copenhagen City Heart Study and the Copenhagen General Population Study. The authors reported no association with increased risk of T2D in the general population, suggesting that ABCA1 dysfunction may impact β-cell function, but is not enough to cause diabetes [24].

It is noteworthy that *ABCA1* has never been identified as a diabetes-associated gene in genome-wide association studies GWAS [62–64]. Associations of *ABCA1* with T2D have only been observed in a small number of candidate gene case–control studies, in Japanese, Mexican and Turkish populations [16–20]. The *ABCA1/*R230C variant was associated with early-onset T2D in two independent small cohorts of the Mexican population [20], was only marginally associated with T2D in Pimas ($P = 0.06$) [41], and was not associated T2D in a case–control study of the Colombian population [23]. Possible explanations for these inconsistencies include differences in study design, gender bias, mean age, population stratification, genetic background, the effect of plasma lipids, and gene-environment interactions, which should be pointed out as possible confounding factors for *ABCA1* association studies. In the present study, it was not possible to directly assess R230C-diet interactions affecting T2D susceptibility because of the low number of premenopausal women with T2D (n = 29). However, interactions affecting T2D-associated metabolic risk parameters (HOMA-IR, VAT/SAT ratio and adiponectin levels) showed the same unfavorable pattern in premenopausal women bearing R230C genotypes with high dietary carbohydrate intake (Figs. 2b, 2c and 3a). To our knowledge, no single *ABCA1* polymorphism has been associated with HOMA-IR or other measures of insulin resistance. The complexity of the process of insulin sensitivity suggests that ABCA1 function is probably only one of many factors affecting glucose uptake by peripheral cells. Although the *ABCA1/*R230C diet interactions affecting HOMA-IR observed here had marginal significance ($P_{interaction} = 0.058$ and 0.075 for dietary fat and carbohydrate intake respectively), our data suggest that gender and diet are factors that added to ABCA1 function participate in the complex process of insulin resistance, and deserve further study.

Circulating adiponectin levels are highly heritable [65] and are inversely associated with insulin resistance and T2D [66, 67]. Although the regulation of serum adiponectin levels is complex, experimental evidence suggests there is a link between ABCA1 and adiponectin. Adiponectin has been found to upregulate *ABCA1* expression in macrophages [68, 69] and to play a role in ABCA1 regulation in hepatocytes [70], while the adipocytes of *Abca1*$^{(ad-/ad-)}$ mice were found to have decreased adiponectin expression [9]. Moreover, although *ABCA1* has not been found to be associated with this trait in GWAS or candidate gene association studies, a dietary intervention study including nopal, chia seed, soy protein and oat reported that individuals with metabolic syndrome bearing the *ABCA1/*R230C variant responded with a greater decrease in body weight and a sharper increase in serum adiponectin concentrations [40]. Thus, the significant interactions between the *ABCA1/*R230C variant and dietary fat/carbohydrate percentages observed in premenopausal women from the GEA study support this link between ABCA1 and serum adiponectin levels. Similarly, increased VAT/SAT ratio is associated with increased metabolic risk and T2D [71], and although *ABCA1* has not been associated with VAT/SAT ratio, it was recently identified as a novel locus associated with body fat distribution with a stronger effect in women [22], and BMI was found to be positively associated with VAT/SAT ratio in premenopausal women from the GEA study bearing the *ABCA1/*R230C [21] .

ABCA1 and liver enzymes

ABCA1 has not been previously associated with nonalcoholic fatty liver disease, AST or ALT levels, and no gene-diet interactions affecting transaminases were observed in the present study. Interestingly, very significant interactions were observed affecting GGT and ALP serum levels, showing the same unfavorable pattern in premenopausal women bearing the R230C variant with higher dietary carbohydrate and lower dietary fat percentages. In clinical practice, serum GGT has been confirmed to be involved in cardiovascular disease mechanisms, and several studies have reported an independent association of higher GGT levels with metabolic syndrome, carotid atherosclerosis and cardiovascular disease [72–75]. On the other hand, increased GGT and ALP levels are also indicators of liver or biliary tract diseases. ABCA1 is expressed in gallbladder and plays a role in cholesterol concentrations in bile [76, 77], and thus higher ALP and GGT levels observed in premenopausal women bearing *ABCA1/*R230C and consuming high carbohydrates might be related to a higher risk of cholestasis or cholelithiasis. Once again, although *ABCA1* has not been reported as a gene associated with cholelithiasis [78], it might be involved in the pathogenesis of the disease in premenopausal women with certain dietary patterns. Future clinical studies are necessary to prove whether the interactions here observed are in fact related to gallbladder disease.

Study limitations

Because this is a cross-sectional design, we cannot infer causality from the results. The sample size is relatively small to identify gene-diet interactions, and statistical power is low, particularly to identify interactions affecting HOMA-IR. Further studies are needed to confirm

these interactions. One of the main limitations is that carbohydrate and fat percentage were estimated from a validated food frequency questionnaire (FFQ). Dietary intake is difficult to measure, and although doubts of the accuracy of FFQs have been raised, they are still widely used as the primary dietary assessment tool in epidemiological studies [79]. The interactions found based on our FFQ that has been previously validated in the Mexican population can be used to design more controlled and accurate dietary intervention studies. Finally, menopausal status was determined by interrogation, and estrogen levels were not measured.

Conclusion

To our knowledge, this is the first study reporting a gender-specific interaction between *ABCA1/*R230C variant and dietary carbohydrate and fat percentages affecting VAT/SAT ratio, GGT, ALP and adiponectin levels and HOMA index. Our study also replicated previously reported *ABCA1*-diet interaction affecting HDL-C levels in Mexican premenopausal women observed in an independent study. Our results show how gene-environment interactions may help further understand how certain gene variants confer metabolic risk, and may provide information useful to design diet intervention studies.

Additional file

Additional file 1: Table S1. Demographic characteristics of the population. **Table S2.** Comparison of biochemical parameters stratified by gender and menopausal status. **Table 3.** Correlation between metabolic parameters and dietary macronutrients according to *ABCA1*/R230C genotypes in premenopausal women. **Table S4.** Comparison of biochemical parameters stratified by *ABCA1*/R230C genotypes in the study population and premenopausal women. **Table 5.** Comparison of biochemical parameters stratified by *ABCA1*/R230C genotypes and carbohydrate percentage tertiles in premenopausal women. **Table 6.** Comparison of biochemical parameters stratified by *ABCA1*/R230C genotypes and fat percentage tertiles in premenopausal women. (DOCX 162 kb)

Competing interests

The authors declare there are no competing interests.

Authors' contributions

CPR, GVA and TVM conceived and designed the study. LJA, RPS, MCGS, AMU and EAA acquired data. LJA, SRH, MCGS and TVM analyzed and interpreted the data. CPR, GVA, SRH, RPS, AC and SCQ critically reviewed the manuscript for important intellectual content. LJA and TVM wrote the manuscript. All authors approved the final version of the manuscript.

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