Post-GWAS Investigations for discovering pleiotropic gene effects in cardiovascular diseases

Alex-Ander Aldasoro

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Par: **Alex-Ander Aldasoro**

Post-GWAS Investigations for discovering pleiotropic gene effects in cardiovascular diseases

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Membres du jury:

Rapporteurs:	**Mme. Belgin SÜSLEYİCİ**	**Professor, Marmara University, Istanbul, Turkey**
	M. Nikolaos DRAKOULIS	**Professor, National & Kapodistrian University of Athens, Greece**
Examinateurs:	**Mme. Sophie VISVIKIS-SIEST**	**DR1, INSERM U1122 ; IGE-PCV, Universite de Lorraine, Nancy, France – (Thesis Director)**
	Mme. Ndeye Coumba NDIAYE	**MCU, Faculté de Pharmacie, Université de Lorraine, Nancy, France – (Thesis co-Director)**
	M. Christian DINA	**IR CNRS, INSERM UMR 915, Université de Nantes, France**
	Mme. Nathalie THILLY	**PUPH, EA 4360 APEMAC, Université de Lorraine, Nancy, France**
	M. Dwaine R. VANCE	**PhD, Randox Laboratories, Co-Antrim, United Kingdom**

Université de Lorraine - UMR INSERM U1122; IGE-PCV - Faculté de Pharmacie - 30, rue Lionnois - 54000 Nancy - France

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Abstract

Cardiovascular diseases (CVD) are complex diseases where many environmental and genetic factors are involved. Due to this, their study needs to take into account different risk factors and the interactions between them, in order to reduce their incidence and mortality. Thanks to the breakthroughs of the Hap Map and Human genome projects and the development of GWAS and PheWAS, the genetic etiology of the CVD has been extensively investigated the last two decades. However, there is still a big room of knowledge waiting to be discovered and alternative approaches are needed in order to keep advancing in the pathophysiology of CVD.

In this thesis, we propose an integrative approach to discover new genetic associations potentially involved in CVD. We chose previous GWAS hits and we focused on phenotypically homogeneous populations, in order to limit as much as possible the heterogeneity of intermediate phenotypes. We also centred our efforts in studying the pleiotropic and gene-gender interaction effects of the genes selected. Another main goal during this thesis was to defend the implementation of personalized genome-based therapy of the results obtained.

By using the above-explained approaches, new pleiotropic effects were discovered in the IL-6R and ABO genes. Within the IL-6R gene we found an antagonistic pleiotropy of this gene when it relates to CRP and lipid trait levels. We also found new pleiotropic effects within the ABO gene, by independently associating the major rs644234*T allele with increased sE-selectin and HDL levels, and decreased ApoE levels. Detection of pleiotropy is a source of new hypothesis and makes easier the development of new drugs, hence improving prevention and personalized medicine within the CVD. In addition, we studied the gene-gender interaction effects, finding some sex-specific associations in two of the genes studied (ABO and GNB3). Further, we centered our efforts in implementing the results obtained during the thesis at the clinical level, with the aim of ameliorating the genetics of personalized medicine. The SNP rs2234246 within the TREM-1 gene was associated with increased levels of its protein and could be used as a predictor or risk biomarker for different diseases (including CVD). Indeed, and due to the high potential of this polymorphism, we applied a European patent and we are planning to start Phase I clinical trials in patients suffering from Septic Shock and Acute Myocardial Infarction. Also the IL-6R haplotype

rs4845628*T/rs4537545*C, which is increasing simultaneously CRP, LDL-C and ApoB levels, could be used in the treatment of personalized medicine. One of the side effects of the drug Tocilizumab, which is targeting the IL-6R gene, is the increase of LDL-C levels in patients, which could lead to a risk of suffering from ischaemic heart disease. In order to prevent these side effects, patients carriers of the haplotype rs4845628*T/rs4537545*C could be treated by analogue drugs or different doses of tocilizumab, which could make the treatment more personalized and hence, more secure and precise.

Following the goals and approaches synthetized above, we discovered new associations between genes of interest and intermediate phenotypes that are involved in CVD and other complex diseases. Our results help to better understand how the studied genes are exerting their effects at the molecular level, ultimately affecting the outcome of the individuals suffering from CVD. Our results will hopefully be taken into account in future personalized treatments.

Key words: Cardiovascular diseases, Pleiotropy, Gene-gender interactions, Genetic epidemiology, Personalized medicine, Intermediate phenotypes.

Résumé

Les maladies cardiovasculaires (MCV) sont d'une étiologie complexe et elles sont soumises à de nombreux facteurs environnementaux ainsi que génétiques. Pour pouvoir mieux comprendre leur pathophysiologie et réduire leur incidence et leur mortalité, l'étude des MCV prend en compte beaucoup de facteurs de risques différents en même temps, ainsi que leurs interactions. Les dernières percées technologiques dans le cadre du Projet Génome Humain, ou du Projet HapMap ont permis de développer de nouvelles approches méthodologiques. Les études d'association pangénomiques ou GWAS et les études d'association panphénotypiques ou PheWAS ont révolutionné l'épidémiologie génétique de la dernière décennie. Malgré les succès obtenus, une stagnation de la réduction de la morbidité et de la mortalité CV a rapidement été atteinte ces dernières années, rendant nécessaire l'identification de nouveaux biomarqueurs en utilisant des approches différentes.

Cette thèse propose une approche intégrative pour découvrir de nouvelles associations génétiques associés avec les MCV et autres maladies complexes. Nous avons d'abord réuni les résultats existants grâce à des GWAS précédents, puis nous les avons étudié au sein de populations phénotypiquement homogènes, avec l'idée de limiter au maximum l'hétérogénéité des phénotypes intermédiaires. Nous avons recherché la pléiotropie de ces gènes ainsi que leurs éventuels effets dus aux interactions gène-genre. De plus, nous avons dirigé nos efforts vers une possible traduction des résultats obtenus dans l'application clinique.

En utilisant des méthodes alternatives au-delà des GWAS classiques, nous avons détecté les effets pléiotropiques de différent gènes (gènes IL-6R et ABO). Concernant le gène IL-6R, nous avons trouvé une pléiotropie antagoniste pour les phénotypes lipidiques et les niveaux de CRP. Nous avons aussi trouvé un nouvel effet pléiotropique dans le gène ABO, où nous avons associé de façon indépendante l'allèle majeur T de rs644234 avec une augmentation des niveaux soluble de E-sélectine et HDL, et avec une diminution des niveaux de ApoE. La pléiotropie est à l'origine de nouvelles hypothèses, et favorise le développement de nouveaux médicaments. Par ailleurs, nous avons trouvé quelques associations gène-genre intéressantes pour certains gènes étudiés (ABO et GNB3). Concernant l'implémentation clinique des connaissances obtenues par cette thèse, le variant génétique rs2234246 dans le gène TREM-1, qui augmente les taux solubles de la protéine TREM-1, pourrait être utilisé comme un

prédicteur ou un marqueur de risque pour différentes maladies (MCV inclues). En fait, et grâce au grand potentiel de ce polymorphisme, nous avons déposé un brevet Européen et nous envisageons de mener des essais cliniques de phase 1 chez les patients souffrant de choc septique et d'infarctus aigu du myocarde. Notre objectif est d'améliorer la médecine personnalisée basée sur des données génomiques. D'autre part, nous avons associé l'haplotype rs4845628*T/rs4537545*C du gène IL6R à des taux élevés de CRP, de LDL-C et d'ApoB. Le produit pharmaceutique tocilizumab, dont la cible est le récepteur IL-6R a comme effet de réduire les taux de CRP, mais un des effets indésirables du tocilizumab est une augmentation du taux de LDL-C chez les patients, ce qui pourrait augmenter le risque de cardiopathie ischémique. Afin d'éviter les effets indésirables, les patients qui ont l'haplotype rs4845628*T/rs4537545*C pourraient être traités par des produits pharmaceutiques analogues ou différents.

En suivant les objectifs et approches synthétisés ci-avant, nous avons découvert, ces trois dernières années, de nouvelles associations entre gènes et phénotypes intermédiaires qui sont impliquées dans les MCV et d'autres maladies complexes. Nos résultats aident à mieux comprendre comment les gènes étudiés exercent leurs effets au niveau moléculaire, en influant finalement sur l'état des patients souffrant de MCV. Nous espérons que nos résultats vont être pris en compte pour faire progresser la médecine personnalisée.

Mots clés : Maladies cardiovasculaires, Pléiotropie, Interactions Gène-Genre, Epidémiologie génétique, Médecine personnalisée, Phénotypes intermédiaires.

List of abbreviations

ANOVA	Analysis of variance
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
B2M	Beta 2 microglobulin
BMI	Body mass index
BRC	Biological Resources Centre
CAM	Cell adhesion molecule
CCPPRB	Comité Consultatif de Protection des Persones dans la Recherche Biomédicale
CD/CV	Common disease / common variant
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart diseases
CMP	Centre for Preventive Medicine
CNV	Copy-number variants
CRP	C-reactive protein
CVD	Cardiovascular diseases
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
ECs	Endothelial cells
EHR	Electronic health record
EMBL-EBI	European Bioinformatics Institute
eQTL	Expression quantitative trait loci
ERT	Oestrogen replacement therapy
FDR	False discovery rate
GNB3	Guanine Nucleotide-Binding protein subunit β3
GWAS	Genome-wide association studies
HCY	Homocysteine
HDL	High density lipoprotein
HLA	Human leukocyte antigen system
HPSG	Heparin sulphate proteoglycans
HWE	Hardy-Weinberg Equilibrium
ICAM-1	Intercellular adhesion molecule 1
ICD	International classification of diseases
IFN	Interferon
Ig	Immunoglobulin
IGE-PCV	'Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire'
Il-1beta	Interleukin 1 beta
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
IL-8	Interleukin-8
IMT	Intima-media thickness
IMT-F	Intima-media thickness of the femoral artery
JAK	Janus kinase
LD	Linkage disequilibrium
LDL	Low density lipoprotein

LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
MAF	Minor allele frequency
mbIL-6R	Membrane bound IL-6R
mbTREM-1	Membrane bound TREM-1
MCP-1	Monocyte chemo attractant protein 1
MHC	Major histocompatibility complex
MI	Myocardial infarction
mRNA	Messenger ribonucleic acid
NCD	Non-communicable diseases
NHGRI	Human Genome Research Institute
Ox-LDL	Oxidized low density lipoprotein
PBMCs	Peripheral blood mononuclear cells
PECAM-1	Platelet endothelial cell adhesion molecule 1
PheWAS	Phenome-wide association studies
PMNs	Polymorphonuclear leukocytes
PSGL-1	P-selectin glycoprotein ligand
QTL	Quantitative trait locus
RA	Rheumatoid arthritis
RCT	Reverse cholesterol transport
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SFS	STANISLAS family study
sIL-6R	Soluble IL-6R
SMCs	Smooth muscle cells
SNP	Single nucleotide polymorphism
STANISLAS	Suivi Temporaire Annuel Non Invasif de la Santé des Lorrains Assurés Sociaux
STAT	Signal transducer and activator of transcription
sTREM-1	Soluble triggering receptor expressed on myeloid cell 1
TC	Total cholesterol
TFBS	Transcription factor binding site
TG	Triglycerides
TNF-α	Tumor necrosis factor alpha
TREM	Triggering receptor expressed on myeloid cell
TREM-1sv	mRNA splicing variant giving the sTREM-1 protein
UTR	Untranslated region
VCAM-1	Vascular cell adhesion molecule 1
VEGF-A	Vascular endothelial growth factor-A
VNTR	Variable-number tandem repeats
VWF	Von Willebrand Factor
WHO	World health organization

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ORIGINAL ARTICLES

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PLoS One. 2017 Aug 3;12(8)

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CCLM (Clinical Chemistry and Laboratory Medicine). Accepted with minor changes.

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Drug Metab Pers Ther. 2017 May 24;32(2):119-127

Summary in French

SITUATION DU SUJET

Les maladies cardiovasculaires (MCV) représentent toujours aujourd'hui la principale cause de mortalité et de morbidité au niveau mondial : elles sont à l'origine d'environ 17,5 millions de décès par an selon les estimations de l'Organisation Mondiale de la Santé (OMS)(1). Parmi ces décès, 7,4 millions sont causés par des affections cardiaques coronaires et 6,7 millions par des accidents vasculaires cérébraux (AVC). Les MCV sont d'une étiologie complexe, et elles sont soumises à de nombreux facteurs environnementaux ainsi que génétiques. Pour pouvoir mieux comprendre leur pathophysiologie et réduire leur incidence et leur mortalité, l'étude des MCV prend en compte beaucoup de facteurs de risques différents en même temps, et leurs interactions (gène-environnement et gène-gène) (2, 3). Durant les 2 dernières décennies, de nombreuses études ont permis de diminuer significativement le taux de mortalité par des MCV dans des pays plus développés (4). Cette réduction a été possible grâce à une meilleure compréhension de la pathophysiologie des maladies complexes par l'identification de facteurs de risque, y compris des facteurs génétiques, ce qui a permis de détecter des individus à risque élevé de développer des MCV.

Malgré les succès obtenus, une stagnation a rapidement été atteinte ces dernières années, rendant nécessaire l'identification de nouveaux biomarqueurs comme nouvelles cibles d'intervention précoce. De nouvelles voies métaboliques telles que l'inflammation et l'adhésion ont ainsi été investiguées et associées à la physiopathologie cardiovasculaire et la voie génétique a été approfondie.

En effet, les facteurs de risque des MCV ont une composante génétique importante et les dernières percées technologiques dans le cadre du Projet 'Génome Humain' (5, 6), ou du Projet HapMap (7, 8) rendent aujourd'hui disponibles plus d'un million de variants génétiques ou single nucleotide polymorphisms (SNPs) qui ont permis de développer de nouvelles approches méthodologiques. Les études d'association pangénomiques ou genome-wide association studies (GWAS) et les études d'association panphénotypiques ou Phenome-wide association studies (PheWAS) ont révolutionné l'épidémiologie génétique de la dernière décennie. Ces approches exploratoires ont donc logiquement suscité de grandes attentes, car

elles ont permis d'identifier plusieurs milliers de SNPs inédits et significativement associés aux MCV et à leurs facteurs de risque. Par ailleurs, l'évolution des analyses de locus de caractères quantitatifs (QTL, quantitative trait loci) a permis la découverte de nouveaux effets pléiotropiques chez des SNPs qui ont déjà été associés avec des MCV. Les effets pléiotropiques montrent comment une même variante génétique peut avoir des effets dans plusieurs caractères phénotypiques. Toutes les nouvelles approches développées facilitent l'étude et la compréhension des voies métaboliques complexes. Elles ont aussi souligné les intérêts cliniques de nouveaux biomarqueurs et cibles pour la prévention des MCV ainsi que pour développer des médicaments.

Malheureusement la plupart des GWAS n'ont pas permis d'identifier des SNPs biologiquement ou cliniquement significatifs. Plusieurs chercheurs défendent l'idée selon laquelle la solution du problème est de faire des GWAS avec des populations et un nombre de SNPs encore plus grands. Néanmoins, cette solution peut être contre-productive dans quelques situations. En effet, pour réduire le nombre de faux positifs au maximum, la valeur limite de la signification statistique (p) utilisée par les chercheurs est très stricte dans les GWAS, et il y a un prix à payer pour cela : de nombreux polymorphismes, pourtant associés à des maladies et/ou facteurs de risque, ne passeront pas la barre de la significativité statistique et vont passer inaperçus pour les chercheurs.

Au lieu de faire des GWAS avec des populations plus grandes, cette thèse propose une approche différente, centrée sur des populations plus réduites mais phénotypiquement homogènes, avec l'idée de limiter au maximum l'hétérogénéité des phénotypes intermédiaires. En utilisant des populations homogènes, la puissance des analyses est majeure comparée aux populations plus hétérogènes, et ainsi plus facile de détecter les associations entre gènes et phénotypes intermédiaires. Par contre, quelques marqueurs génétiques sont significatifs dans des populations homogènes et peuvent ne pas être associés dans des populations génétiquement et environnementalement différentes. L'idée défendue ici est de pouvoir réduire l'incidence et la mortalité des MCV par le biais de la médecine personnalisée, ce qui devrait être la marche à suivre par les cliniciens et la communauté scientifique. En proposant des démarches de médecine personnalisée, et plus spécifiquement de médecine personnalisée basée sur des données génomiques, la communauté scientifique essaie d'individualiser la prévention, le diagnostic et le traitement du patient. Pour ce faire, il faut intégrer la génomique dans la pratique de la médecine. Il est clairement connu que le génome

de chaque personne est unique, tout comme la prédisposition à souffrir de maladies spécifiques, sans oublier les effets combinés des facteurs environnementaux et du mode de vie. Par conséquent, nous sommes allés plus loin que les approches génétiques traditionnelles, en essayant de comprendre les fonctions biologiques des polymorphismes étudiés et leurs effets pléiotropiques. En effet, la pléiotropie joue un rôle important en génétique, pour sa grande implication dans l'évolution des maladies complexes et leur prévention et traitement. Pour s'assurer que les variants de risques détectés sont biologiquement ou cliniquement significatifs, il est nécessaire de démontrer leur expression génétique. Il est aussi important de détecter des nouvelles voies métaboliques, plutôt que d'associer les polymorphismes justes avec les maladies. C'est seulement en faisant cela que l'on pourra utiliser les variants de risque comme cibles thérapeutiques pour traiter les MCV.

Afin d'accomplir ceci, nous avons étudié des gènes avec des effets pléiotropiques qui pourraient avoir une utilité en médecine personnalisée comme des marqueurs diagnostiques, pronostiques et de prévention. Nous avons d'abord réuni les résultats déjà obtenus pour des GWAS précédents et nous avons continué avec des approches complémentaires. Nous nous sommes concentrés sur la relation des polymorphismes localisés dans des gènes d'intérêt et des phénotypes intermédiaires spécifiques qui sont associés aux MCV. Cette approche peut donner une idée plus détaillée sur l'impact des polymorphismes d'intérêt sur les voies métaboliques et comment ils vont finalement être associés à l'évolution des MCV.

Au cours de cette thèse, nous avons mis en pratique des approches explicatives et nous avons centré le sujet plus particulièrement sur les gènes inflammatoires qui sont vraisemblablement impliqués dans les MCV. En fait, pendant les dernières années, les chercheurs ont mis en évidence de nouveaux facteurs de risque des MCV, et notamment l'inflammation qui a pris un rôle central. Les MCV sont considérées, de plus en plus, comme des maladies inflammatoires chroniques. L'inflammation participe à toutes les étapes essentielles des MCV, et principalement dans l'initiation et la progression de l'athérosclérose (9). Les molécules inflammatoires impliquées facilitent le recrutement et la migration transépithéliale des lymphocytes et monocytes, qui en même temps participent à le maintenance locale d'une réponse inflammatoire chronique (10). Ils augmentent, aussi, l'absorption des lipoprotéines de basse densité (LBD) effectuées par les macrophages, et ils promeuvent la migration et la prolifération des cellules musculaires lisses (CML)(11), ce qui va accélérer et aggraver la progression des MCV. Bien que beaucoup d'avancements aient été faits dans l'implication de

l'inflammation dans les MCV, il y a encore un grand manque de connaissance à combler, principalement concernant le composant génétique.

Pendant les trois dernières années, nous avons utilisé différentes populations d'origine française qui sont disponibles au Centre de Ressources Biologiques de notre groupe d'investigation (UMR INSERM U1122 ; Interactions Gène-Environnement en Physiopathologie Cardiovasculaire), avec un grand nombre de phénotypes disponibles, incluant des ADN, ARN et extraits lymphocytaires. La principale population utilisée est la cohorte STANISLAS (SFS, Stanislas Family Study), regroupant 1006 familles d'origine Lorraine. Ces familles sont composées de deux parents ayant au moins deux enfants dont les données à la fois socio-économiques, biologiques et cliniques ont été recueillies. Nous avons aussi collaboré avec d'autres groupes de recherche et avec une entreprise (INOTREM), dans l'optique de pouvoir appliquer nos résultats et découvertes à la médecine personnalisée.

OBJECTIFS

Nous avons poursuivi quatre objectifs majeurs :

1- La validation des polymorphismes qui ont été associés précédemment aux MCV par des GWAS, mais dont les applications au niveau clinique n'ont pas été démontrées.

2- L'étude des effets pléiotropiques que les polymorphismes d'intérêt ont vis à vis d'autres phénotypes intermédiaires impliqués dans les MCV, avec un intérêt particulier sur les gènes inflammatoires.

3- La validation au niveau biologique des polymorphismes étudiés et des associations découvertes en menant des études d'association gène-protéine, ainsi qu'en utilisant des analyses transcriptomiques et des approches bioinformatiques.

4- Participer à la contribution de la médecine personnalisée des MCV en essayant d'appliquer les résultats obtenus en clinique.

RESULTATS

Publication N°1

Le SNP rs2234246 du gène TREM-1 régule l'expression de l'ARNm et de la protéine TREM-1 et est associé aux taux plasmatiques de L-sélectine

Alex-Ander ALDASORO ARGUINANO[1], Sébastien DADÉ[1], Maria STATHOPOULOU[1], Marc DERIVE[2] , Ndeye COUMBA NDIAYE[1], Ting XIE[1], Christine MASSON[1], Sébastien GIBOT[3], Sophie VISVIKIS-SIEST[1,4]

PLoS One. 2017 Aug 3;12(8)

Des taux élevés de la protéine TREM-1 sont associés à un risque accru de MCV et inflammatoires. Des études récentes ont montré que la délétion ou blocage du TREM-1 est associé à une réduction allant jusqu' à 60% de la progression de l'athérosclérose. Jusqu'à présent, il n'existait aucune donnée quant à une éventuelle régulation génétique du taux de la protéine TREM-1. Par ailleurs, il a été suggéré que les récepteurs de la famille TREM régulent le processus d'adhésion cellulaire.

L'objectif de cette étude était de déterminer si les polymorphismes situés dans le gène TREM-1 régulent les taux sériques de TREM-1 et le niveau d'expression de l'ARNm correspondant. De plus, nous avons cherché à identifier des associations entre les polymorphismes de TREM-1 et les taux des différentes sélectines dans le sang.

Parmi les 10 SNPs étudiés, nous avons démontré que l'allèle mineur T de rs2234246 est associé à une augmentation du taux de sTREM-1 dans la population de détection initiale ($n = 30$; $p = 0,003$), et à une expression plus élevée de l'ARNm de TREM-1 ($n = 30$; $p = 0,007$). Ce même allèle est également associé à des taux plus élevés de L-sélectine soluble ($n = 351$; $p = 0,011$). Ces concentrations plus importantes de sTREM-1 et de L-sélectine sont également observées dans la population de réplication ($n = 80$; $p = 0,0007$ et $n = 80$; $p = 0,018$ respectivement).

Nous avons démontré pour la première fois qu'un SNP du gène TREM-1 a un effet sur le niveau d'expression de ce même gène. Ces résultats originaux viennent étayer l'hypothèse selon laquelle TREM-1 a un effet sur les processus d'extravasation et d'accumulation des

monocytes qui entraînent l'athérogenèse et la progression de la plaque d'athérosclérose, peut-être en favorisant l'inflammation et donc une expression plus élevée de sL-sélectine.

Publication N°2

L'haplotype rs4845625*T/rs4537545*C d'IL6R est un facteur de risque augmentant d'une façon concomitante les taux de CRP, de LDL et d'ApoB

Alex-Ander ALDASORO ARGUINANO[1], Elnaz NADERI*[1,2], Ndeye Coumba NDIAYE*[1], Maria STATHOPOULOU[1], Sébastien DADE[1], Behrooz ALIZADEH[1, 2], Sophie VISVIKIS-SIEST¥[1, 3]

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Le récepteur de l'interleukine 6 (IL-6R), dont l'activation provoque les réponses biologiques associées à l'IL6, joue un rôle important dans plusieurs maladies telles que le diabète, l'obésité et les MCV. Nous avons étudié les effets de deux SNPs du loci IL-6R, rs4845625 et rs4537545, rapportés lors d'études antérieures comme étant associés à une élévation du taux de CRP et à un risque de coronaropathie, et ayant des effets controversés sur les traits lipidiques.

Les individus de la cohorte STANISLAS ont été utilisés comme population principale (N = 368), et les résultats ont été répliqués dans une autre population indépendante d'origine française (N = 995).

Nos résultats montrent que les deux SNPs ont un effet antagoniste sur le taux de CRP : l'allèle mineur rs4845625*T est associé à une élévation du taux de CRP (p = 0,011), alors que l'allèle mineur rs4537545*T est associé à une baisse du taux de CRP (p = 0,009). Fait intéressant, l'allèle mineur rs4845625*T est également associé de façon significative à une élévation des taux de LDL-C et d'ApoB (p = 0,007 et p = 0,009 respectivement). L'analyse des haplotypes a démontré que l'haplotype TC, avec l'allèle mineur rs4845625*T, est associé à la fois à des taux élevés de CRP, de LDL-C et d'ApoB, et peut donc être considéré comme un facteur de risque.

Nous avons donc mis en évidence pour la première fois un effet indépendant du polymorphisme rs4845625 sur les taux de LDL-C et d'ApoB, ce qui permet d'expliquer une partie non négligeable de la variabilité de ces traits (3,49 % et 5,57 % respectivement). La portée clinique de ces résultats pourrait être considérable pour les études pharmacogénomiques sur le tocilizumab dont la cible est l'IL-6R.

Publication N°3

La pléiotropie du gène ABO ; les effets du rs644234 sur les niveaux de E-sélectine et les phénotypes lipidiques.

Alex-Ander ALDASORO ARGUINANO[1], Ndeye Coumba NDIAYE[1], Christine MASSON[1], Sophie VISVIKIS-SIEST[¥1, 2]

CCLM (Clinical Chemistry and Laboratory Medicine). Accepté avec modifications.

Le gène ABO a été largement étudié et sa relation avec différentes maladies, y compris l'infarctus du myocarde, le cancer et le diabète, a été confirmée par de nombreuses études. Les effets pléiotropiques du gène ABO ont aussi été démontrés, ce dernier affectant différents phénotypes comme les E et P-sélectines, les triglycérides et le cholestérol total. L'objectif de ce travail est d'étudier les effets du SNP rs644234, localisé dans le gène ABO, avec différents phénotypes associés avec des maladies ou le gène ABO a également été associé.

Nous avons analysé le SNP rs644234 localisé dans le gène ABO, en menant des études d'association avec différents phénotypes lipidiques et la E-sélectine soluble, chez 348 adultes de la cohorte STANISLAS.

L'allèle majeur T du rs644234 était associé avec une augmentation des niveaux soluble de E-sélectine (valeur p= $8.7x10^{-12}$). Selon les phénotypes lipidiques, l'allèle majeur T du rs644234 était associé avec une diminution des niveaux de ApoE (valeur p= 0.001) et de LBD (valeur p= 0.032), alors qu'il était associé avec une augmentation de niveaux de lipoprotéines de haute densité ou LHD (valeur p= 0.013). Cette association avec le LHD était seulement significative chez les hommes (valeur p= 0.001). Nous n'avons pas trouvé d'associations spécifiques au genre pour les autres phénotypes étudiés.

Nous avons confirmé que le gène ABO était un locus majeur pour la variabilité des niveaux de E-sélectine soluble, et nous avons aussi confirmé qu'il affecte les niveaux lipidiques. En outre, nous avons démontré que les effets pléiotropiques sont indépendants entre eux. C'est la première fois qu'une association a été confirmée entre le gène ABO et le taux d'ApoE. L'allèle majeur T de rs644234 a un rôle protecteur vis à vis des MCV.

Publication N°4

Le polymorphisme rs6918289 localisé dans la région génétique de TREM-2 est associé avec les taux de TNF-α et avec l'épaisseur intima-média de l'artère fémorale.

Alex-Ander Aldasoro Arguinano[1], Vesna Gorenjak[1], Maria Stathopoulou[1], Dwaine R.Vance[3], Christine Masson[1], Sophie VISVIKIS-SIEST[¥1, 2]

Soumis dans Scientific Reports.

La protéine TREM-2 est connue pour ses caractéristiques anti-inflammatoires pendant la réponse immunitaire et influence négativement les niveaux d'expression de TNF-α. Les études génétiques ont associé les polymorphismes localisés dans le gène TREM-2 avec des maladies neurodégénératives et des maladies inflammatoires chroniques. Il a été démontré que les niveaux de TREM-2 n'affectent pas seulement les taux de TNF-α, mais aussi la stabilité de la plaque athéromateuse chez des patients avec une sténose carotidienne.

Dans cette étude, nous avons examiné des polymorphismes localisés dans la région du gène TREM-2 et leurs relations avec le taux de TNF-α ainsi qu'avec l'épaisseur intima-média de l'artère fémorale. Les individus de la cohorte STANISLAS étaient utilisés comme population principale (N = 808), et les résultats étaient répliqués dans une autre population indépendante d'origine française (N = 915).

Après avoir analysé chez les enfants de la cohorte STANISLAS un total de 5 SNPs dans la région génétique de TREM-2, il s'est avéré que seul le SNP rs6918289 était associé avec un taux élevé de TNF-α (p-value=0.0003). Plus tard, nous avons montré que l'allèle mineur T de rs6918289 était aussi associé avec une augmentation de TNF-α dans toute la cohorte STANISLAS (p-values= 0.0026). Les résultats étaient répliqués dans une autre population indépendante (p-value= 0.023). Finalement, le même allèle était associé avec une

augmentation de l'épaisseur intima-média de l'artère fémorale dans la Cohorte STANISLAS (p-value= 0.026).

Les résultats de ce travail suggèrent que l'allèle mineur T de rs6918289 peut être considéré comme un facteur de risque et être utilisé chez les patients souffrants de maladies inflammatoires chroniques, comme l'athérosclérose.

Publication N°5

Le polymorphisme associé avec des maladies cardiovasculaires GNB3 C825T a des effets spécifiques selon le genre sur les taux de E-sélectine soluble

Kokoè Mélinda GBADOE, Nazha BERDOUZI, Alex-Ander ALDASORO AGUIÑANO, Ndeye Coumba NDIAYE[§], Sophie VISVIKIS-SIEST[§]

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Le polymorphisme C825T (rs5443) du gène Guanine Nucleotide-Binding protein subunit β3 (GNB3) a été associe avec l'obésité, l'hypertension artérielle, l'athérosclérose, les maladies coronaires et a quelques effets spécifiques selon le genre. Les associations avec des médiateurs inflammatoires comme les molécules d'adhésion cellulaires n'ont pas été étudiées, même s'ils sont fortement concernées dans la pathophysiologie des MCV. L'objectif de cette étude était d'explorer les possibles associations spécifiques au genre du polymorphisme rs5443 chez les différentes molécules solubles d'adhésion cellulaire, comme la E, P et L-sélectine (sE, sP et sL-sélectines).

Dans la population totale issue de la cohorte STANISLAS (N = 771), nous n'avons pas trouvé d'associations significatives entre le SNP rs5443 et les concentrations sériques de sE, sP et sL-sélectines. Néanmoins, une association significative et spécifique au genre a été trouvée entre le rs5443 et la sE-sélectine ($p<0.001$), mais pas pour sP et sL-sélectines. Après avoir ajouté les covariables, l'allèle T était associé de façon significative avec une augmentation des niveaux de sE-sélectine chez les hommes ($\beta=5.03$; $p=0.020$), alors qu'associé avec une diminution significative des niveaux de sE-sélectine chez les femmes ($\beta= -4.46$; $p=0.030$).

Dans notre population, le polymorphisme rs5443 du gène GNB3 a donc des effets spécifiques au genre pour le taux de E-sélectine soluble, avec un effet défavorisant chez les hommes, puisque des taux élevés de sE-sélectine ont été associés avec les MCV. Le lien observé entre ce polymorphisme et la E-sélectine permet d'avoir une meilleure compréhension des effets de rs5443 dans les MCV.

CONCLUSIONS ET PERSPECTIVES

Cette thèse avait comme principal objectif l'étude des effets des gènes d'intérêts sur différents phénotypes intermédiaires connus pour avoir été associés avec des MCV ainsi qu'avec d'autres maladies complexes. Nous avons aussi centré nos efforts dans une possible traduction des résultats obtenus dans l'application clinique. Nous pensons que la médecine personnalisée basée sur des données génomiques est une approche qui a de l'importance car elle peut réduire la mortalité causée par les MCV, principales causes de mortalité et de morbidité au niveau mondial (1).

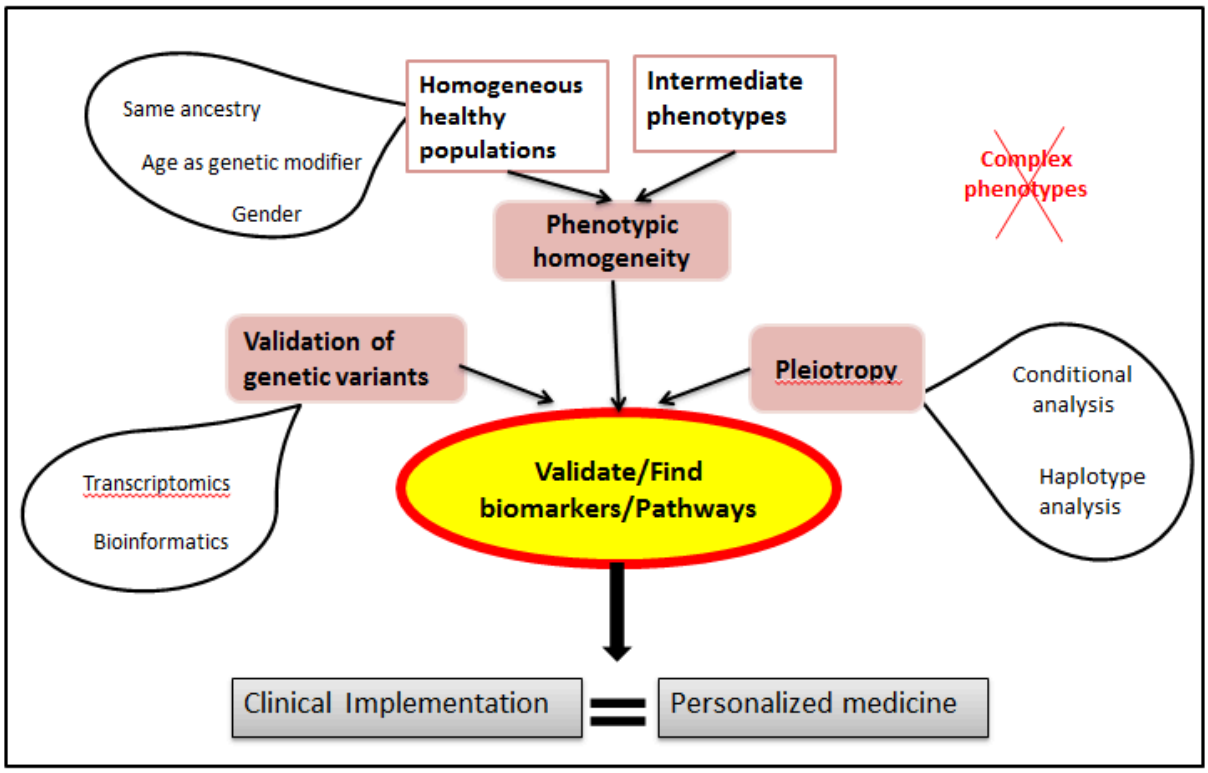
Les approches exploratoires GWAS, PheWAS, QTL et expression QTL (eQTL) ont révolutionné l'épidémiologie génétique des maladies complexes lors de la dernière décennie (12-14) et ont donné beaucoup de renseignements précieux sur la génétique des maladies complexes. Toutefois, 10 ans après la première GWAS (15, 16), il s'avère que les résultats ne sont pas à la hauteur des espérances.

Ces nouvelles approches (GWAS, PheWAS, eQTL…) étaient supposées résoudre la complexité des maladies multifactorielles, mais cela n'a finalement pas été le cas. Des GWAS antérieures ont mis en évidence des milliers de polymorphismes et gènes associés avec différents phénotypes reliés aux MCV. Néanmoins, considérés dans leur ensemble, tous ces gènes expliquent seulement une fraction de l'héritabilité des MCV (17). Ces résultats, insatisfaisants, étaient à l'origine du concept d'héritabilité manquante (18). En fait, les résultats obtenus à partir des GWAS ont une application clinique, jusqu'à présent, très limitée. Malheureusement, la façon dont ces polymorphismes affectent les mécanismes biologiques de ces maladies complexes est encore largement inconnue.

Pendant cette thèse, nous avons utilisé des méthodes alternatives au-delà des GWAS classiques pour détecter les mécanismes qui sont impliqués dans les MCV et autres maladies

complexes (Figure 1). Tout d'abord, nous avons choisi des polymorphismes précédemment associés dans d'autres GWAS et nous avons étudié les phénotypes intermédiaires plutôt que les phénotypes/maladies plus généraux. Nous avons aussi utilisé des populations les plus homogènes possibles et nous avons centré nos efforts pour étudier les effets pléiotropiques et gène-environnement des gènes sélectionnés. En utilisant cette approche, nos résultats ont montré de nouvelles associations entre les gènes et les phénotypes intermédiaires qui sont associés avec les maladies complexes.

Figure 1: Schéma résumant les approches et objectifs de cette thèse.

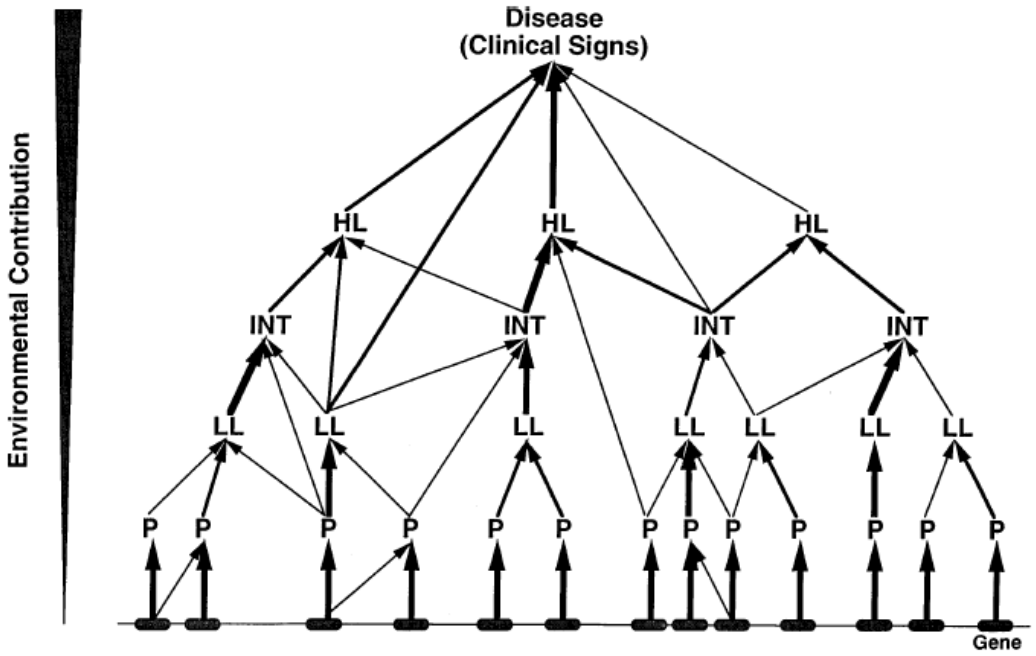


La principale population utilisée était la cohorte STANISLAS, regroupant des familles nucléaires d'origine Lorraine et considérées en bonne santé (sans maladie chronique). Ainsi, nous avons augmenté significativement l'homogénéité de la population, pas seulement au niveau génétique, mais aussi au niveau environnemental. Même si les populations homogènes peuvent faciliter la détection de variants génétiques associés avec des phénotypes intermédiaires, les polymorphismes détectés pourraient avoir beaucoup moins d'effets (ou ne pas en avoir d'effets) dans les autres populations qui sont génétiquement et

environnementalement différentes. Malgré ce désavantage nos résultats ont aidé à découvrir les voies métaboliques et les mécanismes pathophysiologiques où les gènes étudiés sont impliqués. Cette information pourrait être utilisée sur d'autres populations ou champs d'investigation (19). Il serait d'un grand intérêt d'utiliser spécifiquement des individus jeunes de la cohorte STANISLAS, comme nous l'avons déjà fait pendant la première étape de l'étude de TREM-2. Effectivement, l'effet environnemental sur les phénotypes des enfants est moins élevé car leur exposition est moindre comparée aux adultes.

Le fait d'utiliser des phénotypes intermédiaires pour étudier les effets des polymorphismes nous a aidé à découvrir de nouvelles associations impliquées dans les MCV. Le parcours depuis les gènes jusqu'à la maladie est très complexe (Figure 2). Il est beaucoup plus facile de connecter le gène et les phénotypes intermédiaires situés dans la partie inférieure de la pyramide exposée en Figure 2 (montré comme P, LL et INT). En fait, si le phénotype est plus proche du gène, il y aura moins de gènes et autres facteurs qui vont l'influencer. Par ailleurs, les phénotypes qui sont situés dans la partie inférieure de la hiérarchie métabolique et physiologique, seront moins susceptibles aux effets environnementaux (20). Cette approche facilite la détection des associations gène-phénotype réel, ainsi que celles des voies métaboliques impliquées. Quelques GWAS essaient de lier directement les gènes avec les maladies, par contre, dans certains cas, et surtout dans les maladies complexes comme les MCV, les effets des gènes obtenus sont très faibles et il est difficile d'évaluer les résultats obtenus.

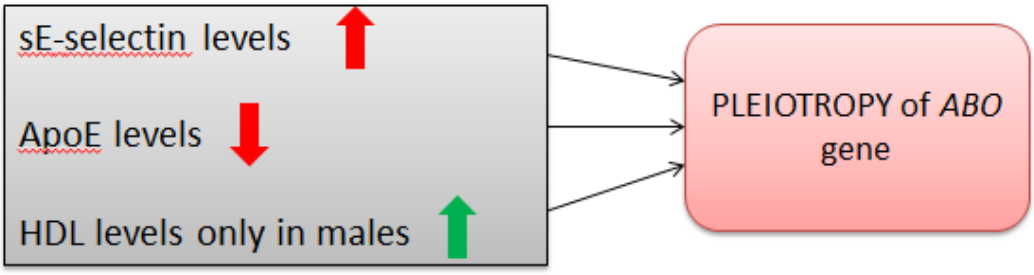
Figure 2: Diagramme de la relation des gènes avec leurs phénotypes, jusqu'aux signes cliniques de la maladie.



Source: Genetics of Complex Disease; Schork.

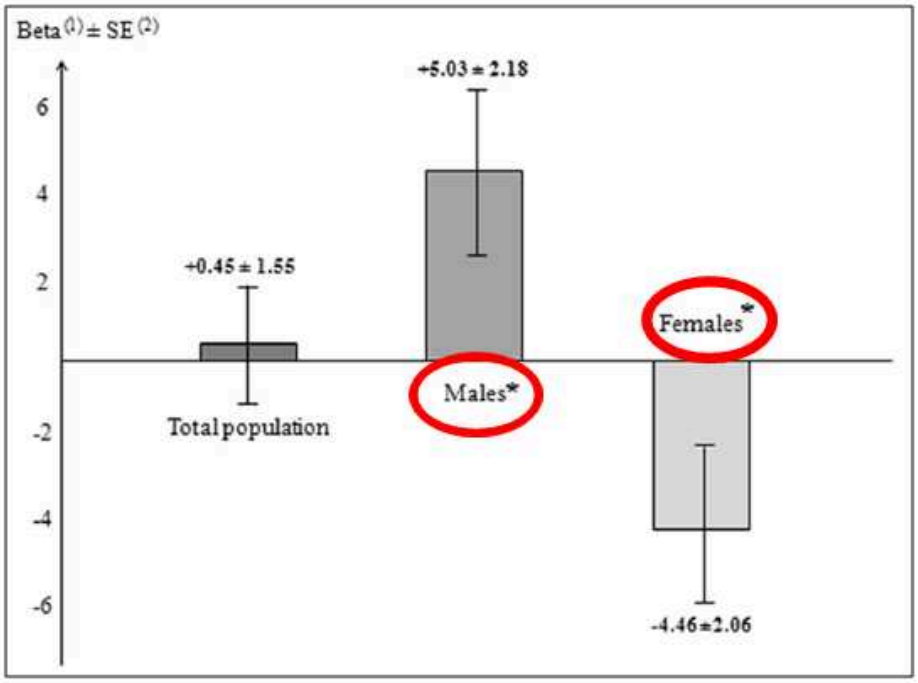
Pendant cette thèse, nous avons aussi détecté les effets pléiotropiques de différent gènes (gènes IL-6R, et ABO). Concernant le gène IL-6R, nous avons trouvé une pléiotropie antagoniste pour les phénotypes lipidiques et les niveaux de CRP. Nous avons aussi trouvé un nouvel effet pléiotropique dans le gène ABO, où nous avons associé d'une façon indépendante l'allèle majeur T de rs644234 avec différents phénotypes (Figure 3). La détection de la pléiotropie dans les gènes, démontrent indirectement qu'ils sont impliqués dans plusieurs processus biologiques, en distribuant leurs produits protéiques dans les différents composants cellulaires et par les interactions protéine-protéine (21).

Figure 3 : Pléiotropie de l'allèle majeur T de rs644234 localisé dans le gène ABO.



De plus, nous avons étudié les effets genre-gène, en utilisant le genre comme un possible modificateur génétique. Nous avons trouvé quelques associations intéressantes dans les gènes étudiés (ABO and GNB3) qui sont spécifiques au sexe. Le polymorphisme étudié dans le gène ABO était associé avec une augmentation du LHD, mais seulement chez les hommes. D'autre part, le polymorphisme rs5443 dans le gène GNB3 était lui associé avec les deux genres pour les niveaux de E-sélectines solubles, mais d'une façon antagoniste (Figure 4). Chose curieuse, dans la totalité de la population, l'association de ce polymorphisme avec les niveaux de sE-sélectine n'était pas significative. Ce fait souligne l'importance de tenir compte du genre quand nous faisons des analyses avec l'objectif de détecter de nouveaux marqueurs de risque des MCV.

Figure 4: Les effets Beta du rs5443 dans le gène GNB3 sur les niveaux de sE-sélectine.



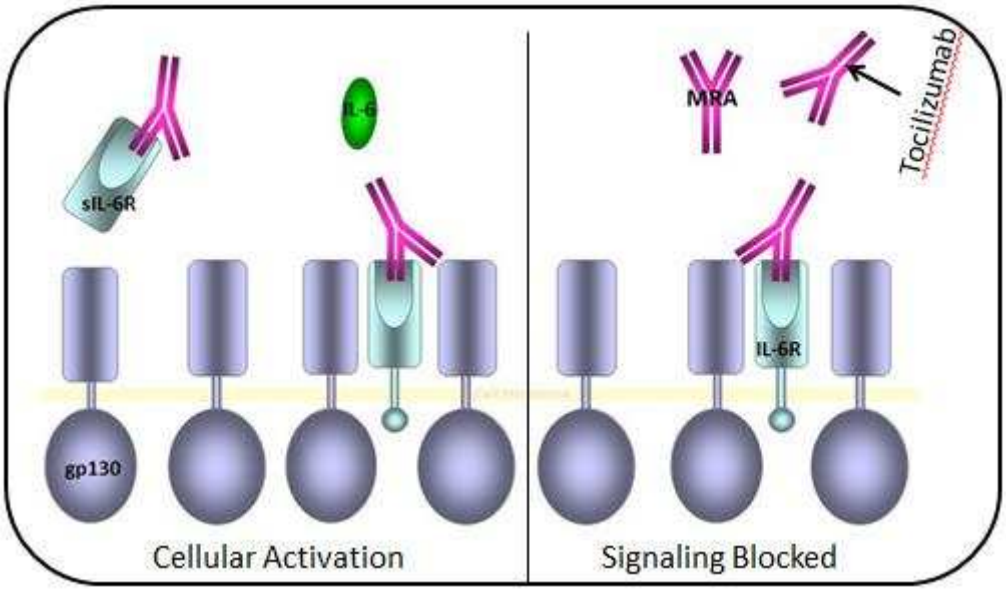
*P-value ≤ 0.05 (niveau significatif)

En détectant la pléiotropie et les effets genre-spécifiques, on comprend mieux les voies métaboliques où le gène est impliqué. Ceci est à l'origine de nouvelles hypothèses, et favorise l'évolution de nouveaux médicaments. Cela a également pour conséquence d'améliorer la prévention et la médecine personnalisée dans les MCV.

De plus, nous avons centré nos efforts dans l'implémentation des connaissances obtenues pendant les trois dernières années au niveau clinique, avec l'objectif d'améliorer la médecine personnalisée basée sur des données génomiques. Nous avons obtenu deux résultats qui peuvent être appliqués dans la clinique. Le premier exemple d'application clinique concerne le polymorphisme rs2234246 dans le gène TREM-1. Ce variant génétique, qui augmente les taux solubles de la protéine TREM-1, pourrait être utilisé comme un prédicteur ou un marqueur de risque pour différentes maladies (MCV inclues). En fait, et grâce au grand potentiel de ce polymorphisme, nous avons déposé un brevet Européen avec nos collaborateurs (entreprise INOTREM et hôpital de Nancy) et nous envisageons de mener des essais cliniques de phase 1 chez les patients souffrant de choc septique et d'infarctus aigu du myocarde.

Le deuxième résultat obtenu qui pourrait avoir une valeur importante dans les études pharmacogénomiques, et pourrait être utilisé aussi dans la médecine personnalisé concerne l'haplotype rs4845628*T/rs4537545*C du gène IL6R. Cet haplotype, est associé à la fois à des taux élevés de CRP, de LBD et d'ApoB. Le produit pharmaceutique tocilizumab est un agent immunosuppresseur dont la cible est le récepteur IL-6R (Figure 6). Il est utilisé de façon répandue pour traiter l'arthrite rhumatoïde. Il a comme effet de réduire les taux de CRP, mais un des effets indésirables du tocilizumab est une augmentation du taux de LBD chez les patients, ce qui pourrait augmenter le risque de cardiopathie ischémique (22). Afin d'éviter les effets indésirables, les patients qui ont l'haplotype rs4845628*T/rs4537545*C, pourraient être traités par des produits pharmaceutiques analogues ou différents.

Figure 6: Blocage de la voie métabolique du IL-6R par Tocilizumab



En suivant les objectifs et approches synthétisés ci-avant, nous avons découvert, ces trois dernières années, de nouvelles associations entre gènes et phénotypes intermédiaires qui sont impliquées dans les MCV et d'autres maladies complexes (23, 24). Nos résultats aident à mieux comprendre comment les gènes étudiés exercent leurs effets au niveau moléculaire, en influant finalement sur l'état des patients souffrant du MCV. Nous espérons que nos résultats vont être pris en compte pour améliorer la médecine personnalisée.

FOREWORD

Cardiovascular diseases (CVD) are the main causes of death and disability worldwide, causing an estimated 17.5 million deaths per year according to the world health organization (WHO) (1). Among those deaths, 7.4 million were caused by coronary heart diseases (CHD) and 6.7 million were due to strokes. Thanks to numerous epidemiological studies, during the past two decades, the number of deaths related to CVD has decreased in high-income countries, including Europe (4). This reduction has been possible through a better understanding of the pathophysiology of these complex diseases and the identification of risk factors, including genetic factors, which allowed the detection of individuals with high risk of suffering from CVD.

Despite these achievements, the reduction of the CVD death rates has reached a plateau, showing to the scientific community that there is still a big room of knowledge waiting to be discovered in the pathophysiology of CVD, and more particularly within the genetic background of these diseases. Innovative investigation methodologies leading to the identification of novel risk biomarkers are needed in order to propose new prevention and treatment strategies and reduce the incidence and mortality from CVD.

For instance, biomarkers involved in the inflammatory state appear to be of particular relevance, as the preliminary results showed the central role that the inflammatory genes have in the early outcome and development of CVD. Because of this, CVD are considered increasingly as chronic inflammatory diseases (9, 25). Focusing on these novel risk biomarkers with a personalized genome-based medicine approach have thus opened a window of opportunity to explore in more depth the etiology of these diseases in order to develop individualized prevention, diagnosis and treatments.

The development of Genome-wide association studies (GWAS) (26) and Phenome-wide association studies (PheWAS) (27) allowed the discovery of many single nucleotide polymorphisms (SNPs) and intermediate phenotypes associated with CVD. This facilitated the identification of individuals at risk of suffering from these diseases and detected metabolic pathways and genes previously unsuspected to be involved in CVD. Indeed the study of complex diseases where many genetic, environmental and lifestyle factors are involved was becoming more and more difficult until the development of these approaches. These approaches will make easier the implementation of the genome-based personalized medicine within the complex diseases.

The clinical implementation of specific polymorphisms conferring risk for certain complex diseases is becoming a reality. As an example, in several types of cancer, the use of this personalized medicine is becoming more and more common, and individuals are being treated differently, depending on the specific genetic mutations of their cancer cells. The management of other diseases, including CVD, rheumatoid arthritis and infectious diseases is also moving towards a more personalized treatment. However a big room of improvement remains in these fields.

In this thesis, we focused primarily on inflammatory genes that are suspected to have pleiotropic effects on other intermediate phenotypes involved in CVD than the targeted inflammatory and adhesion molecules such as lipid levels. We also focused on genes involved in complex diseases that have the potential of being used in future personalized medicine approaches. In order to achieve this, we selected and genotyped a list of SNPs that capture the majority of the genetic variation on our genes of interest, and we performed association studies by further validating the involvement of these polymorphisms not only by gene-protein relations, but also by using transcriptomic and bioinformatic approaches.

In order to develop this work, we used several populations of French origin that are available in the Biological Resource Center of our research group (UMR INSERM U1122; Interactions Gène-Environnement en Physiopathologie Cardiovasculaire), where DNA, RNA, lymphocyte extracts and a variety of phenotypes of interest were already available. The main population used was the STANISLAS Family Study (SFS), a longitudinal cohort composed by 1,006 families (two parents with at least two children) in an apparently good health. SFS was specifically designed to investigate factors related to CVD. We also collaborated with other investigation groups as well as with a company (INOTREM), with the aim of translating our results and discoveries to clinical application and personalized medicine.

CHAPTER I

HYPOTHESIS AND OBJECTIVES

CVD are complex diseases where many environmental and genetic factors take part. Due to this, their study needs to take into account many risk factors at the same time and the interactions between them (gene-environment and gene-gene interactions) in order to better understand their pathophysiology, and reduce their incidence and mortality (2, 3).

The breakthroughs of the 'Human Genome' (5, 6) or the HapMap projects (7, 8) permitted the study of the genetic background of complex diseases by developing new approaches, built on millions of SNPs available to date. GWAS and PheWAS have revolutionized the genetic epidemiology within the last decade and these exploratory approaches brought tremendous expectations. They helped to identify thousands of novel SNPs and different intermediate phenotypes that are statistically associated to CVD and related risk factors. In fact, the development of quantitative trait locus (QTL) mapping made available large data sets that associated previously known SNPs involved in CVD and showed the pleiotropy of some of the genes involved in these diseases. Although the phenomenon of pleiotropy was first noticed 100 years ago, few empirical data were available until the QTL mapping became a reality. All these new technologies facilitate the study and the understanding of complex metabolic pathways and highlight the clinical interest of new biomarkers and targets for preventive tools and drug development.

Unfortunately, many of the GWAS failed to identify clinically and biologically significant associations, pushing some researchers to argue that the solution to this problem is to perform even larger GWAS involving bigger populations and more polymorphisms. However, this solution can be counterproductive in some cases. Indeed, in order to reduce the false positives, the demanding significance cut-off used by many researchers in the GWAS are very strict and there is a price that must be paid for this: many polymorphisms that are actually related to the disease of interest don't reach the cut-off value and pass unperceived to the eyes of the researchers.

During this thesis, instead of performing GWAS with more individuals, we propose a different approach. We focused on using smaller but more homogeneous populations, in order to limit as much as possible the heterogeneity of intermediate phenotypes. Also, we defend that implementation of the personalized medicine might be the goal that clinicians and scientific community need to pursue for reducing the incidence and mortality of complex diseases, including CVD. By doing personalized medicine, and more specifically personalized genome-based medicine, the scientific community tries to individualize prevention, diagnosis

and treatment, by integrating genomics into medical practice. It is known that the genome of each individual is unique, thus, the predispositions for suffering specific diseases are depending on each individual's lifestyle and environment, combined with its genome. Consequently, we went further than traditional genetic studies by trying to understand the biological function of the studied variants and their pleiotropic effects. Indeed, pleiotropy plays a central role in genetics, having broad implications in the development of complex diseases and their prevention and treatment. In order to take full advantage of the detected risk variants, it is necessary to link genetic associations to specific gene expression along with describing novel biological pathways, instead of only associating them with diseases or intermediate phenotypes. Only by doing so could we use those risk variants as targets for treatment of complex diseases.

In order to achieve this, we studied genes with pleiotropic effects that could be used in personalized medicine as markers for prevention, diagnosis and prognosis. We first gathered the results already obtained in GWAS and we proceeded with complementary approaches, by focusing on the relation of the polymorphisms located in these genes of interest and specific intermediate phenotypes that are associated with CVD. This approach could give us a more detailed idea about how the polymorphisms of interest are affecting the pathways that are ultimately involved in CVD development. Indeed, the complex disease genes probably exert their effects by slightly changing the production of specific proteins involved in the biological pathways.

In this thesis, we put in practice the approach explained above by centring more in the inflammatory genes that are presumably involved in CVD. Indeed, during the recent years, investigators have pointed out novel risk factors underlying CVD and among them inflammation has taken a central role, as CVD are nowadays considered as chronic inflammatory diseases. Actually, inflammation mediates all the key steps of CVD and mainly atherosclerosis, from initiation through progression (9). The inflammatory molecules involved facilitate the recruitment and trans-epithelial migration of lymphocytes and monocytes, which participate in the maintenance of a local chronic inflammatory response (10). They also increase the absorption of low density lipoprotein (LDL)-cholesterol by macrophages and promote the migration and proliferation of the smooth muscle cells (SMCs) (11), accelerating and worsening the CVD development. Inflammatory markers are gaining importance in the prediction of the outcome of patients suffering CVD, and although many advances are done,

there is still a big lack of knowledge, mainly concerning the genetic component of those diseases.

We have followed four principal objectives during this thesis:

1- To validate polymorphisms that have been previously associated with CVD in GWAS but investigators failed to explain their clinical applicability.

2- To study the pleiotropic effects that these polymorphisms of interest have on other intermediate phenotypes known to be involved in CVD, having a special focus on inflammatory genes.

3- To biologically validate the polymorphisms studied and the associations found by performing gene-protein association studies, as well as using transcriptomic and bioinformatic approaches, in order to arise the clinical interest of the new biomarkers detected.

4- To try to translate our results in clinical practice with the objective of accelerating and ameliorating the personalized genome-based medicine in CVD.

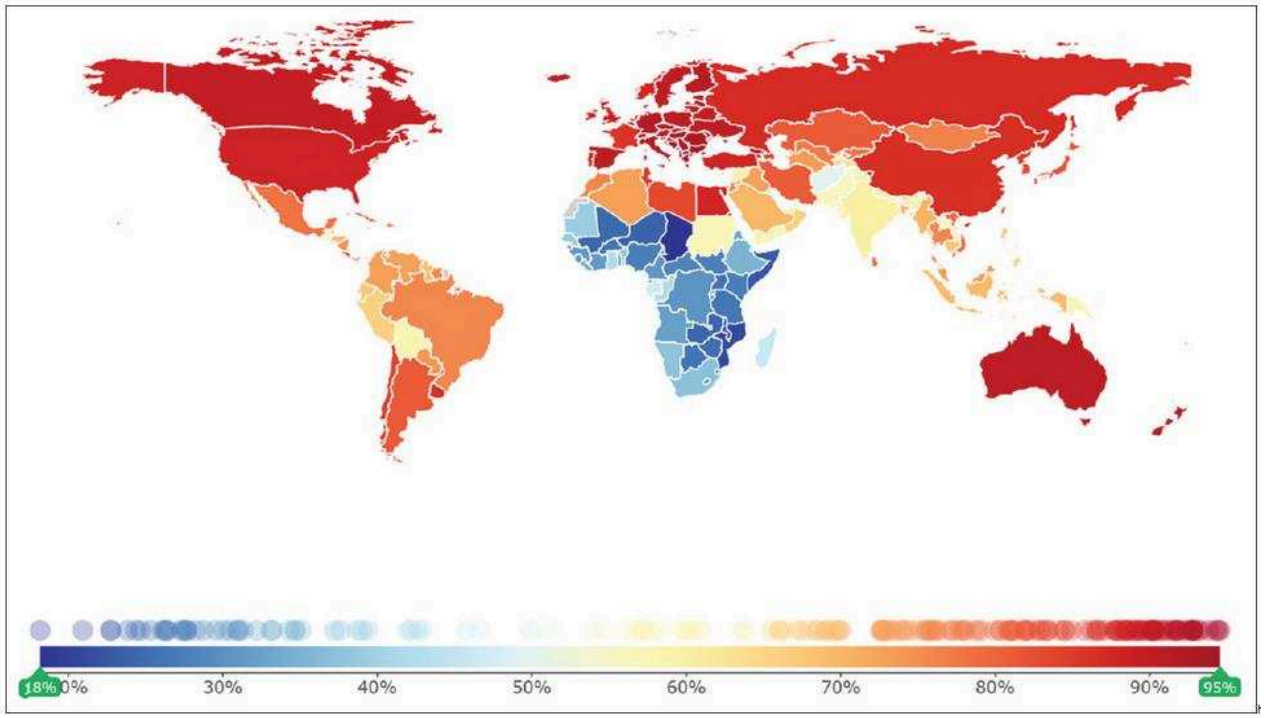
CHAPTER II

INTRODUCTION

1. Epidemiology of cardiovascular diseases

CVD are considered complex non-communicable diseases (NCD), as they are caused by a combination of multiple genes, behavior, physiological and environmental factors. CVD account for most NCD-related deaths annually (17.7 million people), followed by cancers (8.8 million), respiratory diseases (3.9 million), and diabetes (1.6 million) according to the WHO. All together, these diseases are responsible for 40 million deaths each year, representing 70% of all deaths globally, up from 60% in 2000 (Figure 1).

Figure 1: Percentage of deaths from non-communicable diseases by country, 2013.



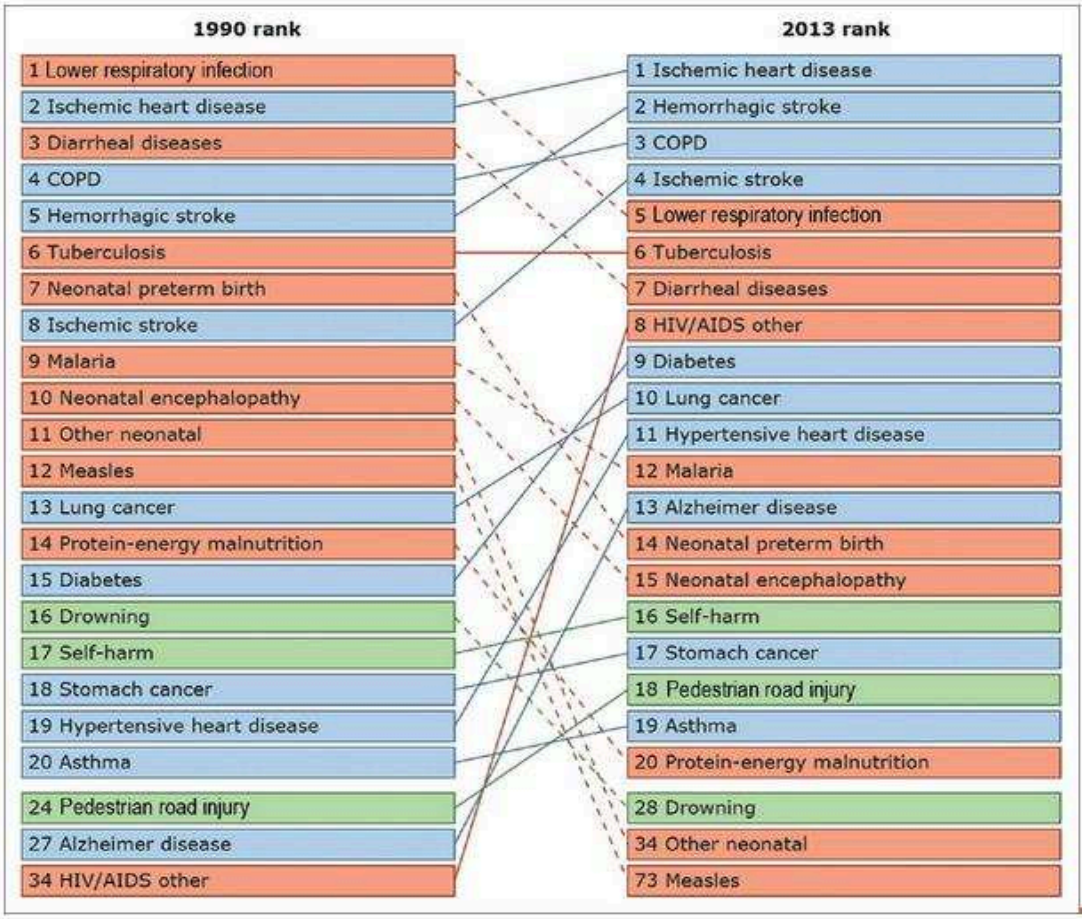
Source: Institute for Health Metrics and Evaluation 2013.

To put things in perspective, the number of people dying from these complex diseases is more than the double of the number of people dying from nutritional deficiencies and all infectious diseases (including malaria, tuberculosis and HIV/AIDS).

During the last two decades, there has been a global epidemiologic shift, affecting all countries regardless of their economic development (Figure 2).

Thus, it is not exaggerated to say that these complex diseases are actually dominating the global human health landscape, being the major challenge of our generation and the coming ones.

Figure 2: Changes in the leading causes of deaths in developing countries from 1990 to 2013.



Source: Institute for Health Metrics and Evaluation

CVD are the leading cause of death and disability in the world and as said previously, cause an estimated 17.7 million deaths per year. Of these deaths, 7.4 million were caused by CHD and 6.7 million were due to strokes. Worldwide, the number of fatalities caused by CVD increased the past few decades. Indeed, in 1990, 25.9% of all deaths were caused by CVD, while in 2013, this percentage raised to 31%, and up to 48% of all NCD deaths, being more than the double caused by cancer (4) (Figures 3 and 4).

Most of the CVD deaths can be prevented by an early detection of the disease, prevention, detection and reduction of risk factors as well as by a continuous surveillance and monitoring of the patients affected.

Figure 3: Distribution of major causes of death globally, including CVD.

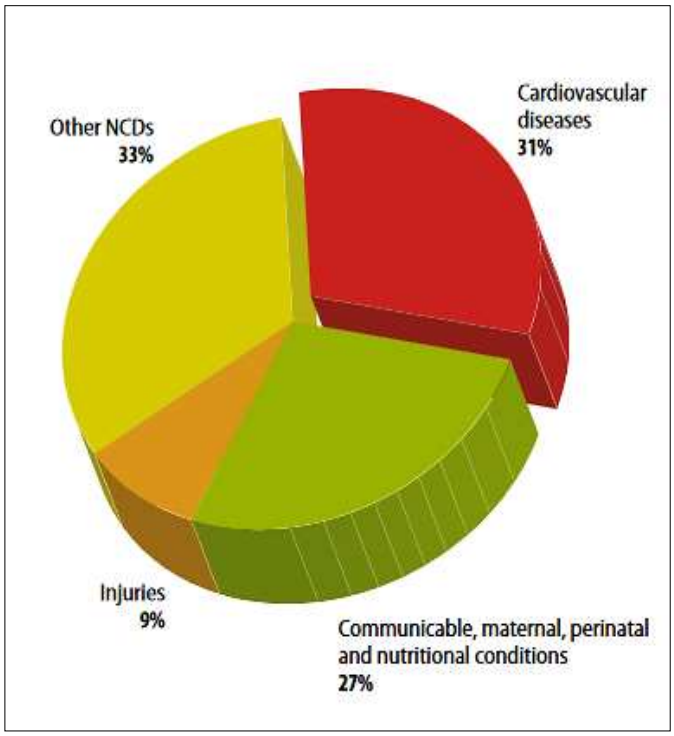
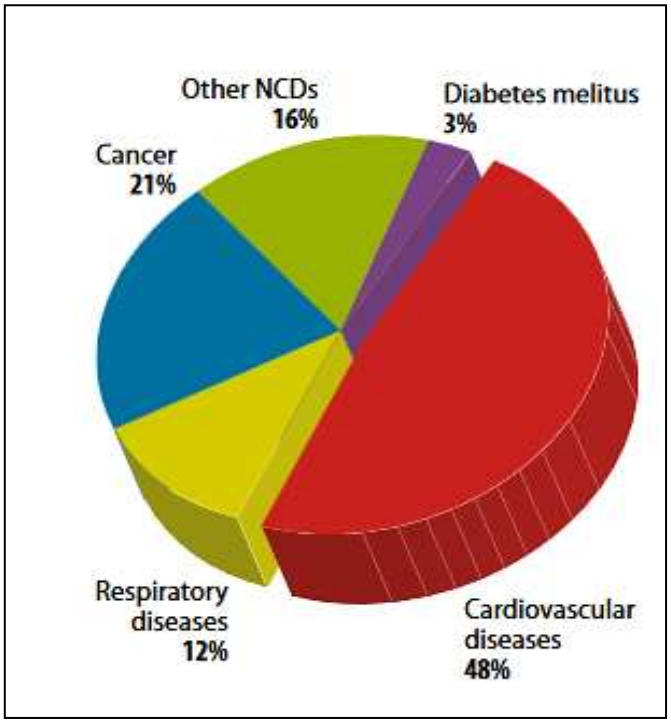


Figure 4: Distribution of causes of death related to non –communicable diseases



Source: Global Atlas on cardiovascular disease prevention and control, 2011. (http://www.world-heart-federation.org)

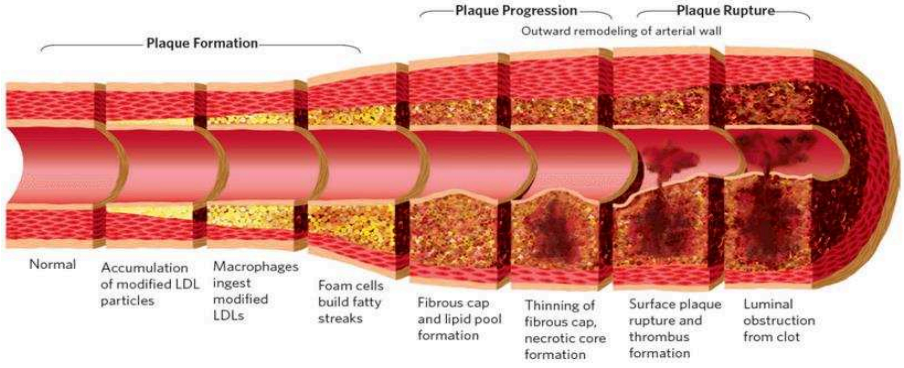
2. Pathophysiology of cardiovascular diseases

CVD are the group of disorders of the heart, vascular diseases of the brain and diseases of blood vessels. Among those diseases, the ischemic heart disease (e.g. heart attack), cerebrovascular disease (e.g. stroke) and diseases of the arteries, including hypertension and peripheral vascular disease, are caused by the underlying disease process known as atherosclerosis (Figure 5). It is the major precursor of CVD, and is a complex pathological process that needs many years to develop until the pathological symptoms appear.

An initial lesion of the intima-media, occurring in the early stages will produce some macrophage lipid infiltration to the blood vessel walls. With time, more cholesterol and fatty material are deposited in the lumen of arteries, and there is an invasion and accumulation of white blood cells (known also as foam cells). The excessive accumulation of white blood cells will produce a chronic inflammatory response and will also reduce the elasticity of the artery walls (due to the calcium and other crystallized remnants present in the foam cells). However, the process will need many years until the first pathological symptoms appear.

The stiffening of the arteries due to a progressive inflammatory state and lipid and foam cells accumulation will eventually produce high blood pressure (hypertension). If the removal of fats and cholesterol from the intima-media of the artery doesn't exceed their accumulation, a hardening will occur and multiple atheromatous plaques will grow, causing irregularities and thickness in the inner surface of the arteries, making it harder for blood to flow through. Ultimately, the rupture of the plaque can happen. The rupture of the fibrous cap will expose thrombogenic material to the circulation and eventually induce thrombus formation in the lumen, stopping blood flow, and leading to death of the tissues fed by the artery in question. This event will cause a heart attack if the thrombus formation develops in a coronary artery and a stroke if it develops in the brain.

Figure 5: Endothelial dysfunction timeline during the progression of atherosclerosis



Source: Advanced Cardiovascular Screening services. https://www.rockcreekwellness.com

The atherosclerosis process is multifactorial and complex, which is a result of a synergy between many risk factors (1, 2, 28). The individual contribution of each risk factor varies widely between different communities, ethnic groups and individuals. However, the overall contribution of these risk factors is very consistent (3). They can be congenital or acquired and modifiable or not, and are divided as classical and non-classical risk factors.

2.1 Classical cardiovascular disease risk factors

The early stages of the atherosclerosis process begin in childhood and adolescence due to different factors (1, 2). Among the classical risk factors is included hyperlipidemia, high blood pressure, age and gender, tobacco use, physical inactivity, harmful use of alcohol, genetic predisposition, diabetes and obesity.

2.1.1 Dyslipidemia

Dyslipidemia is an abnormal amount of lipids in the blood. The most common form of dyslipidemia is hyperlipidemia, when there are abnormally elevated levels of lipids in the blood, and is a major cause of atherosclerosis. Although it can be caused by a genetic predisposition (familial or primary hyperlipidemia), the principal cause of hyperlipidemia is the high dietary intakes of saturated fat (acquired or secondary hyperlipidemia).

2.1.1.1 Total cholesterol and LDL-cholesterol

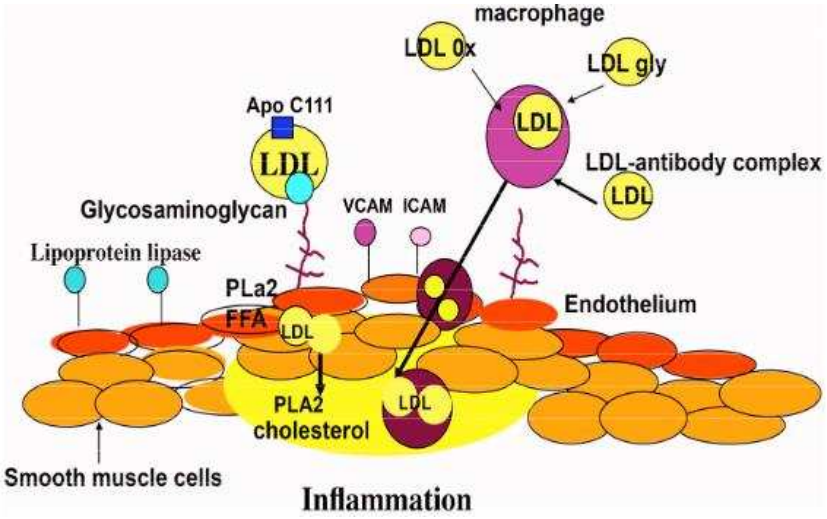
High levels of total cholesterol and specially LDL-cholesterol have shown a close relationship with CVD and atherosclerosis (29). The LDL is the major transporter of both cholesterol and fatty acids. LDL are important in maintaining cholesterol homeostasis, and the direct relation with lower levels of LDL and lower atherosclerosis is been known for a long time now (30). It has been demonstrated that the size of the LDL particles are as important as the total amount of LDL in the blood. Early atherosclerosis is associated with larger LDL particles, rather than small dense LDL (31, 32). One of the possible reasons for this explanation is that LDL particles with higher surfaces are more prone to oxidation, resulting in antibody formation and a subsequent macrophage migration and uptake of LDL particles.

Table 1: LDL-cholesterol levels

<100	Optimal
100-129	Near optimal/above optimal
130-159	Borderline high
160-189	High
≥190	Very high

The LDL particles are prone to attach damaged intima in the arteries, being the major contributor to plaque cholesterol (Figure 6). The attachment to the endothelium happens through the lipoprotein lipase (LPL) (33) and heparin sulfate proteoglycans (HPSG). The macrophages attracted to the site by modified LDL particles will suffer trans-epithelial migration to the intima, and being trapped there will mature into foam cells, accelerating the formation of the lipid-rich atherosclerotic core.

Figure 6. Involvement of the LDL particles in the formation of the atherosclerotic plaque

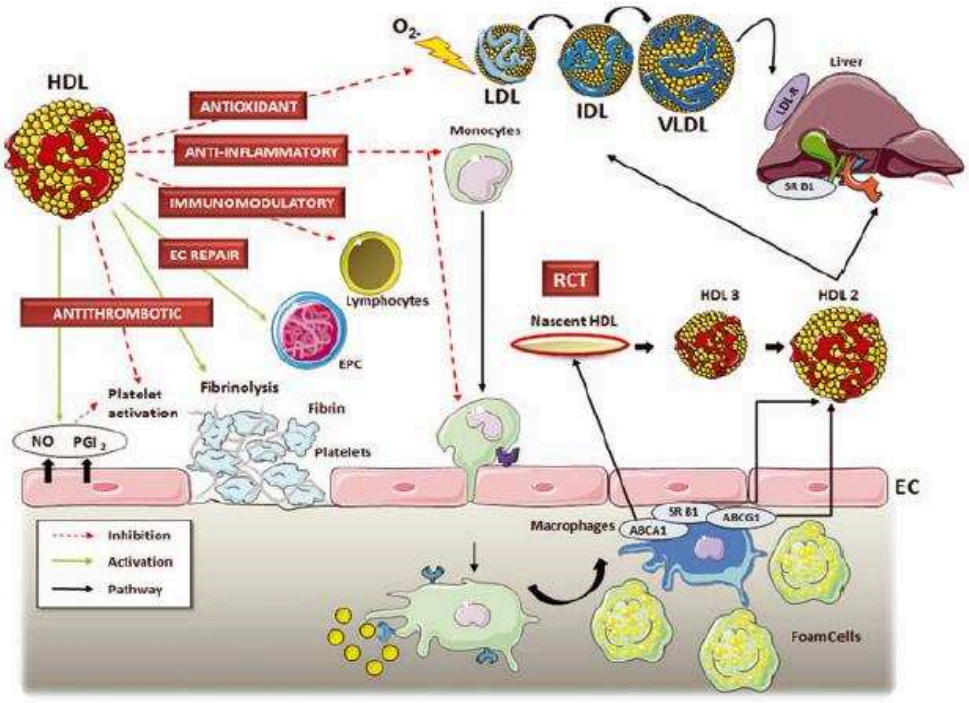


Source: The Open Atherosclerosis & Thrombosis Journal, 2012, Volume 5

2.1.1.2 HDL-cholesterol

Contrary to LDL-cholesterol, the high density lipoprotein (HDL)-cholesterol is inversely associated with the risk of atherosclerosis (34). Indeed, patients with HDL 35 < mg/dL have eight times higher risk of suffering cardiovascular events than individuals with HDL > 65 mg/Dl (35, 36). Also, a meta-analysis showed that for every increase of 1mg/dL in HDL-cholesterol levels, there is a 3.2% reduction in cardiovascular risk in women and 1.9 to 2.3% in men (37). The HDL major function and the reason why is inversely associated with atherosclerosis is the reverse cholesterol transport (RCT). During the RCT process, the HDL particles uptake the cholesterol from macrophages and foam cells located in the atherosclerotic plaque, removing it from the vasculature to transport it to the liver for excretion into bile (38). Recent studies have also demonstrated other abilities by which HDL have anti-atherogenic properties (Figure 7), like anti-inflammatory effects or even protecting LDL from oxidation, among others.

Figure 7**:** HDL anti-atherogenic effects.



Source: LDL vs HDL in atherothrombosis, Badimon & Vilahur

2.1.1.3 Triglycerides

High levels of triglycerides (TG) are associated with cardiovascular disease, even when the levels of LDL-cholesterol are low (39, 40). Although the direct effect of the TG in the atherogenesis is not yet demonstrated, they are closely associated with atherogenic lipoproteins. Some TG-rich lipoproteins (chylomicrons, very low density lipoproteins and intermediate density lipoproteins), are capable of promoting atherogenesis independently of LDL (41). The remnants of these TG-rich lipoproteins are prone to endothelial accumulation and uptake by macrophages, accelerating the foam cells formation and thus, of atherosclerotic plaque (42, 43).

Table 2: Triglyceride levels

<150	Normal
150-199	Borderline high
200-499	High
≥500	Very high

2.1.2 Hypertension

Also called high blood pressure, the hypertension is a long-term medical condition, in which the blood pressure in the arteries is constantly elevated. It is considered a major risk factor for CVD (44) and is a multifactorial process that involves the interaction of genetic and environmental factors. Blood pressure is obtained by measuring the systolic and diastolic blood pressures (SBP and DBP respectively). High blood pressure is considered when an individual has a systolic blood pressure ≥ 140 mmHg and a diastolic blood pressure ≥ 90mmHg (45) (Table 3).

Table 3: Classification of blood pressure readings.

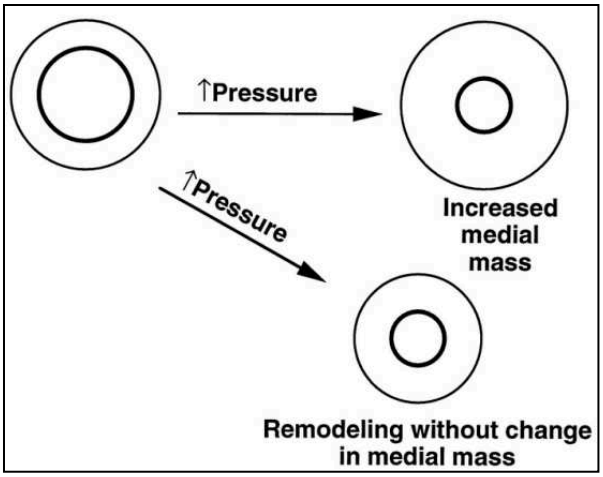
Diagnosis	Systolic *(top)*	Diastolic *(bottom)*
Normal	<120	<80
Prehypertension	120-139	80-89
Stage 1 Hypertension	140-159	90-99
Stage 2 Hypertension	>160	>100
Hypertensive Emergency	>180	>120

Source: https://heartengine.org/

The most important independent risk factor for suffering hypertension is the age, followed by obesity. In the Framingham study, it has been estimated that 10% weight gain is associated with a 6.5 mmHG increase in SBP (46). Other factors significantly increasing the blood pressure are insulin resistance, high alcohol intake and high salt intake.

It is well known that hypertension promotes the formation of atherosclerosis (47), one of the reasons is because the high blood pressure, resistance arteries undergo eutrophic and/or hypertrophic remodeling (Figure 8)(48). Also, inflammation and fibrosis have been shown to be implicated in arterial remodeling during hypertension (49).

Figure 8: Effect of hypertension on smooth muscle cells



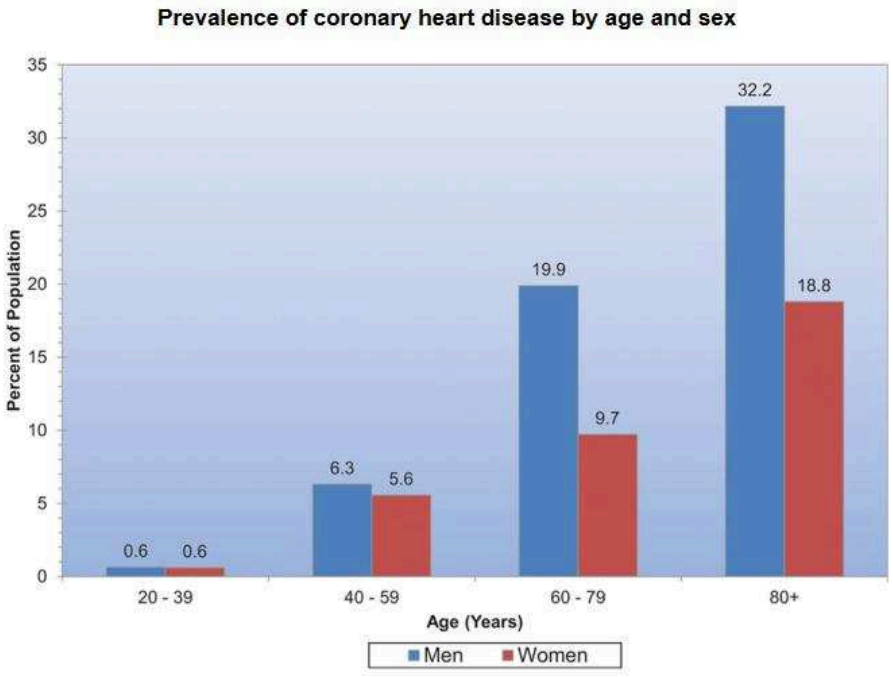
Source: Hypertension and the Pathogenesis of Atherosclerosis, Hypertension, 1995.

2.1.3 Age and Gender

The risk of suffering from CVD increases with age, which is logical if we take into account that older individuals spent more time exposed to all CVD risk factors. In fact, with age, the levels of many of the risk factors involved in CVD increase, including total cholesterol, blood pressure, rigidity of the arteries and obesity among others.

Also, the risk is different according to gender, males having significantly higher risk of suffering CVD than females (Figure 9) (50). Indeed, women develop CVD about 10 years later than men. However, this gender protection reduces once the female passes the menopause. This gender difference can be explained from hormonal differences. Females' more predominant hormone is the estrogen, which has been argued to have anti-atherogenic properties (51). Once female passes the menopause, there is a decrease in the HDL/LDL cholesterol ratio, making them more predisposed for suffering CVD (52, 53).

Figure 9**:** Prevalence of coronary heart disease by age and sex



Source: American Heart Association. Mozaffarian D et al. Circulation. 2015.

2.1.4 Tobacco use

In terms of deaths attributable, tobacco is the second risk factor, to which 9% of global deaths are attributed (WHO; Global Atlas on cardiovascular disease prevention and control). Tobacco consumption is estimated to cause nearly 10% of CVD (28). However, it is an avoidable and highly reversible risk factor. A 50 year follow-up showed that individuals that quitted smoking between 35 and 44 years of age had same survival rates as those who had never smoked (54). According to the pathogenesis of tobacco, smoking activates a wide number of mechanisms that induce atherosclerosis, dyslipidemia, vascular inflammation, thrombosis, endothelial homeostatic loss and abnormal vascular growth (55-57).

2.1.5 Physical inactivity

Another easily preventable risk factor is physical inactivity. It is the fourth leading risk factor for mortality and people who are physically active have reduced risk of death from overall CVD. Being a key determinant of energy expenditure, it is fundamental to energy balance and

weight control, and it helps also to improve blood pressure, lipid profile and insulin sensitivity (58, 59). Beneficial effects of physical activity have also been demonstrated in the endothelial function, by enhancing vasodilatation and vasomotor function in the blood vessels (60).

2.1.6 Obesity

Obesity is a growing health problem worldwide. In 2008, 34% of adults over the age of 20 were overweight with a body mass index (BMI), a measure of weight relative to height) greater than 25 kg/m² (1) (Table 4). Also, according to the WHO, 11.8% of individuals were obese in 2008 (9.8% of men and 13.8% of women) with a BMI greater or equal to 30 Kg/m², compared to 6.4%, in 1980. The imbalance between increased energy and intake (diet) and energy expenditure (physical activity) is the major cause of obesity.

Many studies have reached the conclusion that obesity or overweight is related to CVD and mortality. Indeed, obesity leads to metabolic changes that accelerate the progression of CVD, and is strongly linked with other CVD risk factors such as hypertension, hyperlipidemia, type 2 diabetes and glucose intolerance (61, 62). Also, the adipose tissue is one of the principal producers of pro-inflammatory cytokines and is thought to induce a low-grade chronic inflammation, which is also considered as a risk factor for the pathogenesis of atherosclerosis (63). Indeed, in obese individuals pro-inflammatory molecules and other risk molecules, like the tumor necrosis factor alpha (TNF-α), Interleukin 6 (IL-6), lipoprotein lipase etc., appear in higher concentrations.

Table 4: Weight classifications by body mass index

Classification	**BMI (Kg/m^2)**
Underweight	< 18.5
Normal range	18.5 - 24.9
Pre-obese	25 - 29.9
Obese I	30 – 34.9
Obese II	35 – 39.9
Obese III	≥ 40

2.1.7 Diabetes

Diabetes is a group of metabolic diseases, in which there are high blood sugar levels over a prolonged period of time (fasting plasma glucose value ≥7.0 mmol/l). The most common type of diabetes is the diabetes mellitus type 2, which begins with the cells of the body not responding properly to the insulin produced (insulin resistance). It is considered as major risk factor for CVD, and only in 2008, it was responsible for 1.3 million deaths. The risk of suffering cardiovascular events is between two to three times higher in people with diabetes, this risk being much higher in women (64, 65). In individuals with diabetes, the response to exogenous Nitric Oxide enzyme, a vasodilator, is diminished, avoiding the endothelium cells to relax and thus, increasing the formation of plaques in diabetic patients (66). Also, in diabetic patients, NF-kB production increases, generating oxygen- derived free radicals in vascular smooth muscle, which leads to a endothelial impairment and atherosclerosis related inflammation (67). An early detection and care for diabetes is essential to avoid severe complications that include hearth attacks and stroke.

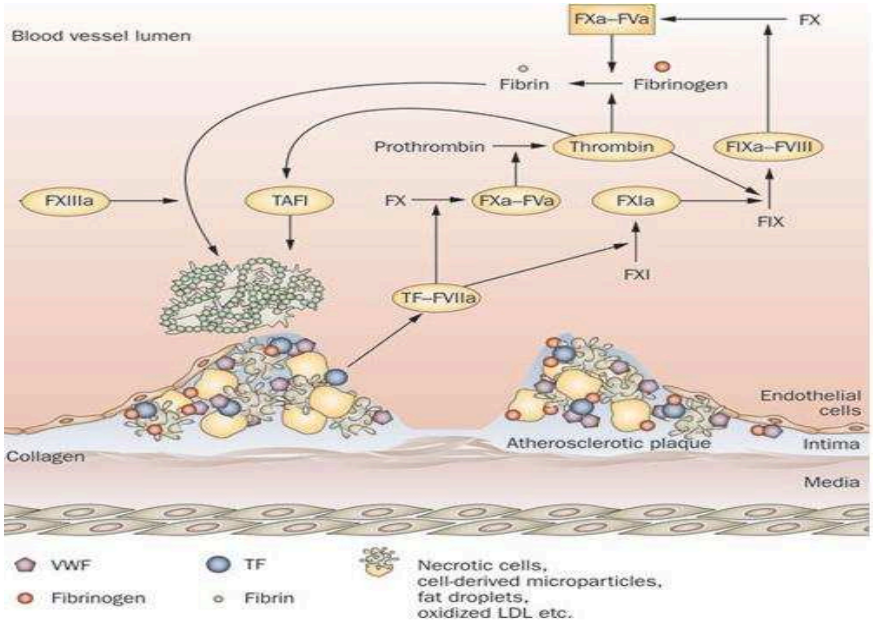
2.2 Novel cardiovascular disease risk factors

The novel cardiovascular risk factors or the non-traditional ones are numerous and still not completely understood. Among novel risk factors, the most important are related with inflammatory molecules, adhesion molecules and thrombogenic factors, all of them being key steps in the development of CVD.

2.2.1 Thrombogenic factors

Thrombosis is the formation of a blood coagulum, resulting in the obstruction of a blood vessel. If thrombosis occurs in an artery, this will affect the blood supply and may lead to damage of the tissue supplied by that artery, resulting in cardiovascular events (68). The mechanisms of coagulation involve activation, adhesion and aggregation of platelets along with deposition and maturation of fibrin and it is a highly conserved process where many molecules are involved (Figure 10). The evidences suggest that arterial thrombosis originate mainly as a consequence of a previously injured atherosclerotic plaque. Some of the factors considered as a risk factor for suffering thrombosis are high levels of fibrinogen, Von Willebrand factor and factor VII, among others (69).

Figure 10: Activation of blood coagulation in arterial thrombus formation.

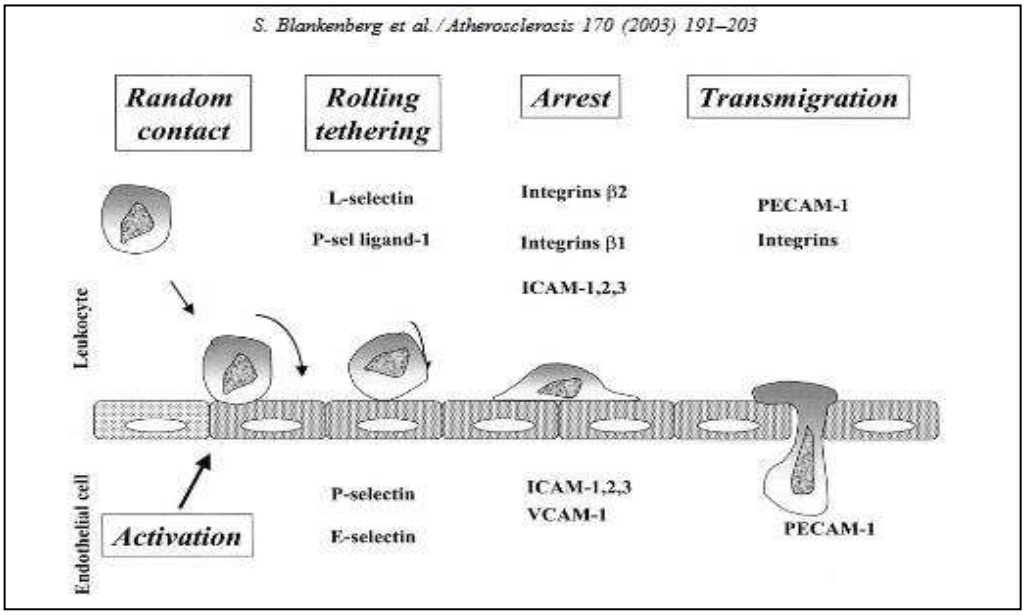


Source: Nature Reviews Cardiology 8(9):502-12; July 2011

2.2.2 Cell Adhesion molecules

Cell adhesion molecules (CAMs) are proteins located in the cell surface that mediate the binding with other cells or with the extracellular matrix. There are many evidences that support the role of the adhesion molecules in the progression of atherosclerosis. Actually one of the early stages of atherosclerosis involves the recruitment of inflammatory cells from the circulation and their trans-epithelial migration into the intima of the arteries (70) (Figure 11), and this process is predominantly mediated by the adhesion molecules. They are expressed on circulating leukocytes and vascular endothelium cells (71), numerous soluble isoforms circulating in the blood have also been detected (72).

Figure 11: Leukocyte-endothelial cell interactions during the initial steps of atherosclerosis, and role of the different adhesion molecules in this process.



Source: S. Blankenberg et al. / Atherosclerosis 170 (2003) 191-203.

Table 5: Adhesion molecules involved in atherosclerosis, their main ligands and functions.

Adhesion molecules	Other names	Ligands	Functions
Selectins/ligands			
P-selectin	CD62P, GMP140	PSGL-1, Lewis X, CD24	Rolling/tethering
E-selectin	CD62E, ELAM1	ESL-1, Lewis X, PSGL-1, L-set	Rolling/tethering
L-selectin	CD62L	Lewis X, CD34, PSGL-1, GlyCAM	Rolling/tethering
E-selectin ligand 1	ESL-1	E-selectin	Rolling/tethering
P-selectin ligand 1	CD162, PSGL-1	P-, L-, E-selectin	Rolling/tethering
Immunoglobulins			
ICAM-1	CD54	αLβ2, αMβ2, αXβ2	Firm adhesion
ICAM-2	CD102	αLβ2, αMβ2	Firm adhesion
ICAM-3	CD50	αLβ2, αDβ2, DC-SIGN	Firm adhesion
VCAM-1	CD106	α4β1, α4β7, αDβ2	Firm adhesion
PECAM-1	CD31	PECAM-1, αVβ3	Endothelial integrity, leukocyte extravasation
Integrins			
Integrin α2/β1	CD49b/CD29, VLA2	Collagen, laminin	Platelet receptor
Integrin α4/β1	CD49d/CD29, VLA4	VCAM-1, FN	Firm adhesion
Integrin αL/β2	CD11a/CD18, LFA1	ICAMs	Firm adhesion
Integrin αM/β2	CD11b/CD18, Mac1	ICAMs,iC3b, FX, FG	Firm adhesion
Integrin αX/β2	CD11c/CD18	ICAM-1, FG, iC3b, CD23	Firm adhesion
Integrin αD/β2	CD11d/CD18	ICAM-3, VCAM-1	Firm adhesion
Integrin α2B/α3	GPIIb/IIIa	vWF, FN, FG, VN, thrombospondin	Platelet receptor
Integrin αV/β3	VNR, CD51/CD61	PECAM-1, VN, FN, FG, vWF	Proliferation, migration
Integrin αV/β5		VN	Proliferation, migration

Source: S. Blankenberg et al. / Atherosclerosis 170 (2003) 191-203

2.2.2.1 Selectins

There are three types of selectins; E-, P- and L-selectins, and they are named according to their expression site: L-selectin is expressed in leukocytes, E-selectin in endothelial cells and P-selectin on platelets. They are responsible of mediating the initial capture, tethering and rolling along endothelium (73). By interacting with their respective ligands, selectins develop weak bonds between leukocytes and the endothelium. That's why an over-expression of these molecules can increase the inflammatory state and affect the integrity of the vascular wall.

2.2.2.1.1 L-Selectin

L-Selectin is expressed constitutively on the majority of circulating leukocytes and mediates the primary lymphocyte rolling and secondary capture, at sites of chronic inflammation (74). It has been demonstrated that lymphocyte migration into the atherosclerotic aorta is regulated by L-selectin (75), giving the L-selectin a potential interest as a biomarker to address atherosclerosis. Inhibition or absence of L-selectin may prevent leukocyte rolling, eventually decreasing the trans-epithelial migration of leukocytes into the atherosclerotic vessels. Indeed L-selectin lymphocytes show a reduction of 50% on their migration capacity into the aorta (75).

2.2.2.1.2 P-Selectin

P-selectin is specifically expressed in platelets, and in a less concentration in endothelial cells. It is stored in α-granules of resting platelets and in weibel-palade bodies of endothelial cells. The P-selectin will bind its receptor, P-selectin glycoprotein ligand (PSGL-1), which is expressed in leukocytes (76) and will approach the recruited cells and favorice the contact of other adhesion molecules, such as integrins. This process induces inflammation and thrombosis. In normal non inflammated endothelium, the P-selectin is not expressed in the membrane of the platelets and endothelial cells, while it is detected on the atherosclerotic endothelium (77). Some authors suggest that P-selectin expression could be one of the earliest events in the development of atherosclerosis. Actually, one study showed that in newborns with a strong family history of myocardial infarction, the P-selectin was over-expressed (78). Also, blocking of P-selecting reduces significantly the monocyte rolling and attachment to the endothelium (79), showing its importance on the recruitment of monocytes to atherosclerosis regions.

2.2.2.1.3 E-Selectin

E-selectin is barely expressed in resting endothelium cells. However, it is transcriptionally induced by some inflammatory cytokines, such as TNF-α or platelet factor 4 (80, 81). Expression of E-selectin has been detected on endothelial cells present in atherosclerotic plaque, as well as, in fibrous and lipid-containing human plaques (82).

2.2.2.2 Immunoglobulin Super Family, Cellular adhesion molecules and Integrins

Among the members of the immunoglobulin (Ig) super family considered being more involved in atherosclerosis are: Intercellular Adhesion Molecule 1 (ICAM-1), Vascular cell adhesion molecule 1 (VCAM-1) and Platelet endothelial cell adhesion molecule 1 (PECAM-1). They all contain a variable number of extracellular immunoglobulin domains, which participate to adhesion sites.

Integrins are heterodimeric molecules formed by non-covalent union of different glycoproteins classified as α and β chains. The integrins that are more relevant in atherosclerosis are the β2 integrins and α4β1 integrins.

2.2.2.2.1 ICAM-1 and β2 integrins

ICAM-1 is constitutively expressed in leukocytes and endothelial cells, and the inflammatory cytokines up-regulate their expression. One of the ligands of ICAM-1 are the leukocyte specific β2 integrins and their union mediate adhesion of leukocytes to activated endothelium, subsequently inducing firm arrest of inflammatory cells at the endothelium (Figure 11). It has been observed an over-expression of ICAM-1 in atherosclerosis sites (83).

2.2.2.2.2 VCAM-1 and α4β1 Integrins

VCAM-1 is principally expressed on endothelial cells and among their ligands is the integrin α4β1. Their union allows a firm adhesion of the recruited cells to the endothelium. As well as for ICAM-1, VCAM-1 increased levels have been detected in human coronary arteries (82). Moreover, at atherosclerosis-prone sites of the endothelium, the expression of VCAM-1 is increased (84). The interaction VCAM-1/Integrin α4β1 is also important for starting the changes in the shape of the cells for allowing the trans-epithelial migration (85).

2.2.2.2.3 PECAM-1

PECAM-1, also known as CD31, is expressed by leukocytes, platelets and endothelial cells (86). They are involved in the trans-epithelial migration of the cells from the lumen of the arteries into the intima (Figure 11). It has also an important role in the neovascularization of atherosclerotic plaques, showing increased levels of expression in atherosclerosis sites (87).

2.2.3 Inflammatory molecules

Although atherosclerosis has been during many years considered only as a lipid storage disease, actually, it is more and more considered as an inflammatory process, which affects medium and large-sized blood vessels throughout the cardiovascular system (9, 25). During the recent years, the principal role of inflammation, mediating all the key steps of atherosclerosis, from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis, has been established.

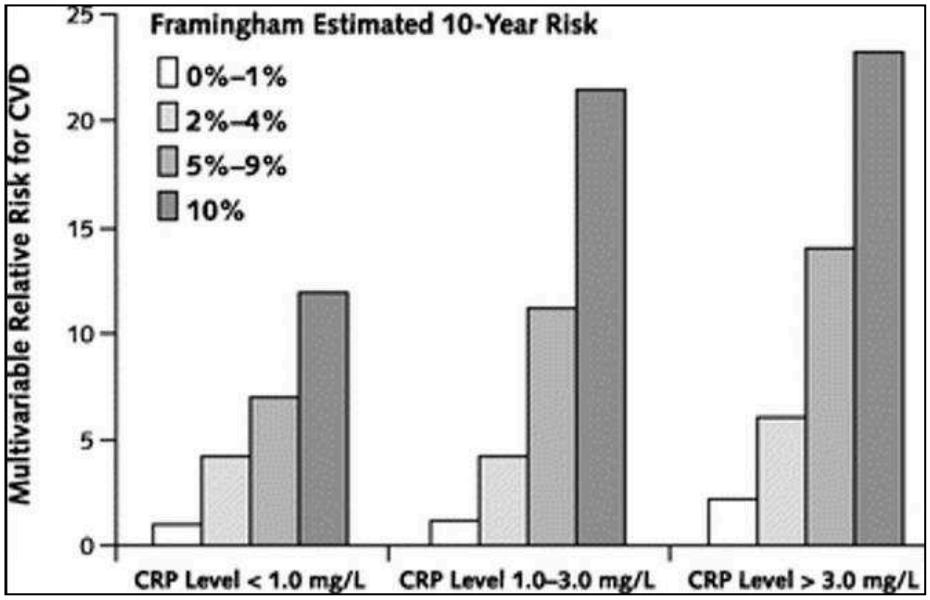
Leukocytes participate in the earliest steps of atherosclerosis. The inflammatory cells from the blood adhere poorly to the normal non-inflamed endothelium. However, the action of the free radicals, along with the modified LDL-cholesterol particles and other factors, make the endothelial monolayer inflamed (10). Thus, they will express higher levels of adhesion molecules (88), making it easier to the lymphocytes and monocytes to adhere to the endothelium. Pro-inflammatory molecules will provide a chemotactic stimulus, making the inflammatory cells prone to suffer trans-epithelial migration to the intima. Once in the intima, the inflammatory cells participate in the maintenance of a local chronic inflammatory response. The inflammatory molecules present will increase the absorption of LDL-cholesterol by the macrophages, thanks to a higher expression of scavenger receptors in their membranes (89). The cytokines produced by these same cells promote the migration and proliferation of the SMCs (11), making the atherosclerotic plaque grow by producing extracellular matrix. In response to this chronic-inflammatory state, the activated macrophages produce matrix metallo-proteinases that will degrade the elastin and collagen, making the fibrous cap of the atherosclerotic lesion thinner and susceptible to rupture. At the same time the macrophages produce pro-coagulant factors that can trigger to thrombosis (90).

Inflammatory markers are gaining importance in the prediction of the outcome of patients suffering CVD, and many novel treatments are focused on limiting the chronic inflammation present in atherosclerosis.

2.2.3.1 CRP

C-reactive protein (CRP) is a ring shaped inflammatory protein found in plasma. The relation between CVD and CRP has been deeply studied in recent years. Many studies have reported the association between the CRP levels and CVD mortality, myocardial infarction (MI) and stroke (Figure 12) (91-93). CRP is present within the atherosclerotic plaques, and has been demonstrated that in patients with atherosclerosis, CRP is capable to directly bind to oxidized LDL-cholesterol particles (94). It also affects the macrophage polarization, leading to their infiltration in the adipose tissue and atherosclerotic lesions (95). Many studies have used the CRP levels as risk biomarker in CVD. The median concentration of CRP levels in healthy individuals is 0.8 mg/L, and concentrations above 3 mg/L are considered high. Indeed, studies using a CRP cut point of 3mg/L have shown a significantly increased risk of cardiovascular events (96, 97). However the variability changes highly among ethnicities, sexes (98) and age (99), polymorphisms and other factors (100), making the use of CRP as a biomarker challenging.

Figure 12: Multivariable relative risk for cardiovascular disease, based on level of CRP and level of absolute predicted 10-year Framingham coronary heart disease risk in the women's health study.

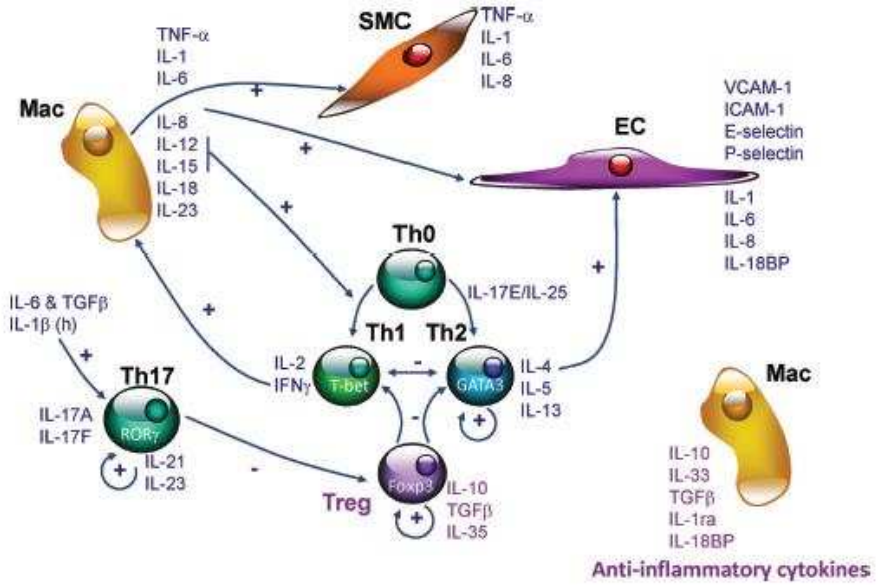


Source: Assessment of CRP in Risk Prediction for Cardiovascular Disease. Donald M. Lloyd-Jones et al.

2.2.3.2 Cytokines

Atherosclerosis is nowadays recognized as a chronic inflammatory disease initiated by risk factors already mentioned, such as hypertension and hyperlipidemia. Among the first steps of atherosclerosis appear the activation of the endothelium and migration of leukocytes and monocytes to the intima of the arteries. The innate and adaptive immune responses in atherosclerosis are regulated by a wide range of cytokines (101-103). Cytokines are a category of small proteins that are important in cell signaling. Among cytokines there are interleukins, chemokines, interferons (IFNs), lymphokines and tumor necrosis factors. Many different cytokines are expressed in atherosclerosis, being classified as pro- or anti-atherogenic, and their role in atherosclerosis is multiple (Figures 13 and table 6).

Figure 13: Cytokines produced by several inflammatory and vascular cells that are participating in atherosclerosis.



Source: Arterioscler Thromb Vasc Biol. 2011;31:969-979

As mentioned before, among cytokines participating in atherosclerosis development are the chemokines. When the endothelial cells are activated, apart from expressing higher numbers of adhesion molecules, they will also release a variety of chemokine's that will cause the recruitment of monocytes and lymphocytes (104, 105). Also the trans-epithelial migration of monocytes is mediated by chemokines (among other molecules), produced by endothelial cells and smooth muscle cells (105). Other cytokines are also present during some of the stages of atherosclerosis progression. In the early stages, TNF-α and IFN-γ are capable to alter the distribution of vascular endothelial cadherin-catenin complexes, disrupting the endothelial junctions (106) and therefore facilitating the trans-epithelial migration of leukocytes to the intima. Once in the intima, the activation of the leukocytes and the differentiation of monocytes into macrophages as well as the formation of foam cells will also be mediated by cytokines (107). In the later stages of atherosclerosis, the cytokines will also play a key role by promoting cell apoptosis and matrix degradation, and hence, by destabilizing the atherosclerotic plaques.

Table 6: Cytokines expressed in human atherosclerotic plaques

Cytokines	Protein	mRNA
TNF-α	+	+
IL-1	+	+
IL-2	+	+
IL-4	+/−	+/−
IL-5	+/−	+
IL-6	+	+
IL-8	+	+
IL-10	+	+
IL-12	+	+
IL-15	+	+
IL-17	+	+
IL-18	+	+
IL-20	+	
IL-21	+	+
IL-23	+	+
IL-27	+	
IL-33	+	+
IL-35	+	
IFN-γ	+	+
TGF-β	+	+
GM-CSF	+	+
M-CSF	+	+

Source: Arterioscler Thromb Vasc Biol. 2011;31:969-979

In the following pages I will summarize the principal cytokines that are considered to orchestrate the different stages of atherosclerosis.

2.2.3.2.1 TNF-α

TNF-α is an important pro-inflammatory molecule already associated with many different diseases. The fact is that TNF-α is participating in a positive regulatory loop , being capable of inducing the expression of other pro-inflammatory molecules (108). Studies performed in TNF-α knockout mice showed a reduced atherosclerosis, associated with fewer foam cells and other pro-inflammatory markers. TNF-α is promoting the apoptosis of macrophages and foam cells, thus, increasing the atherosclerotic lipid core (109). It also produces the apoptosis of smooth muscle cells, which makes the fibrous cap thinner and more prone to thrombosis (110,

111). TNF-α has been also associated with the reorganization of the actin and tubulin cytoskeletons in endothelial cells, a process that open the gaps between the cells and makes easier for the inflammatory cells to suffer trans-epithelial migration (112).

2.2.3.2.2 INF-γ

The pro-atherogenic role of INF-γ has been well studied in mice, where INF-γ has been blocked, atherosclerosis lessions have been reduced and, with less lipid accumulation and more collagen content, the plaque has been made more stable and less prone to thrombosis (113, 114). Indeed, it has been demonstrated that INF-γ is capable of inhibiting the synthesis of collagen by smooth muscle cells (115). On the other hand, in apolipoprotein E (ApoE) knock-out mice, the expression of INF-γ was associated with enhanced atherosclerosis (116, 117). One of the main reasons why the INF-γ is pro-atherogenic is because it is capable to activate monocytes/macrophages and dentritic cells. Also, it is thought that INF-γ promotes the uptake of modified LDL by inducing the expression of the SR that binds phosphatidyl serine and oxidized lipoproteins (107), process that will promote the foam cell formation, dysrupting the intracellular metabolism of cholesterol by macrophages.

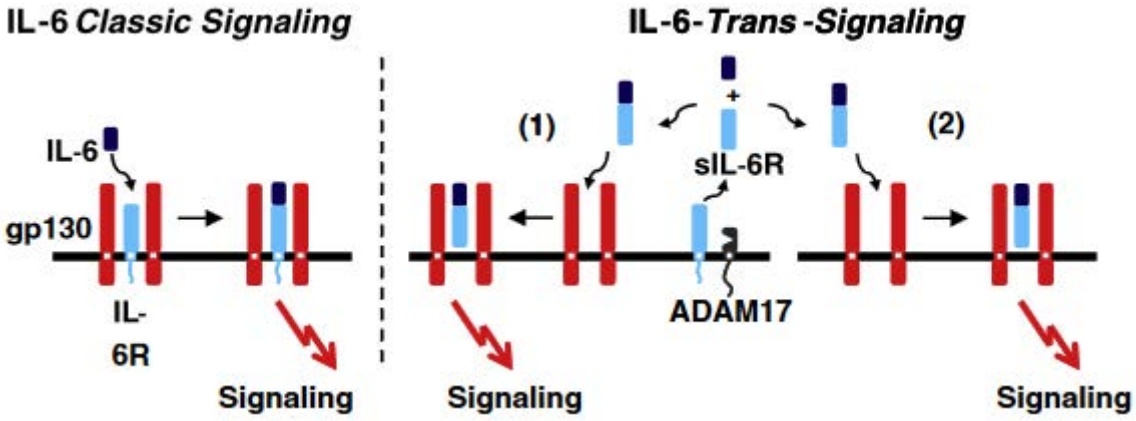
2.2.3.2.3 IL-6

The IL-6 is one of the early and central regulators of inflammation. IL-6 is capable of regulating a wide variety of other inflammatory and anti-inflammatory molecules via its downstream signaling pathways, explaining its pleiotropic effects that not only affect the immune system but also other different pathophysiological processes. The receptor complex that will mediate the signaling activities of IL-6 consists of its direct binding to interleukin-6 receptor (IL-6R) and though the membrane glycoprotein subunit gp130 (Figure 14).

Two types of signaling pathways can be differentiated in IL-6, the classical signaling and the trans-signaling. The classical signaling happens when IL-6 is binding the membrane bound IL-6R (mbIL-6R), this will only happen in cells that express the membrane bound IL-6R, which include hepatocytes, monocytes and T- and B-lymphocytes (118) (Figure 14). The classical signaling is very important in early immune responses. On the other hand, the IL-6 trans-signaling happens when IL-6 binds the soluble form of IL-6R (sIL-6R), which is

generated by alternative splicing (119) or proteolytic cleavage by ADAM17 (120, 121). The IL-6/sIL-6R complex can act in cells where the membrane bound gp130 (ubiquitously expressed) is present, which include cells that do not express the IL-6R (Figure 14). The soluble IL-6R binds the IL-6 with an equal affinity as the membrane bound IL-6R. The trans-signaling pathway is thought to have an important role in the chronic inflammatory disorders, including atherosclerosis and different CVD (122).

Figure 14: Classic- and trans-signaling of IL-6. Only cells that express IL-6R will respond to the classic-signaling, while the cells that only express gp130 can be also responsive via the trans-signaling, where the IL-6/sIL-6R complex is present.



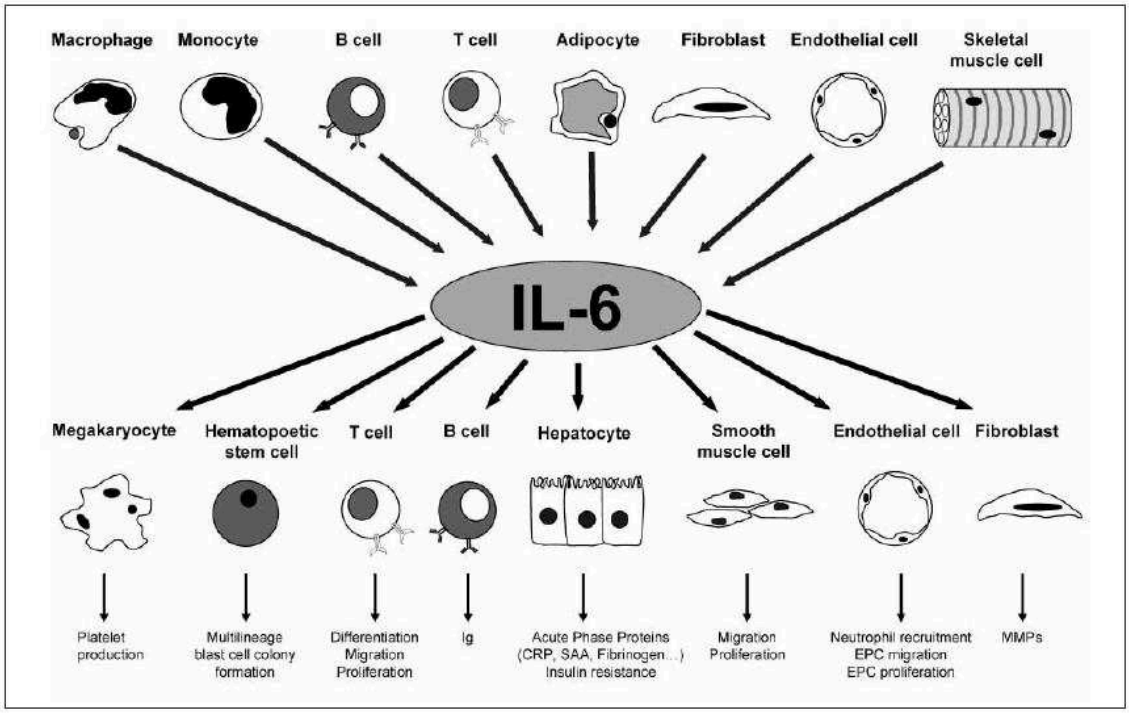
Source: J. Scheller et al. / Biochimica et Biophysica Acta 1813 (2011) 878-888

Once the complex IL-6/IL-6R will be associated with gp130, two gp130 will suffer intracellular dimerization. This will trigger the trans-phosphorylation of two members of the Janus kinase (JAK) family, following the activation of signal transducer and activator of transcription 3 and 1 (STAT3 and STAT1). The activated STAT proteins will then suffer nuclear translocation to act as transcription factors of different genes (123).

The IL-6 is produced by numerous cell types, acting in different parts and processes of the human body (Figure 15). In stress conditions, the IL-6 expression especially increases in monocytes and macrophages. It is also expressed in the adipose tissue, producing a chronic inflammatory state in obesity, which is considered a potential trigger for cardiovascular and

metabolic diseases (124). IL-6 is capable of activating the endothelial cells, which will express more adhesion molecules and chemokine factors, subsequently resulting in a recruitment of neutrophils into the affected area (125). It also promotes the proliferation and migration of T-lymphocytes, endothelial cells and smooth muscle cells (126-128).

Figure 15: Cellular sources and actions of IL-6

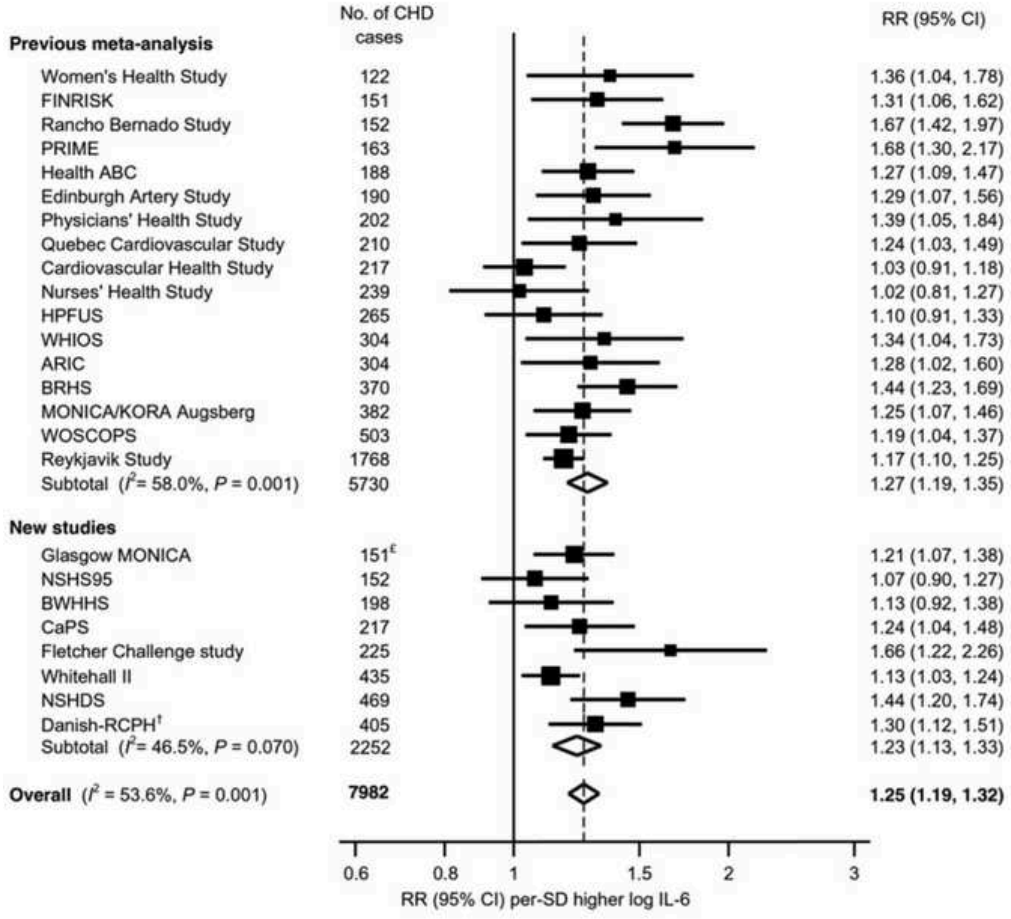


Source: Schuett et al. IL-6 and its signaling in atherosclerosis

Being one of the most important pro-inflammatory molecules, IL-6 and its relation with CVD have been extensively studied. IL-6 perpetuates the chronic inflammation present in atherosclerosis. The involvement of IL-6 in atherosclerotic plaque progression and in plaque destabilization has been demonstrated (129). IL-6 also increases the expression of scavenger receptors, responsible for the absorption of oxidized LDL particles by macrophages, and thus, promotes the formation of foam cells (130). Indeed, IL-6 levels have been used to predict the mortality in patients with unstable coronary artery disease (131). A recently updated meta-analysis using 25 epidemiologic cohorts indicates that the levels of IL-6 are an independent indicator of cardiovascular events in initially healthy individuals (132) (Figure 16). All those

evidences make IL-6 and its regulators attractive targets for detecting individuals at risk for cardiovascular events.

Figure 16: Relationships of plasma levels of IL-6 with risk of CVD in 25 prospective epidemiologic cohorts.



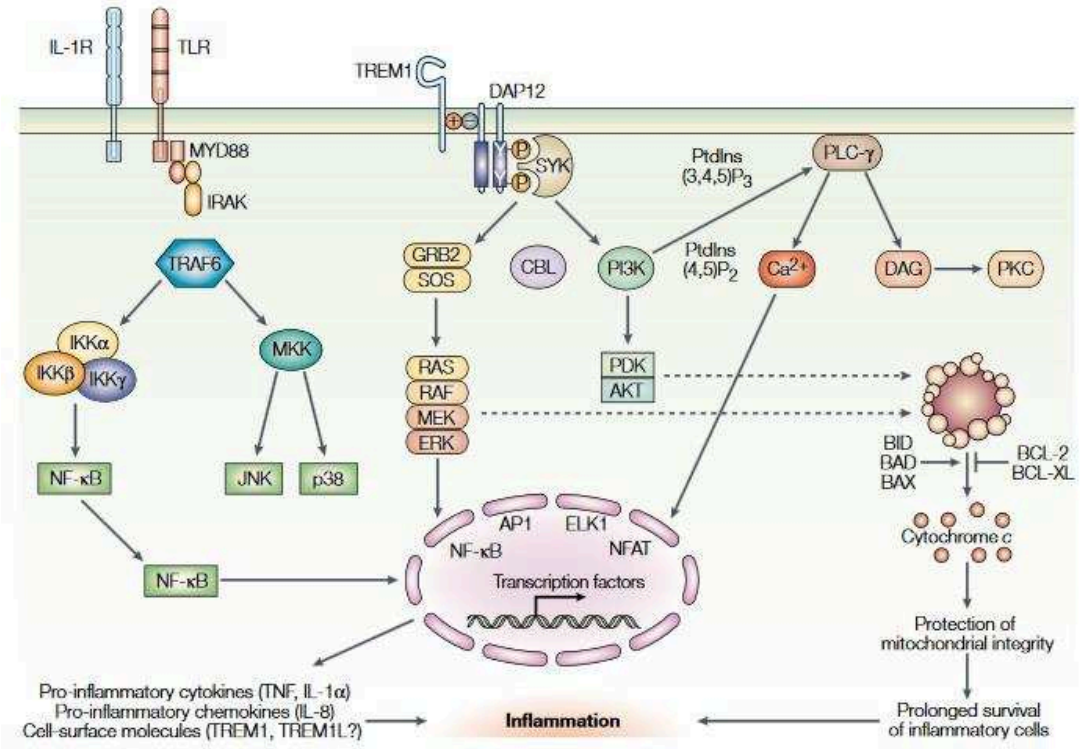
Source: Kaptoge et al, Eur Heart J. 2014;35:578–589

Treatments to inhibit the effect of IL-6 have been already tested. One example is the treatment of patients with low-dose methotrexate used in individuals suffering from rheumatoid arthritis (RA). The results showed a 21% lower risk of future cardiovascular events in patients treated with this anti-IL-6 drug (133). Another example of inhibition of IL-6 is found with the drug called tocilizumab, which has been reported to improve endothelial function and reduce arterial stiffness (134).

2.2.3.3 TREMs (TREM-1 and TREM-2)

Triggering receptors expressed on myeloid cells (TREMs) were discovered 15 years ago (135). Among them, TREM-1 and TREM-2 are considered as activators and both use the same common receptor. While TREM-1 controls the inflammatory response, TREM-2 controls the development and function of dendritic cells, microglia and osteoclasts. TREM-1 and TREM-2 are trans-membrane glycoproteins and have an immunoglobulin domain (135). For the activation of the signaling pathway (Figure 17), the TREM molecules will associate with DAP12, which will suffer phosphorylation, activating the union site for other tyrosine kinases (136). The activation of the TREM pathway will eventually activate different transcription factors. A splicing variant of the TREM-1 mRNA have been found, which lacks the trans-membrane and cytoplasmic domain, generating a soluble TREM-1 protein (sTREM-1) (137).

Figure 17: Scheme presentation summarizing the signaling pathway involving TREM-1, and its inflammatory responses



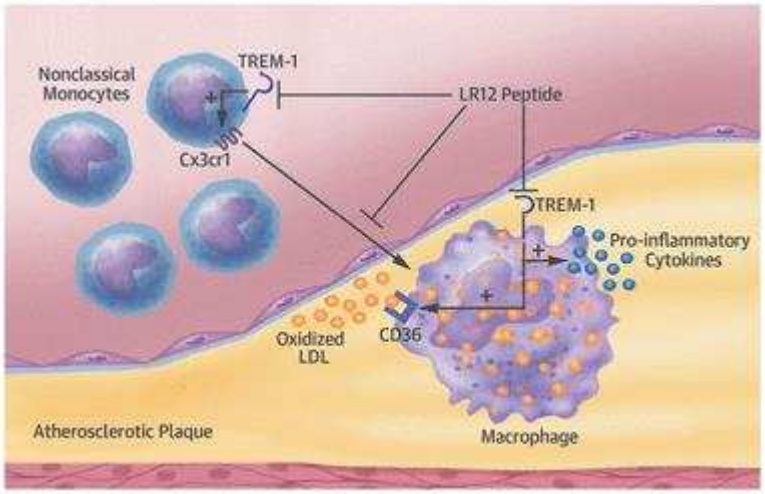
Source: Marco Colonna; TREMs in the immune system and beyond, Nature reviews 2003

The TREM-1 expression is constitutive on neutrophils, monocytes and macrophages, cells that are the principal effectors in innate responses (135). TREM-1 plays a key role in tissues prone to microbial infection such as the lung (expressed in alveolar macrophages) and epithelial cells in human skin (expressed in neutrophilic infiltrates). Increased TREM-1 levels stimulate the production of a wide variety of inflammatory molecules (135, 138), including interleukin-8 (IL-8), a potent chemo attractant for neutrophils), monocyte chemo attractant protein 1 (MCP-1), TNF, IL-6 and IL-1α, along with a rapid neutrophil degranulation and oxidative burst (135, 138). It has been also related with the negative regulation of the anti-inflammatory Interleukin 10.

All above evidences show that TREM-1 can amplify broadly the inflammatory responses with a variety of regulatory functions, making TREM-1 a potential target for therapeutic interventions in human diseases where excessive inflammatory responses are related with a bad outcome of the disease, including septic shock, but also CVD and atherosclerosis. Apart from mediating the inflammatory responses, TREM-1 also regulates monocyte and neutrophil migration to inflammatory sites (139, 140), a process of high importance during atherosclerotic development. Despite being principally studied in septic shock, its importance has been proved in aseptic inflammation such as rheumatoid arthritis (141) and CVD (142).

The levels of the soluble variant of TREM-1 (sTREM-1) are starting to be used as biomarker for predicting the outcome and gravity of different inflammatory diseases, such as critical limb ischemia (143), hemorrhagic fever (144), chronic obstructive pulmonary disease (145), acute pancreatitis (146) or inflammatory bowel disease (147). Levels of soluble TREM-1 have been also correlated with the severity of CVD (142, 148). It has been demonstrated that TREM-1 is involved in post-ischemic myocardial remodeling, by recruiting leukocyte cells to the ischemic heart (148). Indeed, when TREM-1 is pharmacologically inhibited, there is a five-fold decrease in the number of recruited neutrophils (Figure 18) (149). Also, the genetic and pharmacological inhibition of TREM-1 limits the development of early and advanced experimental atherosclerosis in up to 60% (142).

Figure 18: Pro-atherogenic effects of TREM-1 engagement



Source: Joffre, J. et al. J Am Coll Cardiol. 2016;68(25) :2776-93

2.2.4 Hyperhomocysteinemia

The homocysteine (HCY) is an amino acid, homologue of the cysteine. High levels of this amino acid (above 15 µmol/L) results in the medical condition called hyperhomocysteinemia, which has been demonstrated to be an independent risk factor of CVD (150). It has been demonstrated that high levels of HCY induce the migration and proliferation of vascular smooth muscle cells (151, 152), and also activate the endothelial cells (153), thus accelerating the initiation and progression of atherosclerosis. High levels of HCY are also affecting the adhesion and trans-epithelial migration of T cells and monocytes to the vessel wall (154), and it is well known that the chemotaxis and accumulation of leukocytes (such as monocytes and T cells) are critical events in the development of atherosclerosis. It has been argued that this effects of the HCY in the leukocytes migration could be because it induces an increases endothelial surface expression of adhesion molecules, VCAM-1 and E-selectin (154).

Taken together, the studies conclude that HCY is mediating the leukocyte-endothelial cell interaction, which leads to an increased inflammatory state and the consequent worsening of atherosclerosis.

2.2.5 VEGF-A

The vascular endothelial growth factor-A (VEGF-A) encodes a protein that is found as a homodimer. This protein acts specifically on endothelial cells, and has a wide variety of effects such as mediating increased vascular permeability, inducing angiogenesis and endothelial cell growth and promoting cell migration (155). The association of cardiovascular diseases with endothelial dysfunction is well known and as one of the principal molecules affecting endothelial cell proliferation and migration, the VEGF-A molecule is nowadays considered as an emerging risk factor for CVD (156). If there is an insufficient vascular development (process orchestrated by VEGF-A), this could lead to myocardial infarction, from the other side an excessive vascular development is associated with inflammatory diseases and cancer (157).

The VEGF-A has a particularly important role in cancer, actually for the growth of a tumor the angiogenesis is a vital step. That's why the anti-VEGF treatments have been widely studied and used in order to fight against cancers. Indeed 60% of all types of human tumors have an over-expression of VEGF-A (158). However, the anti-VEGF treatments show side effects that are related to CVD, such as hypertension, cardiac ischemia, and arterial thromboembolic events, demonstrating also the importance of the VEGF in CVD (156). However, the involvement of the VEGF-A in CVD is complex and the literature is contradictory. Studies performed in animal models yet show that the VEGF-A promotes neovascularization and inflammation, which produce a progression of the atherosclerosis and plaque instability (159-161).

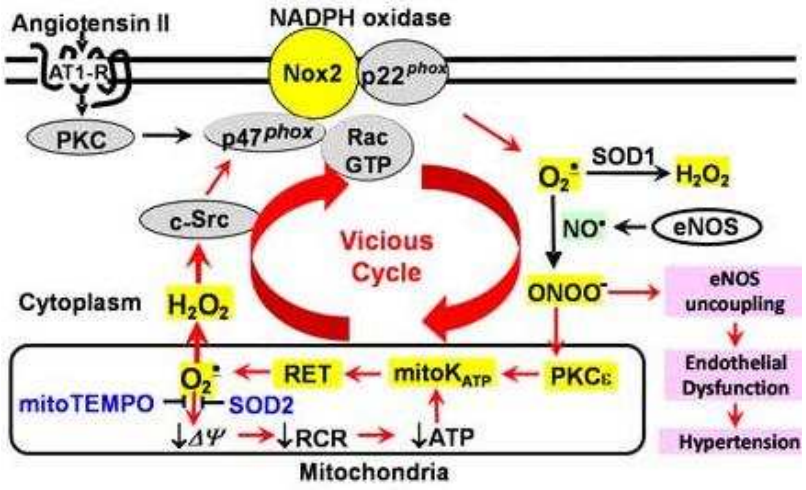
2.2.6 Oxidative stress (free radicals)

Oxidative stress appears when there is an imbalance between the amount of reactive oxygen species (ROS) and the biological system's ability to readily detoxify those reactive species. The ROS are formed as a natural product of the metabolism of oxygen and although have important roles in cell signaling and homeostasis, they have been associated with several diseases, including atherosclerosis (162).

ROS are highly reactive oxygen fragments and previously described cardiovascular risk factors, such as smoking, hypertension and hypercholesterolemia have been demonstrated to

increase the production of ROS (163). Evidences show that vascular NADPH oxidase, which in part is stimulated by angiotensin II, is a major source of production of ROS in arterial smooth muscle cells and endothelial cells, and thus in atherosclerosis (164) (Figure 19).

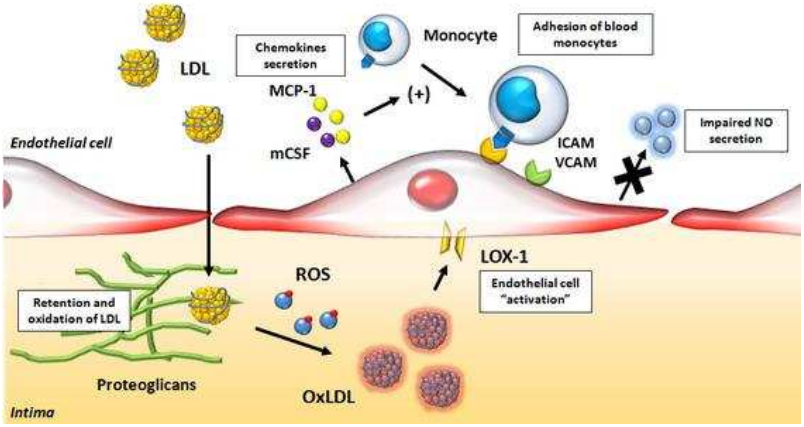
Figure 19: Production of ROS by angiotensin II and NADPH oxidase resulting in endothelial dysfunction and hypertension.



Source: Dikalov Lab. http://www.mc.vanderbilt.edu/labs/dikalovlab/index.html

One of the most studied negative effects of the ROS is the increase of the conversion of LDL to the oxidized LDL (ox-LDL). As explained before, the LDL by itself is a risk factor for atherosclerosis, but its demonstrated that oxidized LDL it become much more atherogenic and increased oxidative stress in vascular cells accelerates this conversion (165). The ox-LDL forms are much more prone to be retained by proteoglycans in the endothelium and increasing concentrations of LDL particles accelerates the atherosclerosis within the walls of arteries. It has been also demonstrated that ox-LDL particles induce expression of adhesion molecules involved in the trans-epithelial migration of monocytes and lymphocytes to the intima media of the arteries (166) (Figure 20). Finally, the final effect of the reactive oxygen species will be endothelial dysfunction and an increased vascular inflammatory response, which will accelerate the atherosclerotic process.

Figure 20: Role of ox-LDL in the endothelial dysfunction and increased inflammatory response



Source: Role of Oxidized LDL in Atherosclerosis. E. Leiva et al.

2.3 Genetics of Cardiovascular diseases

There are inherited genes and SNPs that together with the environmental factors participate in conferring risk of suffering from complex diseases, including CVD. Because of this, although an individual may not be born with a disease, he can have higher risks of acquiring the disease. This is called genetic predisposition or susceptibility. For example, a history of premature atherosclerotic CVD in a parent confers three times increased risk of this same disease in the offspring (167). In addition, CVD tends to manifest differently among different human populations. For instance, south Asians tend to have more hearth attacks as a result of CVD, while African populations tend to have strokes (168).

The understanding of genetic predisposition to CVD is necessary in order to advance through a more precise and effective treatment and management of the CVD in the future. During the last decades, and thanks to the mapping of the human genome, many correlations between specific genes and SNPs and phenotypes involved in CVD have been made. It helped to a better understanding of the actual biologic mechanisms involved in these complex diseases.

2.3.1 Approaches to discover genes for cardiovascular diseases

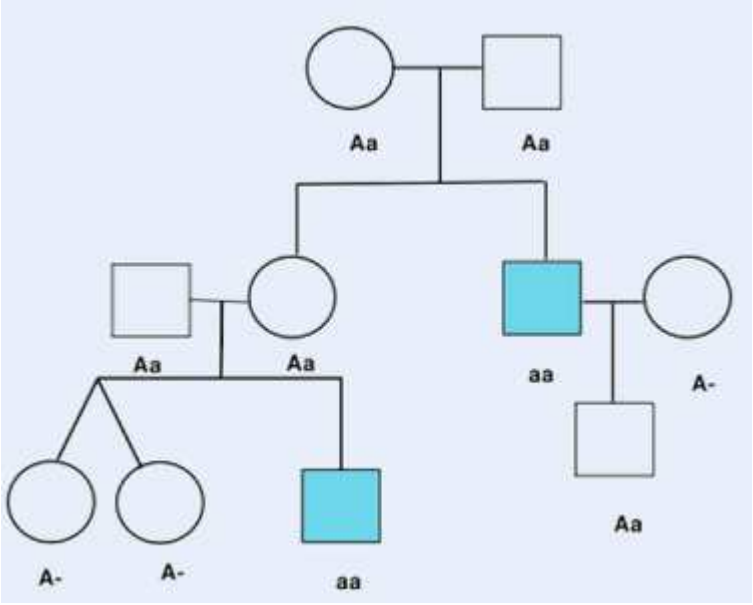
Two major approaches have been used to discover genes that confer risk for cardiovascular diseases in humans: linkage analysis and association studies.

2.3.1.1 Linkage analysis

Some CVD have a simple pattern of inheritance that is consistent with the ratios described by Mendel (Figure 21). Within these CVD, a single gene is conferring a large effect on the risk phenotype, however the frequency of such diseases are low among the human populations. For some of these Mendelian forms of CVD, the linkage analysis has been an effective way to detect the causal gene and mutation. Lehrman and colleages described the first Mendelian CVD in 1985, when they discovered a 5kb deletion which eliminated exons from the low-density lipoprotein receptor gene (LDLR) (169). Same approach of linkage analysis was used

detecting severe familial hypercholesterolemia, Mendelian forms of hypertension, hypertrophic cardiomyopathy, valve defects and hypertrophic cardiomyopathy, among others (170-174).

Figure 21: Example of a Mendelian pedigree pattern



Source: https://opencurriculum.org/5372/mendelian-inheritance/

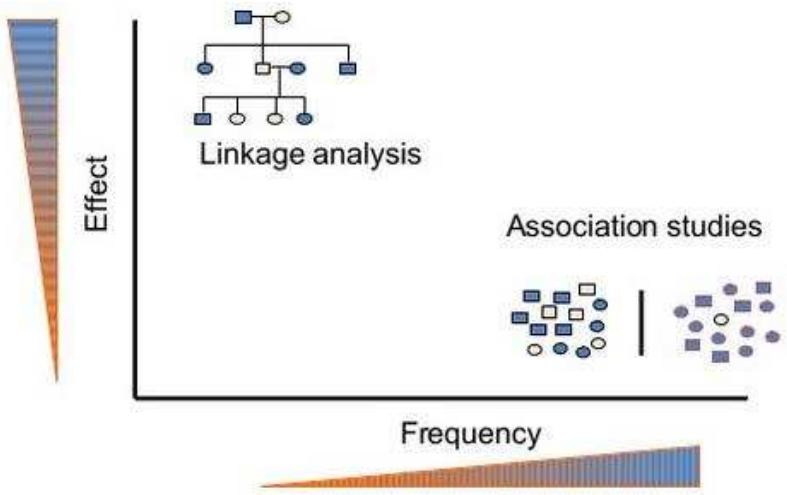
2.3.1.2 Association studies

Most of the CVD don't have simple patterns of inheritance, and several genes, together with environmental factors, are participating in their development. Among the complex traits and diseases that are dependent of several factors are the blood lipid levels, inflammatory molecules, atherosclerosis or myocardial infarction. The human genome (6) and HapMap projects (175) provided an enormous amount of information that were needed in order to genetically map the DNA sequences associated with these complex traits.

Unlike linkage analyses, the association studies are useful to detect more common genetic variants with small effects that are participating in the complex CVD (Figure 22). Nowadays, they are a major tool for identifying genes conferring susceptibility to complex CVD. Genetic

association studies aim to test whether a specific polymorphism is influencing a studied phenotype. Thus, by performing these association studies, we can determine whether a genetic variant is associated with a disease or trait. The most widely tested genetic markers are the SNPs. These are common DNA sequence variants among the different human populations. However, other markers such as microsatellite, insertion/deletions, copy-number variants (CNVs) and variable-number tandem repeats (VNTRs) are also starting to be used.

Figure 22: Differences between the linkage analysis and association studies

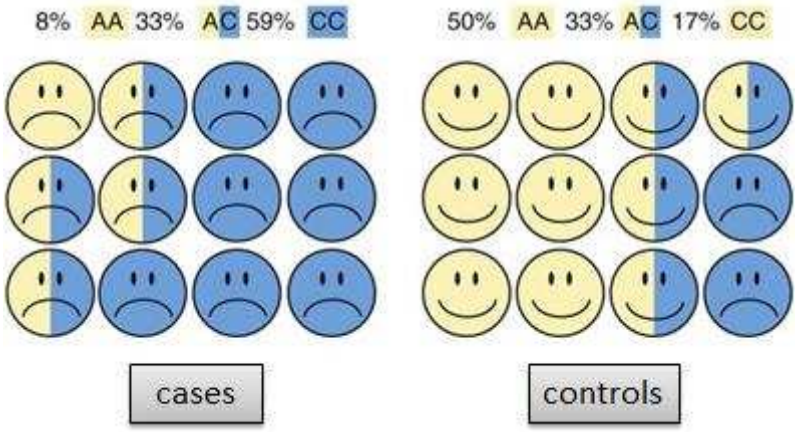


Different approaches are used among the association studies, including case-control association studies, family-based association studies and quantitative trait association studies.

Case-control studies are the most simple and commonly used approaches for genetic associations. The frequency of alleles or genotypes is compared between two groups (Figure 23): the "case" group with individuals diagnosed with a specific trait or disease, and the "control" group composed of unaffected/healthy individuals. Polymorphisms showing allelic frequency differences between the two groups could affect the risk of suffering from the disease being studied. One limitation of the case-control study is that it is susceptible to population stratification (variation of genotype frequencies between different human populations). Sometimes, the selection of an appropriate comparison group may be difficult,

and if populations from different ethnicities or countries are used for the same study, false positives can appear.

Figure 23: Case-control design for a genetic association study

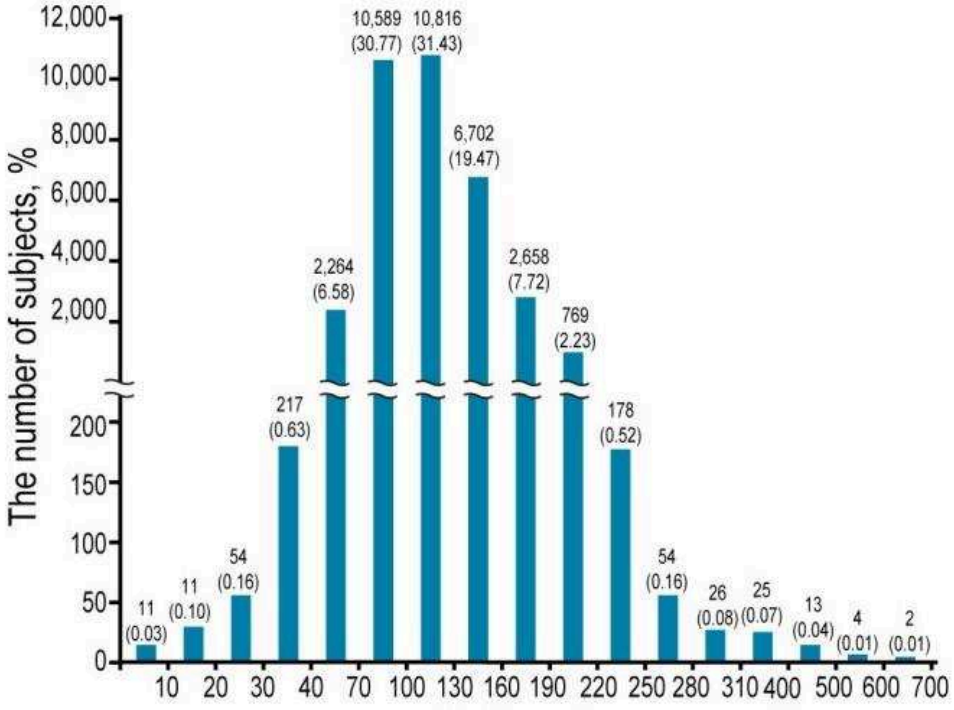


Source: Scott R; genetic susceptibility to periodontal disease

Family-based genetic association studies are another approach allowing to avoid the confounders and false positives that arise because of the population stratification. Considerable power can be gained using family-based association studies. However, it is difficult to gather big number of samples of well-characterized families. The goal is to measure association of genetic markers in families by transmission from parent to offspring. If an allele is increasing the risk of suffering from a disease, that allele is expected to be transmitted from parent to offspring more often in populations with the disease.

A third approach used in association studies is the quantitative trait association. Here, the goal is to measure the mean of differences of a phenotype of interest between genotypes. The traits analyzed by this method are quantitative (concentration levels of a certain molecules in the blood for example), instead of qualitative (presence or absence of a disease in an individual). The variations of these quantitative phenotypes in the population tend to be continuous, approximating a statistical normal distribution (Figure 24). Quantitative trait associations can be performed using an unrelated population sample or family-based populations.

Figure 24: Example of the distribution of LCL-cholesterol concentration, a quantitative trait.



Source: The Korean Journal of Internal Medicine

2.3.1.2.1 Genome-wide Association Studies

GWAS are an examination of a genome-wide set of genetic variants (typically SNPs) in different individuals to see if any variant is associated with a phenotype or disease. This approach is a genetic association study, and asks if the allele of a genetic variant is found more often than expected in individuals with the phenotype of interest. In contrast to previous methods, GWAS investigate the entire genome. Because of this, it is considered a non-candidate-driven approach.

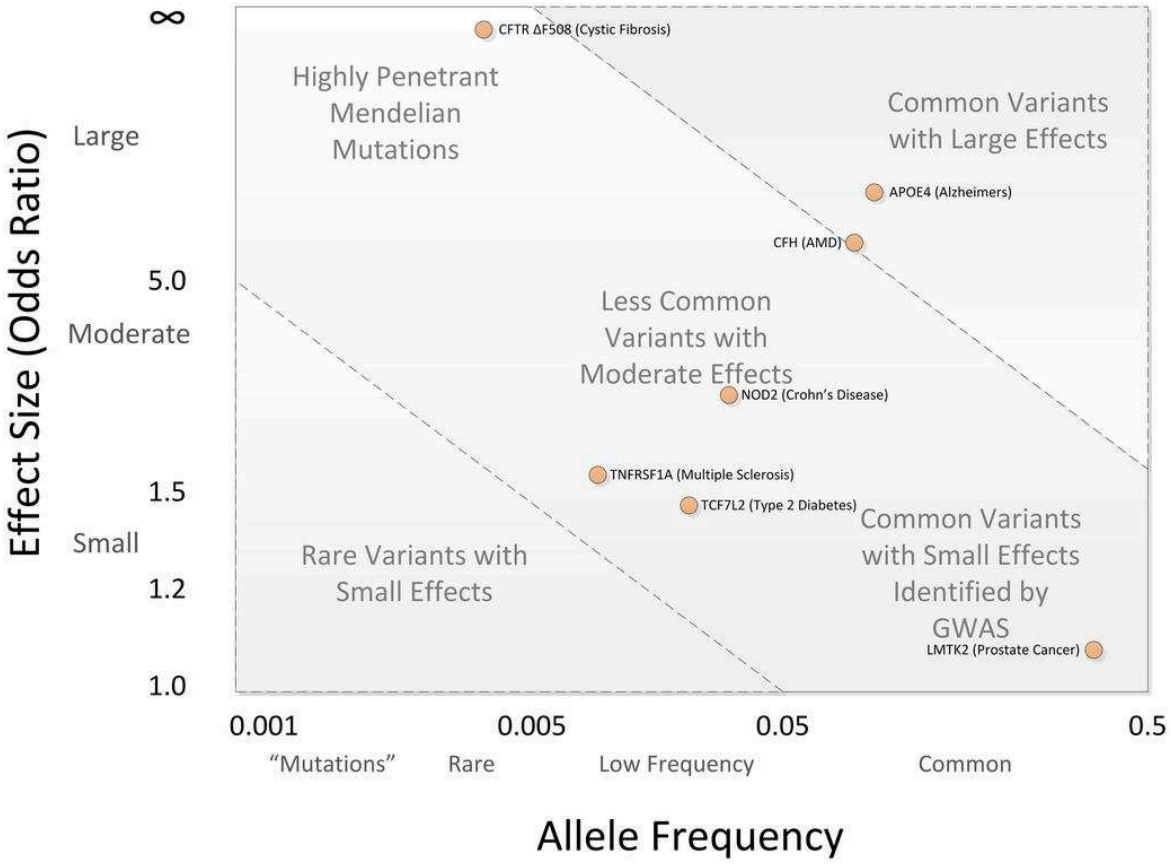
Before the use of GWAS, the genetic linkage in families was the principal method of genetic investigation. Although useful for the study of in single gene disorders (176), this method was not giving positive results in common and complex diseases such as the CVD (177). The development of the human genome (5) and the International HapMap Project (7) almost 15 years ago made available thousands of SNPs. This, together with the improvement of

computational approaches and biobanks repositories of human genetic material, enabled the development of GWAS. Also, the development of the methods of genotyping, by using genotyping arrays, was an important step towards the development of GWAS (178).

The first GWAS was published in 2005 and detected two SNPs associated with age-related macular degeneration (16). More than ten years later, over 3,000 GWAS have examined over 1,800 traits, and thousands of SNPs associations have been found (179). Nowadays, the GWAS are a powerful tool for investigating the genetic architecture of human complex diseases. The typical GWAS methodology includes regression modeling (linear and logistic) with adjustment for relevant covariates such as sex, age, BMI and race or ethnicity. Also, thresholds of significance are adjusted for multiple testing, the most commonly accepted threshold of genome-wide significance being at P **<5.0 x** 10^{-8} (180).

Typically, GWAS detect common genetic variants (SNPs) with small effects in complex diseases (Figure 25). Indeed, the National Human Genome Institute GWAS catalog lists over 3,600 SNPs identified with common diseases or traits, most of them having a small effect in these diseases (normally increasing disease risk between 1.2-2 times) (181). Results obtained by GWAS during the last decade suggest the hypothesis of common disease / common variant (CD/CV), which states that common disorders are likely influenced by genetic variations that are also common in the population. Although the CD/CV hypothesis certainly plays a role in complex diseases, much of the heritability for these conditions is not yet explained.

Figure 25: Spectrum of Disease Allele Effects showing allele frequency and effect size. Highly penetrant alleles for Mendelian disorders are extremely rare with large effect sizes (upper left), while most GWAS findings are associations of common SNPs with small effect sizes (lower right).



Source: William S. Bush; PLOS computational Biology

The final goal of GWAS is to uncover the genetic background of the complex diseases in order to use genetic risk factors for risk prediction of disease susceptibility, and for improving disease prevention as well as treatment strategies. One of the most successful applications of GWAS has been facilitating the understanding of the biological basis of genetic effects. This made possible the development of new pharmacologic therapies, by identifying DNA sequence variations that are associated with drug metabolism and efficacy as well as adverse effects. The advancement in this field accelerated significantly the pharmacogenetics and

gene-based personalized medicine, which aims to individualize prevention, diagnosis and treatment, by integrating genomics into medical practice.

Despite the success of GWAS, it is considered that this approach can be problematic because of the massive number of statistical tests performed. This presents an unprecedented potential for false-positive results (182). Other limitations of GWAS are the lack of well-defined control and study populations, insufficient sample size and control for population stratification. Besides, even if GWAS have associated several SNPs linked with the diseases and phenotypes, they frequently failed to identify clinically and biologically significant associations.

Linkage disequilibrium

Linkage disequilibrium (LD) is the non-random association of SNPs at different loci in a given population. Two SNPs are said to be in LD when the frequency of association of their different alleles is higher or lower than what would be expected if the loci were independent from each other and associated randomly (183).

The rate of LD is depending on multiple factors, such as population size, the number of founding chromosomes in the population, and the number of generations for which the population has existed. Thus, different human populations have different degrees and patterns of LD. Th*e two commonly used measures of LD are D' and* r^2 (8, 184), and the recombination events are scaled between 0 and 1. In genetic analysis the LD is generally showed as r^2. High r^2 values indicates that two SNPs are in strong LD, and thus, one allele of the first SNP is often observed with one allele of the second SNP, meaning that only one of the SNPs needs to be genotyped to capture the allelic variation of that specific genetic locus.

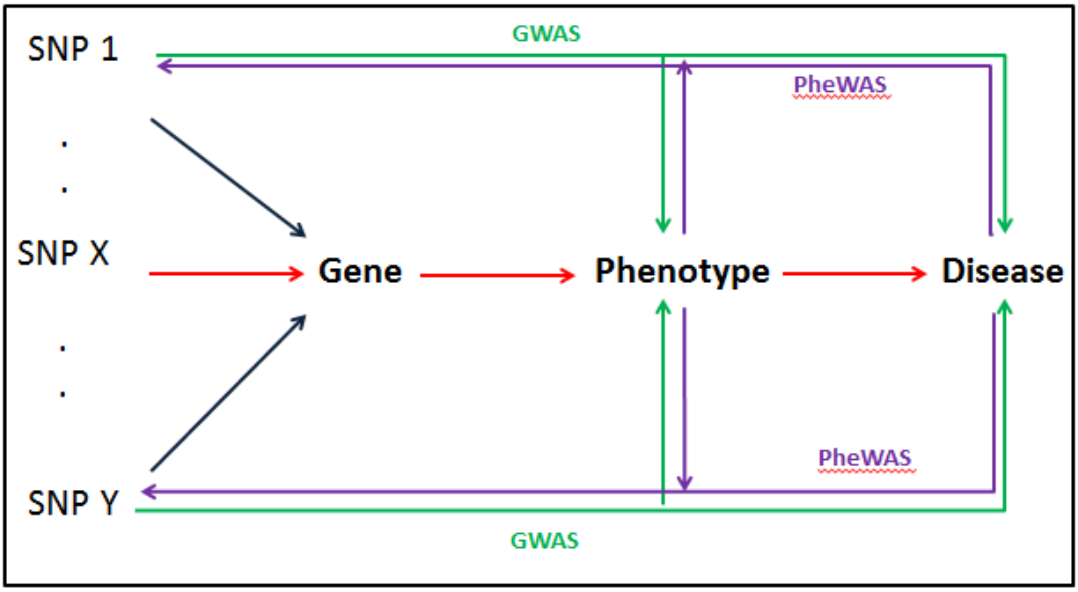
2.3.1.2.2 Phenome-wide Association Studies

As an alternative to GWAS and with the hope of overcoming some of its weak points, another approach was developed during the last years: Phenome-wide association studies. PheWAS can be used to examine the impact of one genetic variant across a broad range of human phenotypes (27, 185). It emerged in part from the availability of dense electronic health

record (EHR) data (186). In these datasets, patient's vital signs (height, weight, blood pressure…), current and past health conditions, as well as laboratory values (lipid profiles, complete blood counts, inflammatory molecules…) are included. Taken together, these data can be used to describe the phenome of a single patient.

The availability of densely phenotyped populations has made it possible to switch from "candidate phenotype" studies designed for certain outcomes to phenome-wide studies. The main difference between GWAS and PheWAS is that while GWAS attempts to relate many genetic variations to clinical traits, PheWAS attempts to relate many phenotypes to a single chosen gene or SNP (Figure 26).

Figure 26: Differences between GWAS and PheWAS approaches.

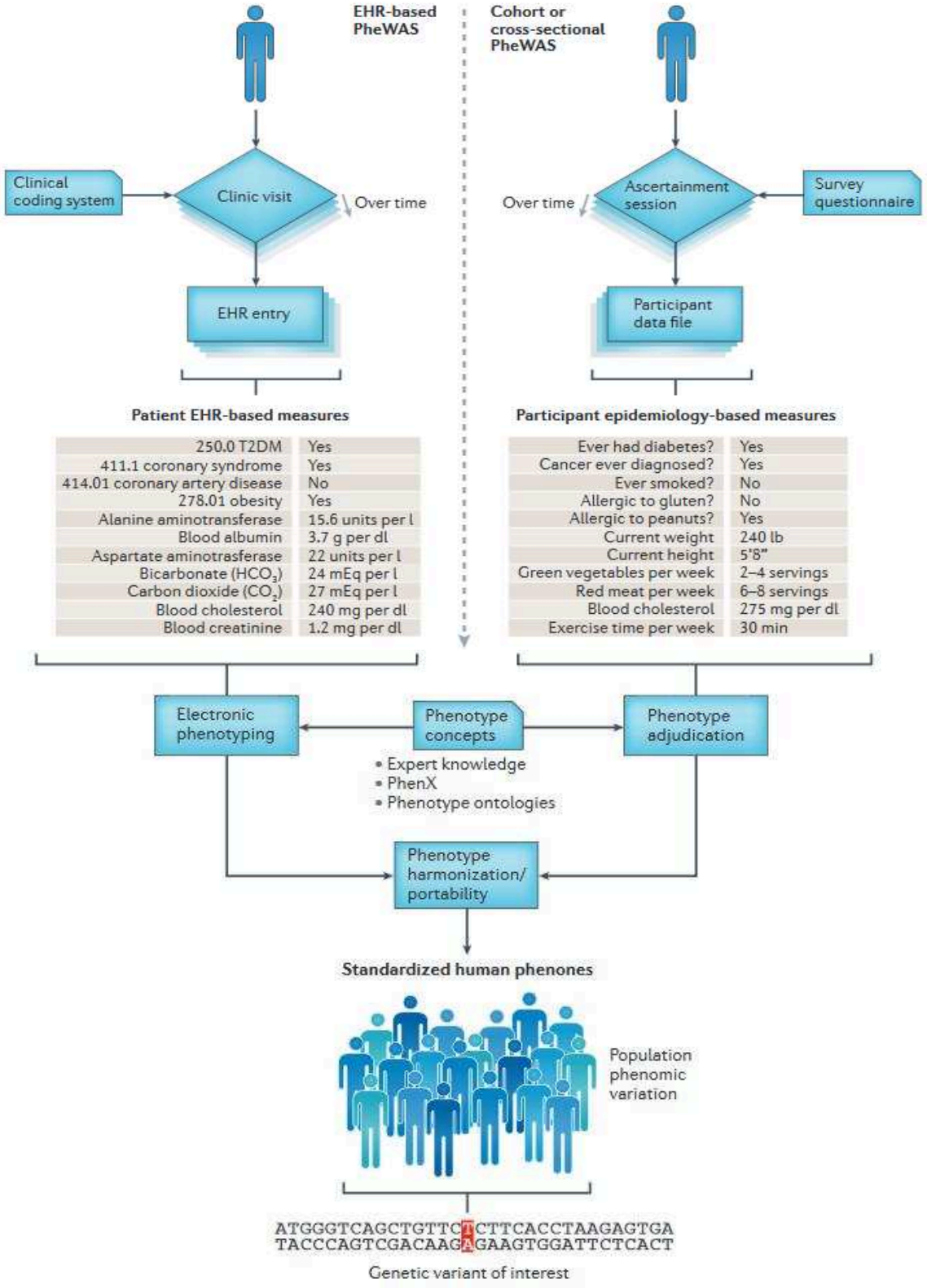


This antagonistic approach from GWAS has several advantages. First, it enables the deeper study of genetic variants already proven to be of special interest in clinical traits (for example those with established molecular functions or previously associated by GWAS with specific disease or phenotypes). Furthermore, analyzing the possible associations of one SNP with several phenotypes, allows to uncover pleiotropic effects of these polymorphisms.

Furthermore, some genetic effects may be dependent on life stages and environmental exposures (187), which can be studied in detail with more complete phenomes.

Current PheWAS methodology is similar to the GWAS methodology. Frequently, logistic regression modelling is performed to allow the adjustment for covariates. Also, significance thresholds are adjusted for multiple testing. The biggest difference from GWAS is the process of characterizing an individual's phenome (Figure 27). Many PheWAS use the EHR data, generally organized by the international classification of diseases (ICD) codes for classifying patients into case control groups depending in their phenome. However, phenomes can also be produced by epidemiological collections, where phenotypes are directly measured by physical examinations and laboratory measures (such as intermediate phenotypes and biomarkers), as well as by performing questionnaires.

Figure 27: Characterization of human phenome for PheWAS. Human phenome characterization requires a broad data collection system, either an EHR system or an epidemiological study (a cohort or cross-sectional study).



Source: Unravelling the human genome-phenome relationships using PheWAS.

PheWAS also have limitations, just as GWAS. The main one is the phenome resolution; the phenome is only as good as the phenotypes within. Classifying the phenotypes using ICD codes helps the investigators to comprehensively assemble a wide spectrum of phenotypes in an efficient and cost-effective manner. However, not every phenotype/ICD9 code is equal, and it is unrealistic to develop sophisticated logic rules to describe every phenotype. Furthermore, differences across populations may affect the ability to validate findings in PheWAS, an even bigger problem than in GWAS. Indeed, differences across populations may go beyond genetics. There may be differences in clinical care and techniques for obtaining intermediate phenotypes and biomarkers can also be significantly different, made by different physicians and across different healthcare providers.

2.3.1.2.3 Candidate gene

Within the genetic association studies, the candidate gene approach focuses on associations between genetic variations of previously chosen genes of interest and phenotypes or disease traits. Traditional candidate gene approach has been widely used for the identification of genes responsible of complex diseases. However it has been largely limited by its dependence on previous knowledge about the biochemical, physiological or functional aspects of possible candidates. These limitations resulted in a fatal information bottleneck, becoming an impediment for further applications of traditional candidate gene approach.

However, the progress made in genomics during the last decade are allowing a better insight into disease mechanisms and highlighting potential regions of interest in the genome. Especially, the development of GWAS and QTL has allowed the detection of common variations across the genome that are located within or near potential candidate genes. Candidate genes are selected based on previously known biological, physiological, or functional relevance to the disease of interest. Also the availability of big genetic databases have facilitated the researchers to find new candidate gene targets (188).

The candidate gene approaches seeks to balance the use of data while attempting to minimize the possibilities of having false positive or negative results (189). It is a powerful approach used to study complex diseases, especially if it is combined with other complementary approaches. However there are also several criticisms of this approach. The principal one is

the difficulty of replicating the results obtained from it, which is thought to be because of population stratification (190).

2.3.2 Missing heritability problem

During the last decade, GWAS, PheWAS and QTL approaches have improved the knowledge about the genetic background of CVD. When GWAS came out, the CD/CV hypothesis was dominating the field (191-193). This hypothesis postulates that few loci of moderate effect and intermediate frequency each explain several per cent of disease risk in a population. The forecast were optimistic, and the scientific community thought that the large genetic contributions of many traits and diseases would soon be mapped and connected to specific genes and their genetic variants.

However, more than a decade later, the genetic background of complex diseases still remain unexplained, and the so-called "missing heritability problem" emerged. Indeed, the polymorphisms detected by GWAS have been able to explain only a small minority of the inferred genetic variance (18, 194).

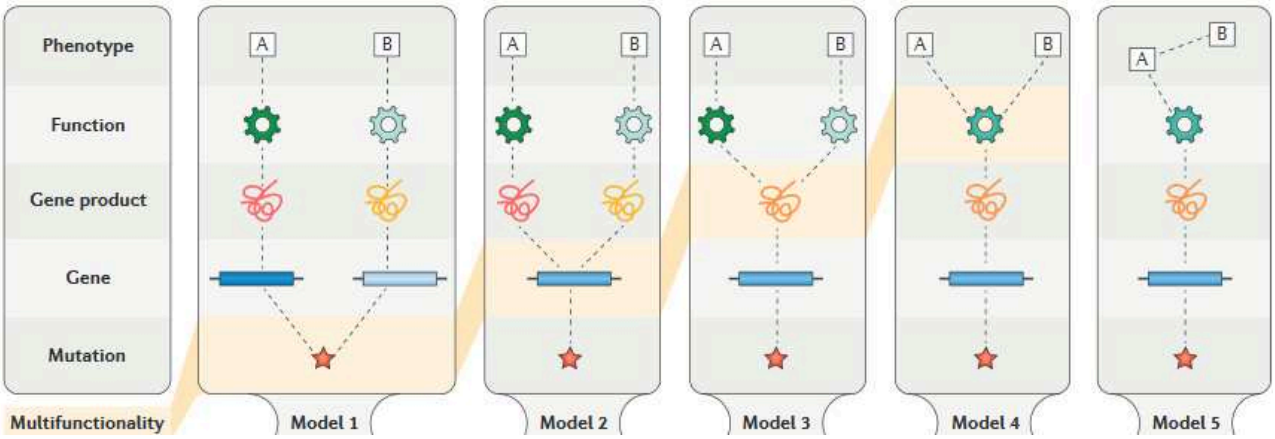
2.3.3 Genetic pleiotropy

Pleiotropy was first noticed and defined by the developmental geneticist Ludwig plate in 1910. It is a common phenomenon, is a central feature in genetics, and has broad implications not only in disease (195), but also for evolution (196, 197), development (198) and ageing (199). Pleiotropy occurs when one gene or polymorphism influences two or more unrelated phenotypes (Figure 28). This can result in comorbidities in which two disease states coexist in the same individual.

Despite the fact that pleiotropy was known for more than a century now, during decades, few empirical data were available. Only during the last years and thanks to the large data sets generated by functional genomics, the scientific community started to uncover the real importance of pleiotropic effects in complex diseases and comorbidities. Indeed, many disease share genetic architecture (200, 201). The databases of GWAS Catalog, the National Human Genome Research Institute (NHGRI) and the European Bioinformatics Institute

(EMBL-EBI) (181) sowed that around 17% of genes or gene regions and 5% of SNPs associated with different traits by GWAS are at least associated with more than one different phenotype (200). Notable overlap of shared disease risk variants and pathways can be found in immune mediated diseases, which proves it's the high number of pleiotopic effects involved (202, 203). There is thought to be a positive relation between pleiotropy and the number of biological processes in which a gene is involved.

Figure 28: Five models of multifunctionality and pleiotropy.



Source: Network biology concepts in complex disease comorbidities.

Although no clear consensus has yet been reached to define pleiotropy consistently in a molecular context, Jessica Xin Hu et al., made a classification of 5 different models of pleiotropy (Figure 28). These five models describe de mechanisms of how one genotype can lead to multiple different phenotypes. Models 1-4 cover mechanisms that have been described as "true" pleiotropy in the literature. Model 1: a mutation that affects multiple genes through, for example, insertions or deletions that span multiple genes or variants that affect the expression of more than one gene. Model 2: a gene with multiple functions through, for example, alternative splicing (204) or exon shipping (205). Model 3: a gene product, such as a protein, that contains multiple domains with separate functions (206) or that has different functions in different tissues (207). Model 4: a gene product with one function that is involved in multiple phenotypes, for example, by being present in multiple tissues where it is involved

in the same biological process. Model 5: a case in which one phenotype leads to a second phenotype through physiological means. This last case is often considered as "mediated pleiotropy" as the genetic factor only has a direct effect on one of the phenotypes.

Antagonistic pleiotropy

Pleiotropy causes compromises among adaptations of different traits, and sometimes a gene may be at the same time harmful and beneficial, because a mutation that is advantageous to one trait may be harmful to another trait. This phenomenon is called antagonistic pleiotropy. It was first described by G.C. Williams in 1957, suggesting that the expression of some genes *are beneficial for the organism's early life (when the fertility is higher), but turning to* decrease fitness later in life, giving an evolutionary explanation for senescence. An example of this phenomenon is the p53 gene, which is a suppressor of cancer, but also suppresses stem cells, which renovates the deteriorated tissue (208).

The detection of genes with pleiotropic effects helps to uncover the biological mechanisms behind complex phenotypes and diseases. Detecting shared genetics and pathways is especially interesting to understand exactly how the etiology of different diseases overlaps. Because of this, the interest in the detection of human pleiotropic genes is increasing (Table 7) (209). Indeed, detecting pleiotropy in genes involved in complex diseases could be of great interest to advance in the development of drug targets (210). Utilization of pleiotropic data could provide a robust approach for identifying new drug candidates, and thus for drug repositioning. Drug repositioning is the process of discovering new indications for existing drugs, and is a process that is becoming very important in drug development, as success rates for novel drugs in clinical trials decrease and costs increase (211). This interest about pleiotropy comes together with the recent emphasis in today's precision medicine, where medical care and prevention are designed to take into account individuals genetic variability, lifestyle and environmental exposures (212).

Table 7: Approaches for investigating pleiotropy and multifunctionality.

Dynamic term	Approaches	Description	Refs
Pleiotropy and multifunctionality	GWAS and joint GWAS	Identifying candidate pleiotropic variants by GWAS	3,166
	PheWAS	Identifying candidate pleiotropic variants using genome-phenome-linked data	66,167
	NetWAS	Identifying proteins with tissue-specific functions	12
	BUHMBOX	Distinguishing pleiotropy and clinical heterogeneity	69
	eQTL and QTL	Empirical data to estimate genome-wide pleiotropy	6
	Mendelian randomization	Identification of mediated pleiotropy	168,169

Source: Network biology concepts in complex disease comorbidities.

Personalized medicine

Also known as precision medicine, personalized medicine is "a form of medicine that uses information about a person's genes, proteins, and environment to prevent, diagnose, and treat *disease" (National Cancer Institute 2011). Within personalized medicine, personalized* genomics plays a vital role. Personalized genomics focuses on the integration of genetics into medical practice, by including the use of variants as markers for diagnosis, prognosis, prevention as well as targets for treatment. During the last years, this concept has been dramatically improved. This has been possible thanks to the development of large-scale genetic databases, new methods of patient characterization (such as proteomics, metabolomics and transcriptomics), and new computational approaches for analyzing large sets of data.

CHAPTER III

RESULTS AND DISCUSSION

Publication 1°

TREM-1 SNP rs2234246 regulates TREM-1 protein and mRNA levels and is associated with plasma levels of L-selectin

Alex-Ander ALDASORO ARGUINANO[1], Sébastien DADÉ[1], Maria STATHOPOULOU[1], Marc DERIVE[2] , Ndeye COUMBA NDIAYE[1], Ting XIE[1], Christine MASSON[1], Sébastien GIBOT[3], Sophie VISVIKIS-SIEST[1,4]

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Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) is a member of a recently discovered immunoglobulin family (135, 139). It increases the inflammatory responses of the neutrophils and monocytes (213) and amplifies the production of pro-inflammatory cytokines (TNF-α and IL-1beta) (214). Furthermore, it has been reported that migrating neutrophils bind to TREM-1 (215), suggesting an important role of this receptor in the recruitment and mobilization of neutrophils and monocytes to the arterial intima (148). Although TREM-1 was initially characterized for its role during the pathophysiology of septic shock (135), there are today increasing evidences that it is also implicated in other acute and chronic inflammatory diseases of non-infectious etiology, such as rheumatoid arthritis (141), atherosclerosis (142), acute myocardial infarction (149) and critical limb ischemia (143). Also, the serum levels of soluble TREM-1 (sTREM-1) correlate with the severity of these diseases (141-143, 145-148, 216, 217). Moreover, the genetic invalidation or pharmacological blockage of TREM-1 results in a reduced inflammatory state and improved outcome in animal models (137, 218). The mechanistic process by which TREM-1 participates in inflammatory cell extravasation during inflammatory diseases remains to be clarified.

Polymorphisms within the TREM-1 gene have been associated with the development of acute inflammation and sepsis, but also with coronary artery disease. Indeed, the polymorphism rs4711668 has been associated with severe coronary atherosclerosis in a Russian population (219). However, it is still unknown whether the levels of sTREM-1 are genetically regulated, and no polymorphism has been yet reported to affect its expression levels. Furthermore, another interesting aspect of TREM-1 regulation is that although there exists a specific splicing variant for the soluble form of the protein (TREM-1sv) (220), some authors argue

that the mechanism of shedding of the membrane bound TREM-1 by metalloproteases is the main contributor for the release of sTREM-1 (215, 221).

Objective

The goal of this study was to investigate whether polymorphisms within TREM-1 are regulating the variation of serum TREM-1 levels (sTREM-1 and TREM-1sv) and the expression levels of their mRNA splicing variants (mbTREM-1 and TREM-1sv). Furthermore, we aimed to point out associations between polymorphisms on TREM-1 and blood levels of E-selectin, L-selectin and P-selectin, which are involved in the early stages of atherosclerotic processes.

Results

Association studies between the 10 polymorphisms in TREM-1 and serum levels of sTREM-1/TREM-1sv

Association analyses were performed in 30 individuals of the SFS (discovery population) for the 10 SNPs and the levels of TREM-1 in serum using the additive, dominant and recessive inheritance models (P-value cut-off was set at 0.05/10=0.005). Only for rs2234246 we found significant results. According to the additive model, the minor allele T of rs2234246 was associated with increased levels of sTREM-1 (p-value = 0.003, $\beta = 0.3$). When the dominant model was used, this association was even stronger (p-value = 0.0003, $\beta = 0.49$), and the variance in the serum TREM-1 levels explained by the model is of 33% in the discovery population. The SNP rs2234246 was then genotyped in 80 additional and independent individuals (replication population). Additive and dominant models confirmed the association between the minor allele T and sTREM-1 levels (p-values = 0.0007 and 0.0017 respectively, $\beta = 0.13$ and 0.22 respectively). The TREM-1 variance explained by the model is of 13% in the replication population. The serum TREM-1sv levels, generated by the mRNA splicing variant TREM-1sv, were also measured in 78 individuals of the replication population. The TREM-1sv levels were detected only in four of the 78 samples studied, allowing no statistical analysis.

Association studies between the rs2234246 in TREM-1 and mRNA expression levels (mbTREM-1 and TREM-1sv)

Association analyses were performed between rs2234246 and the expression levels of the two different alternative splicing isoforms of TREM-1 (mbTREM-1 and TREM-1sv mRNAs) in peripheral blood mononuclear cells (PBMCs) of the 30 individuals of the discovery population (threshold of significance was 0.05/1 SNP/2 phenotypes=0.025). Interestingly, the SNP rs2234246 showed significant association with increased mRNA levels of the splicing form that codes for mbTREM-1 (additive model, p-value = 0.007, $\beta = 0.49$), whereas it was not associated with the levels of mRNA coding TREM-1sv.

Association studies of the rs2234246 polymorphism in TREM-1 with soluble selectins' levels

The SNP rs2234246 was further genotyped in 351 individuals. Association analyses were performed between the SNP rs2234246 and the soluble L, P and E-selectins (sL-, sP- and sE-selectin) levels (cut-off value for significance was set to 0.05/1 SNP/3 phenotypes=0.016). Only the association with sL-selectin was significant (p-value = 0.011, $\beta = 0.05$) and explained a total of 2.1% of the variability of the sL-selectin. The minor allele T of the polymorphism was significantly associated with increased sL-selectin levels. No significant association was observed for sP- and sE-selectin levels. The association between the minor allele T and sL-selectin levels was then confirmed in the 80 individuals of the replication population (p-value = 0.018, $\beta = 0.03$) (cut-off value for significance was set to 0.05, as only 1 SNP was tested) and association explained a total of 4.3% of the variability of the sL-selectin.

Regulatory environment of TREM-1 rs2234246

The rs2234246 polymorphism is positioned halfway between an open chromatin zone and a promoter flanking region. The open chromatin zone is active only in monocytes CD14+, which are present in PBMCs. Furthermore, the promoter flanking region is in an active state only in two cell types: the monocytes CD14+ and CD14+CD16-. The polymorphism is located in the 3'UTR region of the two mRNA splicing variants studied: mbTREM-1 (TREM1-001) and TREM-1sv (TREM1-002).

Epigenetic footprint of TREM-1 rs2234246

We established the epigenetic profiles (methylation / acetylation) of rs2234246 according to the cell type involved. A specific epigenetic pattern emerged in monocytes (CD14+ and CD14+CD16-), vein blood neutrophils, eosinophils and macrophages in particular by the

presence of H3K36me3 and H3K4me1. This specific methylation pattern is not present in the other 18 cell types investigated. Especially the presence of H3K36me3 is interesting, as it is considered as a hallmark of actively transcribed regions. Thus, we speculate that the genetic region where the SNP rs2234246 is located is prone to higher expression levels of the TREM-1 gene.

Potential transcription factor binding sites in the rs2234246 locus

According to the TRANSFAC R.3.4. database, the potential transcription factors binding the locus of the SNP rs2234246 could be different depending on the allele. When the major allele is present (C), the specific potential transcription factors that are able to bind are REL, CAAT and NFY, showing a similarity score of 0.838, 0.801 and 0.771 respectively. When the minor allele is present (T), the specific potential transcription factors that are able to bind are AP4, LMO2 and TAL1, with a similarity score of 0.857, 0.791 and 0.773, respectively.

Discussion

We found that rs2234246, located in the mRNA 3'UTR region of TREM-1, was associated with sTREM-1 protein levels. So far, this is the first polymorphism that has been reported to affect the sTREM-1 levels. It has been widely reported that increased levels of sTREM-1 are correlated to the severity and poor prognosis of the diseases where it is involved (141-143, 145-148, 216, 217). The most recent studies have showed that TREM-1 deletion or blockade is associated with up to 60% reduction of the development of atherosclerosis (142). Thus, the T allele of rs2234246 may be considered as a risk factor, while the C allele may be a protective factor for these diseases. In this context, it is important to note that a previous study has associated the minor allele of the SNP rs2234246 with an increased 3.1 odds ratio for septic shock (222). This finding strengthens our hypothesis on the utility of the polymorphisms located in TREM-1 gene in risk stratification. We have also associated the T allele of the SNP rs2234246 with the expression levels of mbTREM-1 mRNA in PBMCs, conferring functional properties to this polymorphism. Our results, taking also into consideration that TREM-1sv protein levels were not present in the serum, support the hypothesis that the levels of sTREM-1 are controlled post transcriptionally by metalloproteases, rather than by alternatively spliced forms of RNA (215, 221).

In our study, we demonstrated for the first time that the rs2234246 polymorphism is specifically correlated with increased plasma levels of L –selectin which has been showed to

be important in the recruitment of monocytes and neutrophils to sites of acute and chronic inflammation (223). At the same time, the number of studies supporting the importance of TREM-1 in the trans-epithelial migration of neutrophils and monocytes is increasing. Migrating neutrophils in septic patients have been found to bind to TREM-1 (215). Also, it has been previously demonstrated that TREM-1 is crucially involved in leukocyte recruitment after myocardial infarction and atherosclerosis (142, 149) and have been demonstrated a five-fold decrease in the number of recruited neutrophils when TREM-1 was inhibited pharmacologically (149).

The conclusion of our work supports the hypothesis that TREM-1 is involved in the trans-epithelial migration process of leukocytes, more specifically of monocytes, that could be effective through a higher level of inflammation, which could be observed with an overexpression of sL-selectin.

PLOS ONE

RESEARCH ARTICLE

TREM-1 SNP rs2234246 regulates TREM-1 protein and mRNA levels and is associated with plasma levels of L-selectin

Alex-Ander Aldasoro Arguinano[1], Sébastien Dadé[1], Maria Stathopoulou[1], Marc Derive[2], Ndeye Coumba Ndiaye[1], Ting Xie[1], Christine Masson[1], Sébastien Gibot[3], Sophie Visvikis-Siest[1,4]*

1 UMR INSERM U1122; IGE-PCV 'Interactions Gène-Environnement en Physiopathologie Cardiovasculaire', Faculté de Pharmacie–Université de Lorraine, Nancy, France, **2** INOTREM, Nancy, France, **3** Réanimation Médicale, Hôpital Central, Nancy, France, **4** Department of Internal Medicine and Geriatrics, CHU Technopôle Nancy-Brabois, Rue du Morvan, Vandoeuvre-lès-Nancy, France

* sophie.visvikis-siest@inserm.fr

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Abstract

High levels of TREM-1 are associated with cardiovascular and inflammatory diseases risks and the most recent studies have showed that ***TREM-1*** deletion or blockade is associated with up to 60% reduction of the development of atherosclerosis. So far, it is unknown whether the levels of TREM-1 protein are genetically regulated. Moreover, TREM family receptors have been suggested to regulate the cellular adhesion process. The goal of this study was to investigate whether polymorphisms within ***TREM-1*** are regulating the variants of serum TREM-1 levels and the expression levels of their mRNA. Furthermore, we aimed to point out associations between polymorphisms on ***TREM-1*** and blood levels of selectins. Among the 10 SNPs studied, the minor allele T of rs2234246, was associated with increased sTREM-1 in the discovery population (p-value = 0.003), explaining 33% of its variance, and with increased levels of mRNA (p-value = 0.007). The same allele was associated with increased soluble L-selectin levels (p-value = 0.011). The higher levels of sTREM-1 and L-selectin were confirmed in the replication population (p-value = 0.0007 and p-value = 0.018 respectively). We demonstrated for the first time one SNP on ***TREM-1***, affecting its expression levels. These novel results, support the hypothesis that TREM-1 affects monocytes extravasation and accumulation processes leading to atherogenesis and atherosclerotic plaque progression, possibly through increased inflammation and subsequent higher expression of sL-selectin.

Introduction

Atherosclerosis is a multifactorial disease affecting arterial blood vessels due to a chronic inflammatory response. One of the early stages of atherosclerosis is the endothelial adhesion/infiltration of white blood cells to the arterial sites, attracted by different pro-inflammatory molecules [1, 2]. This accumulative process gradually promotes inflammation and, by doing so, accelerates the atherosclerotic progression.

preparation of the manuscript. The specific roles of these authors are articulated in the "author contributions" section. Our commercial affiliation did not play any funding role in our study.

Competing interests: We have read and understood PLOS ONE policy on declaration of interests and declare the following interests: The author Marc Derive and Sebastien Gibot are co-founders of INOTREM SA, a Company developing TREM-1 inhibitors. The INOTREM Company supported the study by contributing in the mentorship external to the core team, as well as contributing to the review & editing of the pre-publication stages. A patent has been filed in Europe in March 24th, 2017 (Patent application number EP17160945.6). The applicants are INSERM (Institut National de la Sante et de la Recherche Medicale), UNIVERSITY OF LORRAINE, Regional University Hospital of Nancy (CENTRE HOSPITALIER REGIONAL UNIVERSITAIRE DE NANCY) and INOTREM SA. The inventors are Sophie Visvikis-Siest, Alex-Ander Aldasoro Arguinano, Marc Derive and Sebastien Gibot. These competing interests do not alter our adherence to PLOS ONE policies on sharing data and materials.

Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) is a member of the recently discovered immunoglobulin family [3, 4]. It increases the inflammatory responses of the neutrophils and monocytes [5] and amplifies the production of pro-inflammatory cytokines (TNFα and IL-1beta) [6]. Furthermore, it has been reported that migrating neutrophils bind to TREM-1 [7], suggesting an important role of this receptor in the recruitment and mobilization of neutrophils and monocytes to the arterial intima [8]. Although TREM-1 was initially characterized for its role during the pathophysiology of septic shock [3], there are today increasing evidences that it is also implicated in other acute and chronic inflammatory diseases of non-infectious etiology, such as rheumatoid arthritis [9], atherosclerosis [10], acute myocardial infarction [11] and critical limb ischemia [12]. Activation of innate immunity and inflammatory cells recruitment and extravasation is a common feature of these diseases in which TREM-1 seems to be a central player [8, 10, 13]. Also, the serum levels of soluble TREM-1 (sTREM-1) correlate with the severity of these diseases [8–10, 12, 14–18]. Moreover, the genetic invalidation or pharmacological blockage of TREM-1 results in a reduced inflammatory state and improved outcome in animal models [19, 20]. The mechanistic process by which TREM-1 participates in inflammatory cell extravasation during inflammatory diseases remains to be clarified. It is well known the important role that the adhesion molecules, and specially the selectins, play during this specific process [21–23]. During the last two decades, the importance of the adhesion molecules in the process of inflammatory cells adhesion and trans-endothelial migration, which contributes to pathological inflammation and thrombosis in many preclinical diseases including atherosclerosis, has been widely studied [21, 24–26]. Thus, TREM-1 could be in close connection with the selectins.

TREM-1 gene polymorphisms have been associated with the development of acute inflammation and sepsis, but also with coronary artery disease. Indeed, the polymorphism rs4711 668, which is located within the *TREM-1* gene has been associated with severe coronary atherosclerosis in a Russian population [27]. However, it is still unknown whether the levels of sTREM-1 are genetically regulated, and no polymorphism has been yet reported to affect its expression levels. Furthermore, another interesting part of TREM-1 regulation is that although there exists a specific splicing variant for the soluble form of the protein (TREM-1sv) [28], some authors argue that the mechanism of shedding of the membrane bound TREM-1 by metalloproteases is the main contributor for the release of sTREM-1 [7, 29].

In the present study, we investigated whether single nucleotide polymorphisms (SNPs) within or near the *TREM-1* gene were associated with soluble TREM-1 (sTREM-1 and TREM-1sv) serum levels and with the expression levels of two TREM-1 splicing isoforms, more precisely, the membrane form (mbTREM-1) and the soluble form (TREM-1sv). Moreover, we investigated the associations between the significant in the above associations SNPs and levels of soluble E-selectin, L-selectin and P-selectin (sE-selectin, sL-selectin and sP-selectin), which are involved in the early stages of atherosclerotic processes.

Material and methods

Ethics statement

The samples are part of a human sample storage platform: the Biological Resources Centre 'Interactions Gène- Environnement en Physiopathologie CardioVasculaire' (BRC IGE-PCV—number BB-0033-00051) in Nancy, East of France. All participants gave a written informed consent. All the populations involved in this study were recruited in accordance with the latest version of the Declaration of Helsinki for Ethical Principles for Medical Research Involving Human Subjects. All the protocols were approved by the local ethics committees for the

protection of subjects for biomedical research: the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB).

Study populations

The population enrolled in this study makes part of the STANISLAS Family Study (SFS) [30]. Participants were of French origin and were apparently in good health, not under lipid-lowering and/or cardiovascular drug treatment and free from chronic diseases. This cohort is a longitudinal family study designed to investigate factors related to cardio-vascular disease. The clinical data of the investigated individuals were obtained at the Centre for Preventive Medicine (CMP) of Vandoeuvre-lés-Nancy. Participants were of European descent and came from the Vosges and the South of Meurthe-et-Moselle, in the East of France. Among them, 30 unrelated individuals were used as discovery cohort for the selection of the *TREM-1* SNPs and for their relation with TREM-1 levels. A population of 351 unrelated individuals was used as discovery cohort for the associations with the adhesion molecules levels.

An independent population (n = 80), available in the Biological Resources Centre 'Interactions Gène- Environnement en Physiopathologie CardioVasculaire' (BRC IGE-PCV, number BB-0033-00051), composed of unrelated adults of French origin was used as replication population for the results of associations of SNPs with TREM-1 and adhesion molecules levels.

During and after the data collection, authors had access to information that could identify individual participants.

Data collection and biological measurements

Body mass index (BMI) was measured using the Quetelet's formula: weight divided by height squared (kg/m^2). Blood samples were taken from the individuals after an overnight fast. Plasma and serum samples for adhesion molecules and TREM-1 measurements were frozen at -80˚C until analysis.

Plasma levels of E-selectin, L-selectin and P-selectin were measured with enzyme-linked immunosorbent assay (R&D Systems, Abington, UK). Serum levels of soluble TREM-1 were measured with a double antibody sandwich ELISA assay (Quantikine Human TREM-1 Immunoassay ELISA Kit; R&D Systems, Minneapolis, MN, USA) using the iMARK Microplate Absorbance Reader (Bio-Rad).

All molecules were measured in duplicate and according to manufacturers' instructions.

TREM-1sv levels were measured using a home-made ELISA assay adapted from Duo-Set ELISA assay (R&D Systems, hTREM-1 Duo-Set ELISA assay) and using a monoclonal anti-TREM-1sv antibody (R&D Systems) as capture antibody. A TREM-1sv recombinant protein was used as standard protein for quantification [28]. The sensitivity of the detection of TREM-1sv was of 15pg/mL.

SNPs selection–Bioinformatics analyses

A bibliographic search of the SNPs within or near *TREM-1* was performed. The HuGE navigator (https://phgkb.cdc.gov/PHGKB/phgHome.action?action=home) and the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) were used for the selection of SNPs for the further investigations. Only SNPs with a minor allele frequency >5% according to HapMap were selected.

To point out if the significant SNPs are located in regulation zones (promoter, enhancer, silencers) of the *TREM-1* gene and are linked to specific epigenetic profiles (acetylation/methylation), bioinformatics analyses were performed comparing different cell types.

The SNPs were localised on the Human genome (GRCh38.p7) using Ensembl browser [31]. Identification of potent regulation zones and establishment of epigenetic profiles according to cell types, were determined using also this browser. Regulation and epigenetic profiles have been obtained using 23 different cell types from 19 healthy and 4 cancerous individuals from Ensembl release 87.

The possible influence of the SNPs on the transcription factor binding site (TFBS) was investigated by using the transcription factor database TRANSFAC R.3.4 [32].

Genotyping

DNA was extracted from all participants, and relative biobanks have been constructed inside the BRC IGE-PCV. The SNPs were genotyped by Genoscreen (http://genoscreen.fr), using a Sequenom iPLEX Gold assay-Medium Throughput Genotyping Technology.

Gene expression investigations

Gene expression analysis was performed on peripheral blood mononuclear cells (PBMCs) of a subsample of 30 individuals from the SFS. PBMCs were isolated by centrifugation on a density gradient of Ficoll. The RNA was extracted from PBMCs with the MagNAPure automate, using the MagNA Pure LC RNA HP isolation kit protocol (Roche Diagnostics, France) and the RNA quality was measured by the NanoDrop 1000 Spectrophotometer (Thermo Scientific). Total RNA was used to generate first-strand complementary DNA (cDNA) with the C1000 Thermal cycler (Bio-Rad) and the cDNA Synthesis Kit (Bio-Rad). The reactions conditions were as follows: 30 minutes at 42˚C, followed by 5 minutes at 85˚C.

The mbTREM-1 and the TREM-1sv were investigated. The primers used for the amplification of mbTREM-1 were as follows: forward primer, 5′-GTGACCAAGGGTTTTTCAGG-3′; reverse primer, 5′-ACACCGGAACCCTGATGATA-3′. The primers used for the amplification of TREM-1sv were as follows: forward primer, 5′-GTGGTGACCAAGGGGTTC-3′; reverse primer, 5′-AGATGGATGTGGCTGGAAGT-3′. The reaction conditions were as follows: Initial denaturation of 94˚C for 2 min, followed by 40 cycles of denaturation at 94˚C for 30 seconds, annealing at 58˚C for 20 seconds, extension at 70˚C for 20 seconds. For the absolute quantification of the 2 splicing forms of *TREM-1*, corresponding mRNA levels were normalized to the mRNA levels of beta 2 microglobulin (B2M) gene. The housekeeping gene was simultaneously amplified to check the RNA integrity and to verify that the same amounts of template were used in all cases. The absolute quantification of the 2 splicing forms of the *TREM-1* and the housekeeping gene were performed using Lightcycler Carousel-Based System (Roche Diagnostics) and SYBR Green I reaction Kit. Negative controls were used for each reaction and the specificity of the products was confirmed by polyacrylamide gel electrophoresis as well as by melt curve analysis. All experiments were performed in duplicate.

No PBMCs were available for the replication population and therefore this analysis was performed only in the SFS individuals.

Statistical analyses

All investigated molecules (intermediate phenotypes) were log transformed in order to normalise their distribution before analyses. The normality of distribution was tested by Kolmogorov-Smirnov test. Hardy-Weinberg equilibrium was tested using the chi-square test. The SNPs-mRNA and soluble TREM-1 levels associations were assessed through linear regression adjusted for age, gender and BMI under three inheritance models (additive, dominant and recessive) and using the minor allele as the reference allele. The association of the most significant SNPs with selectins levels were tested using similar models. Association studies were

performed using the Plink Software [33]. Populations' characteristics were determined using the SPSS statistical software version 20.0 (SPSS, Inc, Chicago, Illinois). Bonferroni correction was applied in order to adjust the multiplicity of tests and avoid the type 1 error. Cut-offs were 0.05/ number of SNPs for the association of the selected SNPs with soluble TREM-1 levels, 0.05/ number of SNPs/2 for the associations of the significant SNPs with the 2 mRNA levels and 0.05/number of SNPs/number of selectins for the associations of the significant SNPs with the selectins' levels.

Results

HuGE navigator and NCBI dbSNP database searches resulted to the selection of 10 SNPs located within or near *TREM-1* with a minor allele frequency >5%. The 10 SNPs were genotyped in 30 individuals from the SFS. No significant deviation from Hardy-Weinberg equilibrium was observed for the studied polymorphisms (Table 1).

Association studies between the 10 polymorphisms in ***TREM-1*** and serum levels of sTREM-1/TREM-1sv

For this first decisional step, we used a discovery and a replication population. Demographic and clinical characteristics of the studied individuals included in each population are summarized in the Table 2.

Association analyses were performed in 30 individuals of SFS (discovery population) for the 10 SNPs and the levels of TREM-1 in serum using the additive, dominant and recessive inheritance models (P-value cut-off was set at 0.05/10 = 0.005). Only for rs2234246 we found significant results. According to the additive model, the minor allele T of rs2234246 was associated with increased levels of sTREM-1 (p-value = 0.003, β = 0.3, data available in Table 3 and Fig 1). When the dominant model was used, this association was even stronger (p-value = 0.0003, β = 0.49), and the variance in the serum TREM-1 levels explained by the model is of 33% in the discovery population.

The SNP rs2234246 was then genotyped in 80 additional and independent individuals (replication population). Additive and dominant models confirmed the association between the minor allele T and sTREM-1 levels (p-values = 0.0007 and 0.0017 respectively, β = 0.13 and 0.22 respectively, data available in Table 3 and Fig 2). Threshold of significance was 0.05 (as only 1 SNP was tested). The TREM-1 variance explained by the model is of 13% in the replication population.

Table 1. Characteristics of the polymorphisms studied in 30 individuals of SFS.

SNP	Minor allele	Minor allele frequency (MAF)	Chromosome	HWE P
rs3789204	A	0.265	6	0.667
rs7768162	A	0.317	6	0.912
rs7772334	T	0.460	6	0.948
rs728488	T	0.368	6	0.505
rs612399	C	0.377	6	0.989
rs2234246	T	0.416	6	0.763
rs2234237	A	0.086	6	0.632
rs13211886	T	0.089	6	0.350
rs6910730	G	0.098	6	0.825
rs9471554	C	0.313	6	0.425

Demographic and clinical characteristics of the studied individuals are summarized in the Table 2.

https://doi.org/10.1371/journal.pone.0182226.t001

Table 2. Demographic and clinical characteristics of the individuals.

	SFS sub-sample	SFS Total	Replication Population
Sample size [% female]	30 [47%]	351 [48.43%]	80 [52.5%]
Age (years) [S.D]	47.07 [5.3]	44.09 [4.3]	47 [8.1]
BMI (kg/m^2) [S.D]	26 [4.69]	24.96 [3.76]	26.7 [3.69]
sTREM-1 (pg/ml) [S.D]	213.47 [70.24]	-	352.38 [87.88]
TREM-1sv (pg/ml) [S.D]	-	-	39.08 [32.01]
L-selectin (mg/l) [S.D]	948.68 [293.2]	1018.65 [280.8]	888.19 [74.67]
P-selectin (mg/l) [S.D]	132 [31.94]	137.93 [43.45]	-
E-selectin (mg/l) [S.D]	44.93 [11.68]	53.56 [27.07]	-
CRP (mg/L) [S.D]	1.41 [1.45]	1.83 [2.95]	-

https://doi.org/10.1371/journal.pone.0182226.t002

Table 3. Statistical associations of the 10 polymorphisms studied with the serum levels of TREM-1 and mRNA levels according to the different inheritance models. P-value thresholds for TREM-1 protein levels are P<0.005 for the discovery population and P<0.05 for the replication population. P-value threshold for mRNA levels is <0.025.

Polymorphisms	Model	sTREM-1 (protein) discovery (N = 30)			sTREM-1 (protein) replication (N = 80)			TREM-1sv (mRNA) (N = 30)			mbTREM-1 (mRNA) (N = 30)		
		P-value	β	S.E	P-value	β	S.E	P-value	β	S.E	P-value	β	S.E
rs3789204	Additive	0.090	0.25	0.14	-	-	-	-	-	-	-	-	-
	Dominant	0.090	0.25	0.14	-	-	-	-	-	-	-	-	-
	Recessive	-	-	-	-	-	-	-	-	-	-	-	-
rs7768162	Additive	0.024	0.21	0.08	-	-	-	-	-	-	-	-	-
	Dominant	0.037	0.28	0.12	-	-	-	-	-	-	-	-	-
	Recessive	0.139	0.28	0.18	-	-	-	-	-	-	-	-	-
rs7772334	Additive	0.105	-0.17	0.10	-	-	-	-	-	-	-	-	-
	Dominant	0.226	-0.20	0.16	-	-	-	-	-	-	-	-	-
	Recessive	0.195	-0.23	0.17	-	-	-	-	-	-	-	-	-
rs728488	Additive	0.191	-0.15	0.11	-	-	-	-	-	-	-	-	-
	Dominant	0.103	-0.38	0.22	-	-	-	-	-	-	-	-	-
	Recessive	0.471	-0.11	0.16	-	-	-	-	-	-	-	-	-
rs612399	Additive	0.063	-0.21	0.11	-	-	-	-	-	-	-	-	-
	Dominant	0.193	-0.20	0.15	-	-	-	-	-	-	-	-	-
	Recessive	0.098	-0.38	0.22	-	-	-	-	-	-	-	-	-
rs2234246	Additive	**0.003**	0.30	0.09	**0.0007**	0.13	0.03	0.874	0.02	0.17	**0.007**	0.49	0.16
	Dominant	**0.0003**	0.49	0.11	**0.001**	0.21	0.06	0.225	0.31	0.24	**0.002**	0.79	0.23
	Recessive	0.440	0.17	0.22	0.017	0.15	0.06	0.215	-0.40	0.31	0.250	0.42	0.35
rs2234237	Additive	0.337	-0.15	0.15	-	-	-	-	-	-	-	-	-
	Dominant	0.337	-0.15	0.15	-	-	-	-	-	-	-	-	-
	Recessive	-	-	-	-	-	-	-	-	-	-	-	-
rs13211886	Additive	0.953	0.01	0.19	-	-	-	-	-	-	-	-	-
	Dominant	0.953	0.01	0.19	-	-	-	-	-	-	-	-	-
	Recessive	-	-	-	-	-	-	-	-	-	-	-	-
rs6910730	Additive	0.337	-0.15	0.15	-	-	-	-	-	-	-	-	-
	Dominant	0.337	-0.15	0.15	-	-	-	-	-	-	-	-	-
	Recessive	-	-	-	-	-	-	-	-	-	-	-	-
rs9471554	Additive	0.054	0.28	0.13	-	-	-	-	-	-	-	-	-
	Dominant	0.054	0.28	0.13	-	-	-	-	-	-	-	-	-
	Recessive	-	-	-	-	-	-	-	-	-	-	-	-

https://doi.org/10.1371/journal.pone.0182226.t003

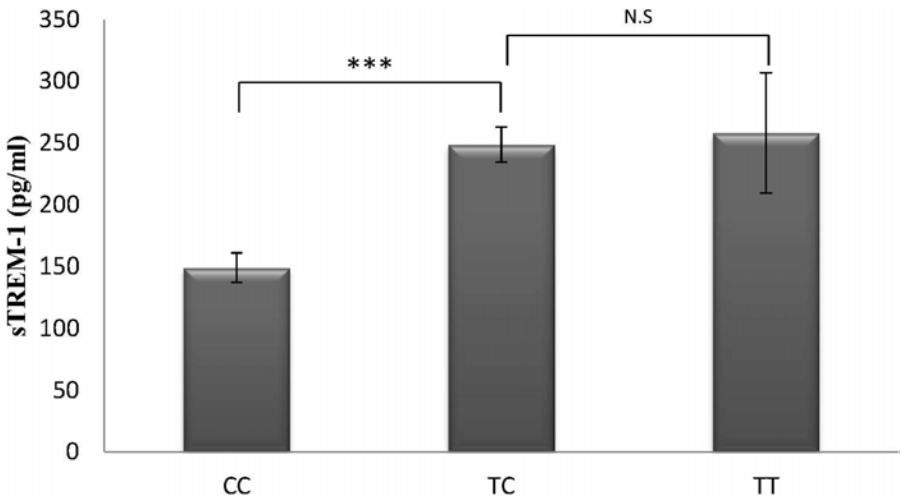

Fig 1. Mean values of sTREM-1 levels according to the different genotypes of rs2234246 (CC vs TC vs TT) in the discovery population. Thin bars show standard errors. CC; Homozygous for the major allele of the rs2234246. TC; Heterozygous for the rs2234246. TT; Homozygous for the minor allele of the rs2234246. The significance between genotypes is showed as follows; N.S.; >0.05, * p<0.05, ** p<0.01, *** p<0.001.

https://doi.org/10.1371/journal.pone.0182226.g001

The serum TREM-1sv levels, generated by the mRNA splicing variant TREM-1sv, were also measured in 78 individuals of the replication population. The TREM-1sv levels were detected only in four of the 78 samples studied, allowing no statistical analysis.

Association studies between the rs2234246 in ***TREM-1*** and mRNA expression levels (mbTREM-1 and TREM-1sv)

Association analyses were then performed between the rs2234246 SNP and the expression levels of the two different alternative splicing isoforms of *TREM-1* (mbTREM-1 and TREM-1sv mRNAs) in PBMCs of the 30 individuals of the discovery population (threshold of significance was 0.05/1

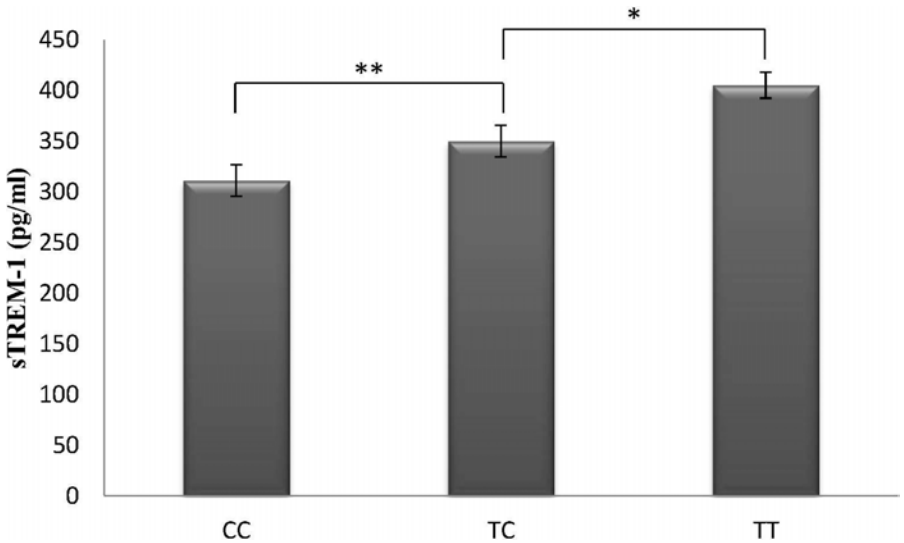

Fig 2. Mean values of sTREM-1 levels according to the different genotypes of rs2234246 (CC vs TC vs TT) in the replication population. Thin bars show standard errors. CC; Homozygous for the major allele of the rs2234246. TC; Heterozygous for the rs2234246. TT; Homozygous for the minor allele of the rs2234246. The significance between genotypes is showed as follows; N.S.; >0.05, * p<0.05, ** p<0.01, *** p<0.001.

https://doi.org/10.1371/journal.pone.0182226.g002

SNP/2 phenotypes = 0.025). Interestingly, the SNP rs2234246 showed significant association with increased mRNA levels of the splicing form that codes for mbTREM-1 (additive model, p-value = 0.007, β = 0.49, data available on Table 3), whereas it was not associated with the levels of mRNA coding TREM-1sv. The TREM-1sv protein was only present in four samples out of the 78 samples studied, not showing increased expression in the presence of the SNP rs2234246.

Association studies of the rs2234246 polymorphism in *TREM-1* with soluble selectins' levels

The SNP rs2234246 was further genotyped in 351 individuals (discovery population, characteristics in Table 2). Association analyses were performed between the SNP rs2234246 and the sL-, sP- and sE-selectin levels (cut-off value for significance was set to 0.05/1 SNP/3 phenotypes = 0.016). Only the association with sL-selectin was significant (p-value = 0.011, β = 0.05) and explained a total of 2.1% of the variability of the sL-selectin. The minor allele T of the polymorphism was significantly associated with increased sL-selectin levels. No significant association was observed for sP- and sE-selectin levels.

The association between the minor allele T and sL-selectin levels was then confirmed in the 80 individuals of the replication population (p-value = 0.018, β = 0.03) (cut-off value for significance was set to 0.05, as only 1 SNP was tested) and association explained a total of 4.3% of the variability of the sL-selectin. All results are available in the Table 4.

Regulatory environment of *TREM-1* rs2234246

Using bioinformatics analyses, we established the regulatory environment of SNP rs2234246 (S1 Fig). The rs2234246 polymorphism is located at 41276002 bp on the forward strand. It is positioned halfway between an open chromatin zone and a promoter flanking region. We can note that the open chromatin zone is active only in monocytes CD14+, which are present in PBMCs. Furthermore, the promoter flanking region is in an active state only in two cell types: the monocytes CD14+ and CD14+CD16-. It can be observed as well that the polymorphism is located in the 3'UTR region of the two mRNA splicing variants studied: mbTREM-1 (TREM1-001) and TREM-1sv (TREM1-002).

It is important to mention also that in the non-leukocyte cell types, the neighbourhood of *TREM-1* gene show only repressed or inactive areas, suggesting that these areas are not subject to regulations (results not shown).

Epigenetic footprint of *TREM-1* rs2234246

Using bioinformatics tools, we established the epigenetic profiles (methylation / acetylation) of rs2234246 according to the cell type involved (S2 Fig). A specific epigenetic pattern emerged in monocytes (CD14+ and CD14+CD16-), vein blood neutrophils, eosinophils and macrophages in particular by the presence of H3K36me3 and H3K4me1. This specific methylation

Table 4. Effects of the polymorphism rs2234246 located within the *TREM-1* gene on the serum levels of the studied selectin molecules.
MAF = Minor allele frequency of the rs2234246. Cutoff value of significance: 0.016 in discovery and 0.05 in replication population.

Population	Phenotypes	β	S.E	P-value
Discovery population (MAF: 0.498)	sL-selectin (mg/l) (n = 351)	0.05	0.02	**0.011**
	sP-selectin (mg/l) (n = 311)	0.02	0.02	0.435
	sE-selectin (mg/l) (n = 351)	0.01	0.04	0.685
Replication population (MAF: 0.487)	sL-selectin (mg/l) (n = 80)	0.03	0.01	**0.018**

https://doi.org/10.1371/journal.pone.0182226.t004

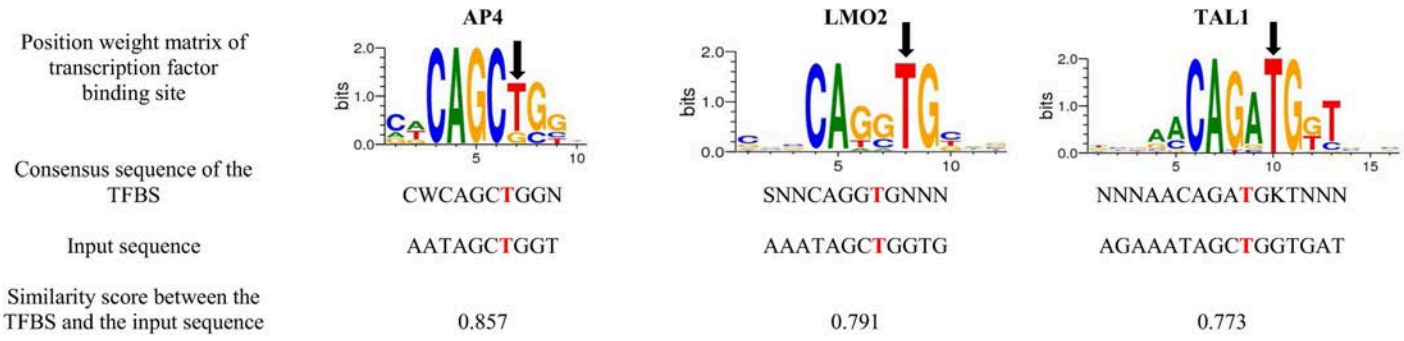

Fig 3. Specific transcription factor binding sites for the minor allele T of the SNP rs2234246.

https://doi.org/10.1371/journal.pone.0182226.g003

pattern is not present in the other 18 cell types investigated. Especially the presence of H3K36me3 is interesting, as is considered as a hallmark of actively transcribed regions. Thus, we speculate that the genetic region where the SNP rs2234246 is located is prone of higher expression levels of the *TREM-1* gene.

Potential transcription factor binding sites in the rs2234246 locus

The bioinformatics results showed also differences in the potential transcriptional factors binding the locus according to the different alleles of the SNP rs2234246.

When the minor allele is present (T), the specific potential transcription factors that are able to bind are AP4, LMO2 and TAL1, with a similarity score of 0.857, 0.791 and 0.773, respectively. Regarding the position weight matrix, we can see that the minor allele T is a highly conserved nucleotide in the TAL1 binding site, having a height of 2.0 bits. In the case of the transcription factor binding site LMO2 and AP4, the T allele is also important with a height >1.7 and >1.2 bits, respectively (Fig 3).

When the major allele is present (C), the specific potential transcription factors that are able to bind are REL, CAAT and NFY, showing a similarity score of 0.838, 0.801 and 0.771 respectively. The position weight matrix shows that the minor allele C is highly conserved in the case of the CAAT and NFY binding sites (height >2 bits and >1.7 bits, respectively). In the binding site of REL, the C allele is also a conserved nucleotide with a height >1.2 bits (Fig 4).

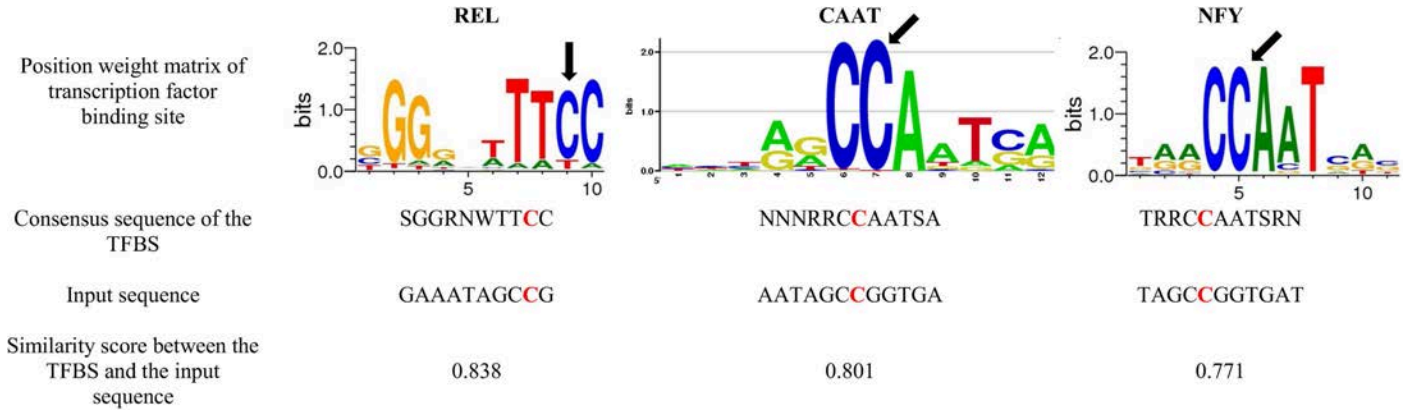

Fig 4. Specific transcription factor binding sites for the major allele C of the SNP rs2234246. The consensus sequence (fixed) of the transcription factor binding sites means: S = C or G, W = A or T, R = A or G, Y = C or T, K = G or T, M = A or C, N = any base pair. The input sequence was 38pb length, centred on the SNP of interest rs2234246. Only TFBS harbouring a nucleotide in its consensus sequence in coherence with the SNP of interest were selected.

https://doi.org/10.1371/journal.pone.0182226.g004

Discussion

TREM-1 protein levels have been related to numerous diseases where inflammation and inflammatory cell extravasation play central roles, such as atherosclerosis [10], acute myocardial infarction [11], and critical limb ischemia [12]. All these diseases have an important genetic component [34, 35], and despite all advances in treatment and prevention, they remain the leading cause of death worldwide [36]. This makes necessary the detection of genetic polymorphisms that could uncover novel metabolic pathways involved in the pathophysiology of these diseases, and therefore, improve their prevention and treatment [37, 38].

After a bibliography search of the SNPs within or near *TREM-1*, a total of 10 polymorphisms were selected and further analysed. Among them, we found that rs2234246, located in the mRNA 3'UTR region of *TREM-1*, was associated with sTREM-1 protein levels. So far, this is the first polymorphism that has been reported to affect the sTREM-1 levels. The minor allele T of this polymorphism was strongly associated with increased sTREM-1 levels, both in the discovery and the replication populations (p-values = 0.003 and 0.0007 respectively), explaining a large percentage of its phenotypic variation (33% and 13% in the discovery and replication population respectively).

It has been widely reported that increased levels of sTREM-1 were correlated to the severity of above-mentioned inflammatory diseases and poor prognosis [8–10, 12, 14–18], and the most recent studies have showed that *TREM-1* deletion or blockade is associated with up to 60% reduction of the development of atherosclerosis [10]. Thus, the T allele of rs2234246 may be considered as a risk factor, while the C allele may be a protective factor for these diseases. Given the high frequency of the polymorphism in the general population (about 50%), this result could be of high interest in further personalized medicine strategies for stratifying patients according to their risk to the above pathologies. In this context, it is important to note that a previous study has associated the minor allele of the SNP rs2234246 with an increased 3.1 odds ratio for septic shock [39]. This finding strengthens our hypothesis on the utility of the polymorphisms located in *TREM-1* gene in risk stratification. Another SNP located also within the TREM-1 gene (rs4711668) has been associated with severe coronary atherosclerosis in a Russian population [27]. However, it is not known whether this polymorphism is affecting the TREM-1 protein levels and although it is close to our SNP of interest, the 2 SNPs are not in strong linkage disequilibrium between them ($r^2 = 0.46$).

We have also associated the T allele of the SNP rs2234246 with the expression levels of mbTREM-1 mRNA in PBMCs (p-value = 0.007 $\beta = 0.49$). This result is conferring functional properties to this polymorphism. However, we didn't differentiate the monocyte subsets, and the expression of TREM-1 as well as the effect of the SNP rs2234246 could vary according the different monocyte subtypes. It is important to note that according to our bioinformatics analysis, the polymorphism is located in the 3'UTR region of the two mRNA splicing variants studied (S1 Fig). Interestingly, the polymorphism rs2234246 was related to an increase in the expression level of the mRNA coding mbTREM-1, but it was not related to the mRNA that codes TREM-1sv, nor to the TREM-1sv protein levels itself, which was not present in the serum. Two hypotheses have been proposed for the origin of soluble TREM-1: (1) splicing of different variants of alternative mRNA, which generates the TREM-1sv [40] and (2) shedding of mbTREM-1 by metalloproteases, which generates the sTREM-1. Our results, taking also into consideration that TREM-1sv protein levels were not present in the serum, support the hypothesis that the levels of sTREM-1 are controlled post transcriptionally by metalloproteases, rather than by alternatively spliced forms of RNA [7, 29].

According to our bioinformatics-epigenetics results, the rs2234246 has a specific H3K36me3 and H3K4me1 methylation epigenetic patterns in two groups of monocytes

(CD14+ and CD14+CD16-), which are present in PBMCs. The presence of H3K36me3 is especially interesting as it is considered to be a hallmark of actively transcribed gene bodies. Thus, we speculate that the genetic region where the SNP rs2234246 is located, is prone of higher expression levels of the *TREM-1* gene [41]. However, this is only an assumption, as we were not able to perform experiments to confirm this effect. At the same time, the regulation profile of *TREM-1* shows an open chromatin zone and active promoter flanking regions only in CD14 + monocytes. The open chromatin zones are functionally related to transcriptional activity where DNA is accessible for the binding of proteins such as transcription factors [42]. Moreover, the SNP rs2234246, by the fact that it is located in the 3'-UTR of the mRNA, which is rich in regulatory regions that post-transcriptionally could influence gene expression [43, 44]. The effect of rs2234246 polymorphism on the sTREM-1 levels could be explained by several hypothetical mechanisms: (i) Post-transcriptional regulation of many pro-inflammatory mediators is controlled by adenosine and uridine-rich elements (AREs) [45]. AREs regions, located in the 3'-UTR of the mRNA can promote mRNA decay, affect mRNA stability, or activate translation. (ii) According to the TRANSFAC R.3.4. Database, the potential transcription factors binding the locus of the SNP rs2234246 could be different depending on the allele. The minor allele T is potentially associated with the matching of the transcription factors AP4, LMO2 and TAL1, while the major allele C is potentially associated with the matching of the transcription factors REL, CAAT and NFY. The type of transcription factors or even the affinity of those for the different polymorphisms of the rs2234246, could explain the changes in the expression levels of the *TREM-1* gene. However, we didn't have experimental data that could confirm these possible direct affinity changes depending on the different alleles of the rs2234246, thus further experiments are needed to confirm this assumption.

It has been previously suggested that the receptors of the TREM family are regulating the cellular adhesion of macrophages and neutrophils *via* the phosphorylation of DAP12, which leads to activation of calcium sensitive kinases [6, 46]. At the same time, the number of studies supporting the importance of TREM-1 in the trans-epithelial migration of neutrophils and monocytes is increasing. Migrating neutrophils in septic patients have been found to bind to TREM-1 [7]. It has been previously demonstrated that TREM-1 is crucially involved in leukocyte recruitment after myocardial infarction and atherosclerosis [10, 11]. We demonstrated a five-fold decrease in the number of recruited neutrophils when TREM-1 was inhibited pharmacologically [11]. Also, in acute respiratory infections, TREM-1 is required for the trans-epithelial migration of neutrophils into the lung [13]. Despite these arguments, so far, the process by which TREM-1 contributes to this trans-epithelial migration is unknown. In our study, we demonstrated for the first time that TREM-1 is regulating one adhesion molecule.

We found that the rs2234246 polymorphism is specifically correlated with increased plasma levels of L -selectin. The soluble L-selectin levels are thought to represent a homeostatic effort to limit excessive inflammation. Indeed, they are correlated with the severity of inflammatory diseases, including cardio-vascular diseases [47, 48]. Although, once in a soluble form, they may have reduced adhesion, migration and trans-epithelial migration capacity, it can be postulated that TREM-1 increases inflammation in general, leading to L-selectin shedding as a downstream effect. Especially, L-selectin, has been showed to be important in the recruitment of monocytes and neutrophils to sites of acute and chronic inflammation [49]. The role of the selectins in inflammatory processes is well established, and the selectin-mediated adhesion and signalling contribute to different cardio-vascular diseases [21]. The selectins, have been shown to contribute to atherosclerosis [24, 25] and arterial thrombosis [50]. According to our results, the minor allele T of the rs2234246 could act as a risk factor as it is correlated with an increase of 2.1–4.3% of sL-selectin [47, 48, 51]. It would be also interesting to further investigate the

possible associations between the membrane bound L-selectin levels and rs2234246. Unfortunately, in this work we were not able to address this issue.

The conclusion from our bioinformatics and transcriptomic analyses supports the hypothesis that TREM-1 is involved in the trans-epithelial migration process of leukocytes, more specifically of monocytes, that could be effective through a higher level of inflammation, which could be observed with an overexpression of sL-selectin.

One of the advantages of our research is that we limited the potential confounders by using a homogeneous population coming from the region of Lorraine in France as a discovery population. Because of this, we have been able to reduce the enormous cardio-vascular-related heterogeneity of the population, by linking genes of interest to intermediate phenotypes and not to the disease. This approach can represent better the real biological pathways where the gene of interest is involved. Moreover, we replicated the results in an independent population, while simultaneously bioinformatics and bibliographical data also strengthen our results.

One limitation of our study is that we were not able to realize gene expression analysis in the PMNs and more specifically in neutrophils. As well as the monocytes, the neutrophils are also expressing TREM-1 protein. However, we were not able to have direct experimental data about the effect of the SNP rs2234246 in neutrophils due to lack of biological material. Future studies are needed to confirm these possible effects. Also, we couldn't make the distinction among the different subgroup of monocytes and TREM-1 expression, and the effect of the SNP rs2234246 may be different in those subgroups. A more specific study including the monocyte subsets would significantly improve the biological relevance of the SNP rs2234246. In conclusion, our study led to the discovery of one polymorphism (rs2234246) strongly affecting sTREM-1 protein levels and associated to an increase in the levels of the mRNA coding mbTREM-1 in PBMCs. Since no association was established with splicing mRNA and TREM-1sv was not detected in the serum of the individuals, it seems that, the levels of sTREM-1 are controlled post transcriptionally by metalloproteases. Interestingly, we demonstrated for the first time that *TREM-1* rs2234246 polymorphism can also modulate the sL-selectin levels via a higher inflammatory state, suggesting that TREM-1 acts in the trans-epithelial migration process of leukocytes and more specifically of monocytes through expression of sL-selectin.

Supporting information

S1 Fig. Regulation profile of the *TREM-1* gene in different cell types expressing the protein TREM-1. The polymorphism rs2234246 is located at 41276002 bp on the forward strand (vertical red line). It is positioned halfway between an open chromatin zone and a promoter flanking region. It can also be observed that the polymorphism is located in the 3'UTR region of the two mRNA splicing variants studied: mbTREM-1 (TREM1-001; ENST00000244709.8) and TREM-1sv (TREM1-002; ENST00000334475.10) and in an intron zone of another *TREM-1* transcript: TREM1-006 (ENST00000589695.1) (VB: Vein blood).
(DOCX)

S2 Fig. Epigenetic profile of rs2234246 in leukocytes cell types: Specific histone methylation patterns (VB: Vein blood).
(DOCX)

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Author Contributions

Conceptualization: Alex-Ander Aldasoro Arguinano, Sophie Visvikis-Siest.

Data curation: Maria Stathopoulou, Ndeye Coumba Ndiaye, Sophie Visvikis-Siest.

Formal analysis: Alex-Ander Aldasoro Arguinano, Sébastien Dadé, Ndeye Coumba Ndiaye.

Funding acquisition: Sophie Visvikis-Siest.

Investigation: Alex-Ander Aldasoro Arguinano, Sébastien Dadé, Maria Stathopoulou, Ndeye Coumba Ndiaye, Christine Masson, Sébastien Gibot.

Project administration: Sophie Visvikis-Siest.

Resources: Sébastien Gibot, Sophie Visvikis-Siest.

Software: Alex-Ander Aldasoro Arguinano, Maria Stathopoulou, Ndeye Coumba Ndiaye.

Supervision: Marc Derive, Sébastien Gibot, Sophie Visvikis-Siest.

Validation: Alex-Ander Aldasoro Arguinano, Maria Stathopoulou, Christine Masson, Sophie Visvikis-Siest.

Visualization: Alex-Ander Aldasoro Arguinano, Sébastien Dadé.

Writing – original draft: Alex-Ander Aldasoro Arguinano, Sébastien Dadé.

Writing – review & editing: Alex-Ander Aldasoro Arguinano, Sébastien Dadé, Maria Stathopoulou, Marc Derive, Ndeye Coumba Ndiaye, Ting Xie, Sébastien Gibot, Sophie Visvikis-Siest.

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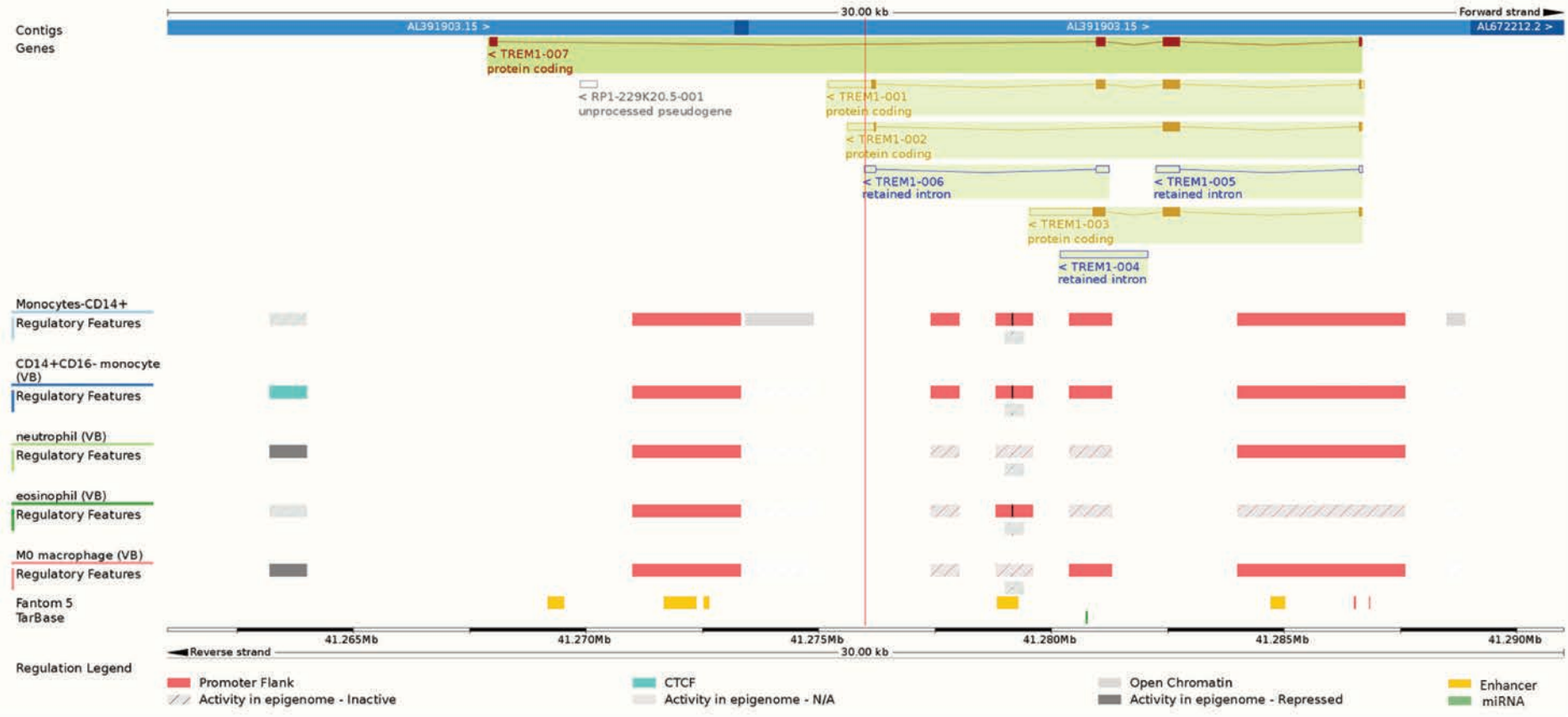
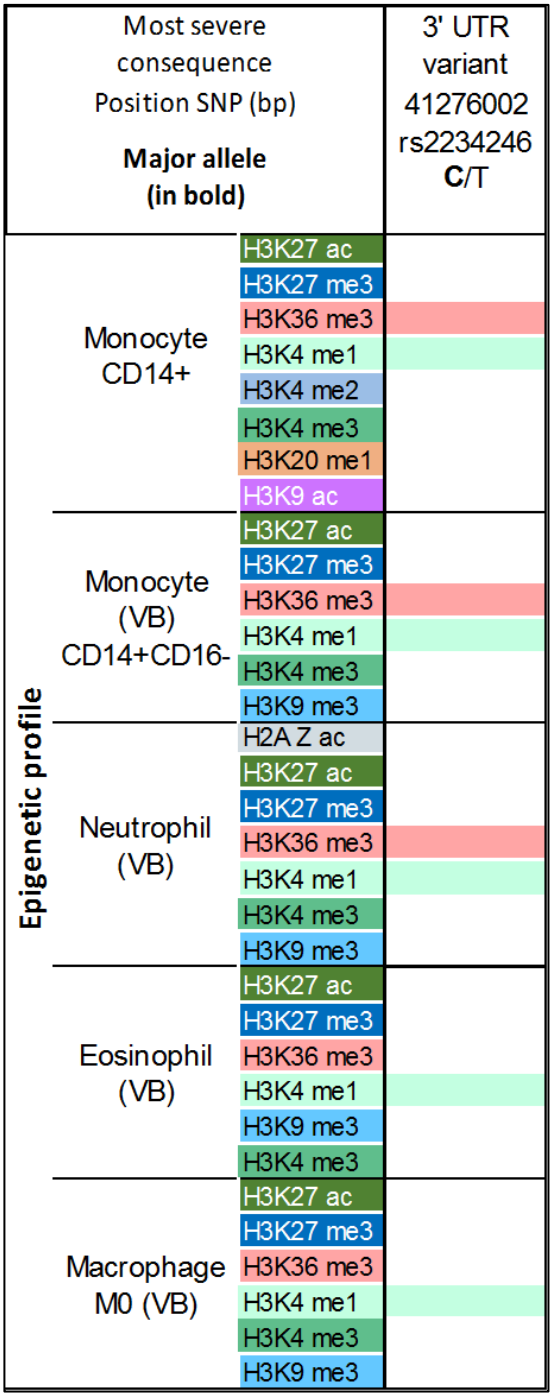
Supplementary Figure 1. Regulation profile of the *TREM-1* gene in different cell types expressing the protein TREM-1. The polymorphism rs2234246 is located at 41276002 bp on the forward strand (vertical red line). It is positioned halfway between an open chromatin zone and a promoter flanking region. It can also be observed that the polymorphism is located in the 3'UTR region of the two mRNA splicing variants studied: mbTREM-1 (TREM1-001; ENST00000244709.8) and TREM-1sv (TREM1-002; ENST00000334475.10) and in an intron zone of another *TREM-1* transcript: TREM1-006 (ENST00000589695.1) (VB: Vein blood).



Supplementary Figure 2. Epigenetic profile of rs2234246 in leukocytes cell types: specific histone methylation patterns (VB: Vein blood)



List of cell types investigated:

1. Monocytes CD14+
    1.1 CD14+ CD16- monocyte from venous blood
    1.2 CD14+ CD16- monocyte from cord blood
2. T cell
    2.1 CD4+ ab T cell from venous blood
    2.2 CD8+ ab T cell from cord blood
    2.3 CM CD4+ ab T cell from venous blood
3. Human leukemic T-cell line with p53 (Notch) mutation
4. Endothelial progenitol cell (EPC) from venous blood
5. 5.1 GM12878 (Human B-lymphocyte cell line
    5.2 Naïve B cell from venous blood
6. Human embryonic stem cells (H1ESC)
7. Human mammary epithelial cells (HMEC)
8. Skeletal muscle myotubes differenciated from the HSMM cell line
9. Human umbilical vein endothelial cell line (HUVEC)
10. Human epithelial carcinoma cells (HeLa)
11. Human hepatocellular liver carcinoma cell line (HepG2)
12. Normal human epidermal keratinocyte cell line (NHEK)
13. Human myelogenous leukaemia cell line (K562)
14. 14.1 M0 Macrophage from cord blood
    14.2 M0 Macrophage from venous blood
    14.3 M1 Macrophage from cord blood
    14.4 M1 Macrophage from venous blood
    14.5 M2 Macrophage from cord blood
    14.6 M2 Macrophage from venous blood
15. Mesenchymal stem cells from venous blood (MSC)
16. Normal human astrocytes
17. 17.1 Human fetal lung fibroblast
    17.2 Human adult dermal fibroblast
    17.3 Human adult lung fibroblast
18. Osteoblasts (NHOst)
19. Eosinophil from venous blood
20. Neutro myelocyte from bone marrow
21. 21.1 Neutrophil from venous blood
    21.2 Neutrophil from cord blood
22. Erythroblast from cord blood

Publication N°2

IL6R haplotype rs4845625*T/rs4537545*C is a risk factor for simultaneously high CRP, LDL and ApoB levels

Alex-Ander ALDASORO ARGUINANO[1], Elnaz NADERI*[1,2], Ndeye Coumba NDIAYE*[1], Maria STATHOPOULOU[1], Sébastien DADE[1], Behrooz ALIZADEH[1, 2], Sophie VISVIKIS-SIEST[¥1, 3]

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Interleukin-6 (IL-6) is a well-documented pro-inflammatory cytokine that is considered multifunctional, as it is involved in various processes. It requires the specific IL-6 receptor (IL-6R) to exert its biological functions. As IL-6R is the molecule that mediates directly the biological action of IL-6, it is clearly of major clinical significance, indeed, IL-6R has been involved in biological pathways where blood lipids and inflammation biomarkers are significantly implicated (224-227).

More than 10 IL-6R polymorphisms have been linked with IL-6 levels (228-230). Among them, 2 being also associated with antagonistic effects on risk for CHD (225, 228, 231) and for C-reactive protein (CRP) levels (230, 232): rs4845625 and rs4537545, both SNPs are in weak LD (r^2=0.48). The minor rs4845625*T allele has been associated with increased CRP and CHD risk (225) while the minor rs4537545*T allele has been associated with decreased CRP and CHD risk (228, 231). Also, both polymorphisms have been associated with triglycerides levels, with contradictory results obtained in different studies (224, 233-235).

Objective

The goal was to investigate the effects of two SNPs, within the IL-6R loci, previously, associated with CRP and coronary heart diseases risk, and shown to have contradictory effects on lipids traits: SNP rs4845625 and SNP rs4537545.

Results

<u>Associations of SNPs rs4845625 and rs4537545 in the IL-6R gene with CRP levels</u>

The 2 SNPs have antagonistic effects on the CRP serum concentrations in the SFS. The minor rs4845625*T allele is associated with increased levels of CRP (p-value = 0.011, β = 0.182). The minor rs4537545*T allele has the opposite effect of that of rs4845625*T, as it is associated with decreased levels of CRP (p-value = 0.009, β = -0.189).

Associations of rs4845625 and rs4537545 in the IL-6R gene with lipid traits

In the discovery population, the minor rs4845625*T allele was significantly associated with increased levels of LDL-C and ApoB levels (p-value = 0.007and p=0.009 respectively). The association with LDL-C was confirmed in the replication sample of adults (p-value = 0.035). The rs4845625*T allele was not significant for the TC in the discovery individuals from SFS, but in the replication sample, it was associated with increased TC levels (p-value = 0.011, β = 0.019, Table 4). In the association studies performed for the SNP rs4537545, none of the lipid phenotypes was significant in the discovery and replication populations.

IL-6R Haplotypes associations with lipid traits and CRP levels

Three main haplotypes of rs4845625/rs4537545, H1 (CT), H2 (TC) and H3 (CC), were present in the two populations investigated, with the following frequencies: H1= 40.6%, H2=41.6% and H3=17.6%.. The results previously obtained when performing association studies separately with each SNP were confirmed in the haplotype studies. The haplotype 1 (CT) was associated with decreased levels of CRP (p-value=0.01) while the haplotype 2 (TC) was associated with increased levels of CRP (p-value=0.007). The combination of both major alleles of the 2 SNPs (haplotype 3: CC) was not associated with CRP levels, as the average effect of both SNPs results in no significant changes in CRP levels. The haplotype 2 (TC), having the minor rs4845625*T allele, was related with increased levels of LDL-C in the discovery population, while a nominal significance was observed in the replication population (p-value=0.015 and p-value=0.024 respectively). The same haplotype (TC) was nominally related with increased ApoB levels in the discovery population (p-value=0.022) while the haplotype 3 (CC) was nominally associated with decreased levels of ApoB (p-value=0.024). The haplotype 2 (TC) was associated with increased levels of total cholesterol in the replication population (p-value=0.008), this association was not significant in the discovery population.

Discussion

We have confirmed the associations between the SNPs rs4845625 and rs4537545 with CRP levels, as the minor rs4845625*T allele increases CRP levels, while the minor rs4537545*T allele decreases CRP levels, confirming previous results. This antagonistic effect of the two SNPs has been already related with the risk of CHD. The minor rs4845625*T allele has been associated with a 4% increase in CHD risk in the CARDIoGRAM-C4D Consortium (225) while the minor rs4537545*T allele has been associated with a 5% decrease in the risk of CHD (228, 231). Concerning the effects of the 2 SNPs on lipid metabolism traits, we demonstrated for the first time an association of the minor rs4845625*T allele with increased ApoB and LDL-C levels, consequently, conferring to this allele the notion of risk factor. This is in concordance with the previous results where this SNP has been associated with increased risk of CHD (225), atrial fibrillation (236) and subclinical atherosclerosis (237). The SNP rs4845625 was not associated with the other lipid traits studied. Although some studies have associated this SNP with TG, the results have been contradictory, relating its minor allele with increased or decreased TG (224, 233-235). High LDL levels are the main cause of endothelium injury, but in presence of high CRP levels, there is an acceleration in the progression of CHD, as the CRP affects the LDL uptake by macrophages, facilitating the formation of foam cells (238). We also performed haplotype analysis of the two polymorphisms, which allowed us to obtain details on the size effect of each variant on the phenotypes studied. The haplotype analysis showed that the association of the SNP rs4845625 with lipid levels is independent from rs4537545 (although the 2 SNPs are physically relatively close one to the other, having a LD of r^2=0.48) and confirmed our previous separate SNPs analyses results. So far, this is the first time that an haplotype analysis investigates associations of IL-6R gene polymorphisms with lipid traits and that one frequent (41,6%) haplotype in the population (haplotype 2) can be designed as risk factor for simultaneously increasing LDL-C, ApoB and CRP levels.

Our results, especially those concerning the haplotype 2 (TC), may have important value in pharmacogenomics studies given the fact that IL-6R gene is a target of the pharmacological agent tocilizumab (239), an immunosuppressive drug widely used for the treatment of rheumatoid arthritis and which competitively inhibits IL-6R, resulting in a reduction of CRP and fibrinogen concentrations (240). One of the side effects of tocilizumab is the increase of LDL-C levels in patients (22), which could lead to a risk of suffering from ischemic heart disease. Moreover, the tocilizumab blocks both classic and trans-signalling pathways and,

looking at the complex biology of IL-6, the extended blockage of this cytokine should be extensively studied. So far, it is not clear whether those blood lipids changes reflect mechanism-based effects of IL-6R modification, or tocilizumab specific effects. Nonetheless, our results could also give interesting clues on the functionality of the drug.

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ORIGINAL ARTICLE

IL6R haplotype rs4845625*T/rs4537545*C is a risk factor for simultaneously high CRP, LDL and ApoB levels

AA Arguinano[1], E Naderi[1,2,4], NC Ndiaye[1,4], M Stathopoulou[1], S Dadé[1], B Alizadeh[1,2] and S Visvikis-Siest[1,3]

Interleukin 6 receptor (IL-6R), mediating IL-6's biological functions, plays an important role in different diseases such as diabetes, obesity and cardio-vascular diseases. In this study, we investigated the effects of two single nucleotide polymorphisms (SNPs), within the *IL-6R* loci, previously associated with C-reactive protein (CRP) and coronary heart diseases risk, and with controversial effects on lipids traits: SNP rs4845625 and SNP rs4537545. The results showed that both investigated SNPs were antagonistically related with CRP levels; the minor rs4845625*T allele was associated with increased CRP levels (*P*-value = 0.011), while the minor rs4537545*T allele was associated with decreased CRP levels (*P*-value = 0.009). Interestingly, the minor rs4845625*T allele was significantly associated with higher low-density lipoprotein cholesterol (LDL-C) and ApoB levels ($P = 0.007$ and $P = 0.009$ respectively). Haplotype analysis showed that the TC haplotype, having the minor rs4845625*T allele, was related simultaneously with increased levels of CRP, LDL-C and ApoB levels, thus could be considered as a risk factor. Our investigation detects for the first time an independent effect of rs4845625 on LDL-C and ApoB traits, explaining an important range of those traits variability (3.49 and 5.57% respectively). Our findings might be of high clinical significance in pharmacogenomics studies of tocilizumab for which *IL-6R* is target.

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INTRODUCTION

Interleukin-6 (IL-6) is a well-documented pro-inflammatory cytokine that is considered multifunctional, as it is involved in many different processes. It requires the specific IL-6 receptor (IL-6R) to exert its biological functions. The binding of IL-6 to its receptor will trigger the recruitment of two gp130 molecules. Once the receptor complex activated, the intracellular regions of gp130 will initiate a signal transduction cascade by associating with JAK/Tyk tyrosine kinases and STAT protein transcription.[1] This is the so-called classical IL-6 signalling, which is a key step in early immune responses. However, the IL-6R is only expressed on a limited cell types like monocytes and T and B lymphocytes. In addition to the membrane-bound IL-6R, a soluble form (sIL-6R) is present in body fluids, which can be generated by alternative splicing[2] or by proteolytic cleavage by ADAM17.[3] The IL-6/sIL-6R soluble complex binds to the cell surface gp130, initiating the signal transduction cascade. This is the so-called IL-6 trans-signalling pathway. As the gp130 is ubiquitously expressed, by this mechanism, the sIL-6R can have effect on cells that cannot bind IL-6 by themselves. As IL-6R is the molecule that mediates directly the biological action of IL-6, it is clearly of major clinical significance; indeed, IL-6R has been involved in biological pathways where blood lipids and inflammation biomarkers are significantly implicated.[4–7]

More than 10 *IL-6R* polymorphisms have been linked with IL-6 levels.[8–10] Among them, two being also associated with antagonistic effects on risk for coronary heart diseases (CHD)[5,8,11] and for C-reactive protein (CRP) levels[10,12]: rs4845625 and rs4537545, both single nucleotide polymorphisms (SNPs) are in weak linkage disequilibrium (LD) ($r^2 = 0.48$). The minor rs4845625*T allele has been associated with increased CRP and a 4% increase in CHD risk in the CARDIoGRAM-C4D Consortium[5] while the minor rs4537545*T allele has been associated with decreased CRP and a 5% decrease in the risk of CHD.[8,11] Also, both polymorphisms have been associated with triglycerides levels, with contradictory results obtained in different studies.[4,13–15] Further investigations are needed in order to clarify the associations of the two SNPs with CRP and lipid metabolism and the common or independent underlying mechanisms. Consequently, in this investigation, we aimed to point out the associations of these two *IL-6R* polymorphisms with CRP and lipids levels and identify potential associations with functional biological processes.

RESULTS

The phenotypic characteristics of the studied populations are shown in Table 1 and the numbers of individuals with each genotype are shown in Table 2.

The genotypes of the polymorphisms were in Hardy–Weinberg equilibrium (data not shown).

Associations of SNPs rs4845625 and rs4537545 in the *IL-6R* gene with CRP levels

Serum CRP levels according to the two *IL-6R* SNPs are shown in Table 3. The two SNPs have antagonistic effects on the CRP serum concentrations in the STANISLAS Family Study (SFS). The minor

[1]INSERM UMR U1122; IGE-PCV 'Interactions Gène-Environnement en Physiopathologie Cardio-vasculaire', Faculté de Pharmacie, Université de Lorraine, Nancy, France; [2]Department of Epidemiology, University Groningen, University Medical Center Groningen, Groningen, The Netherlands and [3]Department of Internal Medicine and Geriatrics, CHU Technopôle Nancy-Brabois, Rue du Morvan, Vandoeuvre-lès-Nancy, France. Correspondence: Dr S Visvikis-Siest, INSERM UMR U1122; IGE-PCV, Faculté de Pharmacie, Université de Lorraine, 30 rue Lionnois, 54000 Nancy, France.
E-mail: sophie.visvikis-siest@inserm.fr
[4]These two authors contributed equally to this work.
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rs4845625*T allele is associated with increased levels of CRP (*P*-value = 0.011, $\beta = 0.182$). The minor rs4537545*T allele has the opposite effect of that of rs4845625*T, as it is associated with decreased levels of CRP (*P*-value = 0.009, $\beta = -0.189$).

Associations of rs4845625 and rs4537545 in the *IL-6R* gene with lipid traits

In the discovery population, the minor rs4845625*T allele was significantly associated with increased levels of low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B (ApoB) levels (*P*-value = 0.007, $\beta = 0.052$ and $P = 0.009$, $\beta = 0.044$ respectively, Table 3). The association with LDL-C was confirmed in the replication sample of adults (*P*-value = 0.035, $\beta = 0.024$, Table 4). The rs4845625*T allele was not significant for the total cholesterol (TC) in the discovery individuals from SFS, but in the replication sample, it was associated with increased TC levels (*P*-value = 0.011, $\beta = 0.019$, Table 4). In the discovery population, the minor rs4845625*T allele explains 3.49 and 5.57% of the LDL-C and ApoB variances respectively. In the replication sample, whereas the variance explained by this allele dropped to 0.44% in the case of LDL-C, the association remained significant. The variance explained was 0.63% in the case of TC in the replication population. In the association studies performed for the SNP rs4537545, none of the lipid phenotypes was significant in the discovery and replication populations (Table 5).

Table 1. Phenotypic characteristics of the studied populations

	SFS	*Replication population*
Sample size (% female)	368 [50.3%]	995 [50.4%]
Age (years) (s.d.)	43.56 [5.58]	55.45 [11.1]
BMI (kg m^{-2}) (s.d.)	24.74 [3.6]	26.61 [3.47]
LDL-C (mmol l^{-1}) (s.d.)	3.49 [1.04]	3.6 [0.86]
ApoB (g l^{-1}) (s.d.)	1.01 [0.23]	—
Total cholesterol (mmol l^{-1}) (s.d.)	5.71 [1.01]	5.95 [0.99]
Triglycerides (mmol l^{-1}) (s.d.)	1.32 [2.04]	—
CRP (mg l^{-1}) (s.d.)	1.54[2.9]	—

Abbreviations: BMI, body mass index; CRP, C-reactive protein; LDL-C, low-density lipoprotein cholesterol; SFS, STANISLAS Family Study.

Table 2. Number of individuals with each genotype for the polymorphisms studied

Polymorphism	*Genotype*	*Discovery population (N)*	*Replication population (N)*
Rs4845625	CC	134	331
	CT	171	497
	TT	63	167
Rs4537545	CC	122	335
	CT	180	492
	TT	66	168

IL-6R haplotypes associations with lipid traits and CRP levels

Three main haplotypes of rs4845625/rs4537545, H1 (CT), H2 (TC) and H3 (CC), were present in the two populations investigated, with the following frequencies: H1 = 40.6%, H2 = 41.6% and H3 = 17.6%. The beta effects and *P*-values of each haplotype for the studied phenotypes are given in Table 6. The results previously obtained when performing association studies separately with each SNP were confirmed in the haplotype studies.

The haplotype 1 (CT) was associated with decreased levels of CRP (*P*-value = 0.01) while the haplotype 2 (TC) was associated with increased levels of CRP (*P*-value = 0.007). The combination of both major alleles of the two SNPs (haplotype 3: CC) was not associated with CRP levels, as the average effect of both SNPs results in no significant changes in CRP levels.

The haplotype 2 (TC), having the minor rs4845625*T allele, was related with increased levels of LDL-C in the discovery population, while a nominal significance was observed in the replication population (*P*-value = 0.015 and *P*-value = 0.024 respectively). The same haplotype (TC) was nominally related with increased ApoB levels in the discovery population (*P*-value = 0.022) while the haplotype 3 (CC) was nominally associated with decreased levels of ApoB (*P*-value = 0.024). The haplotype 2 (TC) was associated with increased levels of total cholesterol in the replication

Table 3. Associations of the SNPs rs4845625 and rs4537545 (*IL-6R*) with CRP levels (units in mg l^{-1}) in the discovery population

SNP	*MAF*	*TT*	*TC*	*CC*	*Total*	β	*Standard error*	P-*value*	*Variance explained*
Rs4845625	0.40	2.55 (0.67)	1.43 (0.17)	1.22 (0.15)	1.55 (0.15)	0.182	0.071	0.011	2.48%
Rs4537545	0.42	1.09 (0.17)	1.42 (0.18)	2.01 (0.36)	1.56 (0.15)	−0.189	0.072	0.009	2.38%

Abbreviations: CC, homozigous for the major allele; MAF, Minor allele frequency; SNP, single nucleotide polymorphisms; TC, heterozigous for both SNPs; TT, homozigous for the minor allele. *P*-value threshold is $P < 0.008$.

Table 4. Associations of the minor allele T of the SNP rs4845625 (*IL-6R*) with blood lipids levels

Population	*Phenotypes*	β	*Standard error*	P-*value*	*Variance explained (%)*
SFS	LDL (mmol l^{-1})	0.052	0.019	**<u>0.007</u>**	3.49%
$N = 368$	ApoB (g l^{-1})	0.044	0.017	**0.009**	5.57%
MAF = 0.40	TC (mmol l^{-1})	0.021	0.012	0.09	—
	TG (mmol l^{-1})	0.013	0.038	0.725	—
Replication	LDL (mmol l^{-1})	0.024	0.011	**0.035**	0.44%
Population	ApoB (g l^{-1})	—	—	—	—
$N = 995$	TC (mmol l^{-1})	0.019	0.007	**<u>0.011</u>**	0.63%
MAF = 0.42	TG (mmol l^{-1})	—	—	—	—

Abbreviations: LDL-C, low-density lipoprotein cholesterol; SFS, STANISLAS Family Study; SNP, single nucleotide polymorphism; TC, total cholesterol; TG, triglycerides. Significant *P*-values are highlighted in bold and with an underline, and nominal *P*-values are highlighted in bold. *P*-value threshold is $P < 0.008$ for the discovery population and $P < 0.016$ for the replication population.

Table 5. Associations of the minor allele T of the SNP rs4537545 (*IL-6R*) with blood lipid levels

Population	*Phenotypes*	β	*Standard error*	P-*value*	*Variance explained (%)*
SFS	LDL (mmol l^{-1})	−0.021	0.019	0.296	—
N = 368	ApoB (g l^{-1})	−0.011	0.017	0.528	—
MAF = 0.42	TC (mmol l^{-1})	0.000	0.012	0.986	—
	TG (mmol l^{-1})	−0.005	0.038	0.088	—
Replication	LDL (mmol l^{-1})	−0.010	0.011	0.364	—
Population	ApoB (g l^{-1})	—	—	—	—
N = 995	TC (mmol l^{-1})	−0.010	0.007	0.190	—
MAF = 0.41	TG (mmol l^{-1})	—	—	—	—

Abbreviations: LDL-C, low-density lipoprotein cholesterol; SFS, STANISLAS Family Study; SNP, single nucleotide polymorphism; TC, total cholesterol; TG, triglycerides. *P*-value threshold is $P < 0.008$ for the discovery population and $P < 0.016$ for the replication population.

Table 6. Association of *IL-6R* haplotypes with differences in lipid traits and CRP levels

Phenotype	*Haplotype*	*SFS (N = 368) Beta effect*	P-*value*	*Replication (N = 995) Beta effect*	P-*value*
LDL	H1 (CT)	−0.0223	0.271	−0.0101	0.374
	H2 (TC)	0.0479	**0.015**	0.0255	**0.024**
	H3 (CC)	−0.0461	0.083	−0.0268	0.074
ApoB	H1 (CT)	−0.0108	0.544	—	—
	H2 (TC)	0.0397	**0.022**	—	—
	H3 (CC)	−0.0529	**0.023**	—	—
TG	H1 (CT)	−0.0105	0.795	−0.0165	0.464
	H2 (TC)	0.0163	0.681	0.0091	0.686
	H3 (CC)	−0.0183	0.730	0.0132	0.659
TC	H1 (CT)	−0.0004	0.975	−0.0101	0.193
	H2 (TC)	0.0198	0.124	0.0203	**0.008**
	H3 (CC)	−0.0344	**0.046**	−0.0173	0.088
CRP	H1 (CT)	−0.194	**0.010**	—	—
	H2 (TC)	0.197	**0.007**	—	—
	H3 (CC)	−0.0133	0.894	—	—

Abbreviations: CRP, C-reactive protein; LDL-C, low-density lipoprotein cholesterol; SFS, STANISLAS Family Study; SNP, single nucleotide polymorphism; TC, total cholesterol; TG, triglycerides. Haplotype defining SNPs = (rs4845625, rs4537545). Frequency of the haplotypes: H1 = 40.6%, H2 = 41.6%, H3 = 17.6%. Significant *P*-values are highlighted in bold and with an underline, and nominal *P*-values are highlighted in bold. *P*-value threshold is set at $P < 0.016$.

population (*P*-value = 0.008), this association was not significant in discovery population.

In silico re-sequencing and functional analysis

Identification of the LD variants associated with the two *IL-6R* SNPs residing in the vicinity of *IL-6R* led to 287 SNPs in LD with the two SNPs at $r^2 \geqslant 0.2$ as shown in LD plot (Figures 1 and 2). Out of 287 LD-SNPs, 63 had $r^2 \geqslant 0.8$ and were used in the next analysis. expression quantitative trait loci analysis identified seven expression probes that were significantly associated with the two SNPs and the 68 LD-SNPs (with $r^2 \geqslant 0.8$) at false discovery rate < 0.05. Seven expression probes belonging to five genes (Table 7) were used in the final step. These five genes were analysed further, and revealed 24 significantly biological functions (at false discovery rate < 0.05; Supplementary Data). Seventeen of the 24 significantly enriched terms were involved in the regulation of the JAK-STAT pathway cascade; thus, we categorised them in one biological function group as JAK-STAT pathway. The enrichment scores of each biological function group and their corresponding significance were obtained from the hypergeometric probability test (Supplementary Data). The JAK-STAT pathway showed a significant enrichment score of $P = 4.5 \times 10^{-8}$.

Bioinformatics analyses

Comparing different cell types, bioinformatics analyses were performed to point out if the selected SNPs are located in regulation zones (promoter, enhancer, silencer) of IL6R gene on the human genome using Ensembl browser[16] (Supplementary Data).

The rSNPBase, a database for curated regulatory SNPs, was used to investigate SNPs of interest.

DISCUSSION

In this investigation, we stressed our analyses on two in weak LD ($r^2 = 0.48$) *IL-6R* polymorphisms that had been previously associated with antagonistic effects with CRP levels[11,12] and with risk of CHD[5,8,11] and with evidence of controversial links with lipid metabolism traits: SNPs rs4845625 and rs4537545, in order to clarify underlying mechanisms. Previously found associations of the two *IL-6R* polymorphisms with lipid metabolism, CRP and CHD are summarised in Table 8.

We have confirmed the associations between the SNPs rs4845625 and rs4537545 with CRP levels. The minor rs4845625*T allele increases CRP levels (*P*-value = 0.011), while the minor rs4537545*T allele decreases CRP levels (*P*-value = 0.009), confirming the previous results. In fact, the antagonistic SNPs effects on CRP levels have been described previously in healthy and pathological populations.[8,10–12,17] This antagonistic effect of the two SNPs has been already related with the risk of CHD. The minor rs4845625*T allele has been associated with a 4% increase in CHD risk in the CARDIoGRAM-C4D Consortium[5] while the minor rs4537545*T allele has been associated with a 5% decrease in the risk of CHD[8,11] (note that the rs4537545 is in strong LD with the SNP rs2228145).

These antagonistic effects of rs4845625 and rs4537545 could explain the previously described controversy of results associating them with lipids traits (Table 8). It is well known that CRP and lipid levels are both associated with CHD.[18] Indeed cardio-vascular diseases are a combination of dyslipidaemia and chronic inflammation.[18] High LDL levels are the main cause of endothelium injury, but in presence of high CRP levels, there is an acceleration in the progression of CHD, as the CRP affects the LDL uptake by macrophages, facilitating the formation of foam cells.[19]

Concerning the effects of the two SNPs on lipid metabolism traits, we demonstrated for the first time an association of the minor rs4845625*T allele with increased ApoB and LDL-C levels, consequently, conferring to this allele the notion of risk factor. This is in concordance with the previous results where this SNP has been associated with increased risk of CHD,[5] atrial fibrillation[20] and subclinical atherosclerosis.[21] In the individuals of SFS, this risk

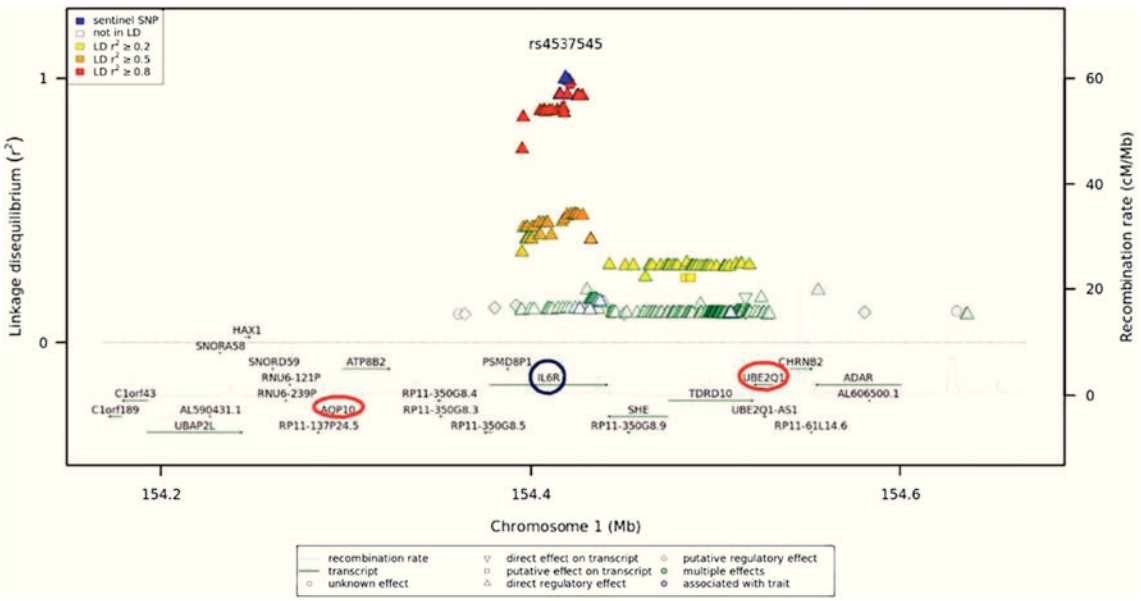

Figure 1. Regional LD plot for rs4537545 that is represented as a blue diamond. The other SNPs are colour-coded according to the strength of LD (as measured by r^2) with rs4537545 and the circles show the genes which are associated with IL-6R in expression data sets.

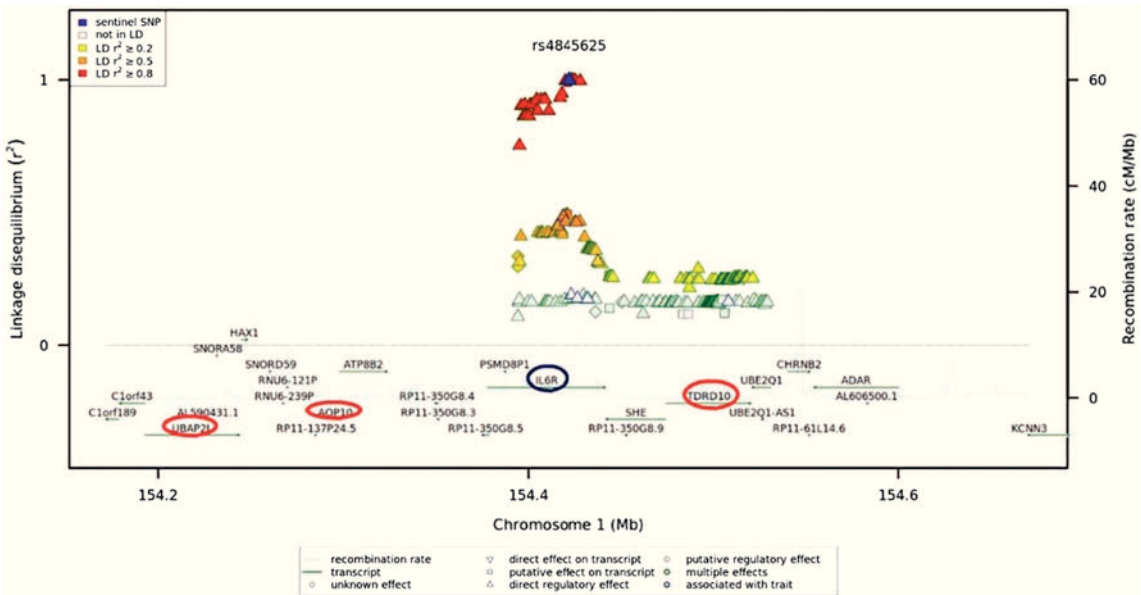

Figure 2. Regional LD plot for rs4845625 that is represented as a blue diamond. The other SNPs are colour-coded according to the strength of LD (as measured by r^2) with rs4845625 and the circles show the genes which are associated with IL-6R in expression data sets ADDIN

allele explains 3.49 and 5.57% of the total variances of LDL-C and ApoB levels respectively (% higher than that of most genome-wide association studies). In the replication population, the minor allele was still a risk factor; however, the variance explained in LDL-C dropped to 0.44% (ApoB levels were not available in the replication population). This drop in the explained variance could be due to the fact that the replication population is older (mean age of 55.45 [11.1] years versus 43.56 [5.58] years in the discovery population). An accumulation of the environmental exposures over age may increase the phenotypic variance of complex traits making more difficult the detection of genetic associations in age-advanced populations.

The SNP rs4845625 was not associated with the other lipid traits studied. Although some studies have associated this SNP with triglycerides (TG), the results have been contradictory, relating its minor allele with increased or decreased TG.[4,13–15] Other studies have failed to find associations between the polymorphism rs2228145, which is in strong LD with the SNP rs4537545, with the LDL-C and TC levels. Only one study has found a weak association between the SNP rs2228145 and TC/HDL (*P*-value = 0.01) and LDL/HDL (*P*-value = 0.02) ratios[22] and this association was found only in teenager girls.

We also performed haplotype analysis of the two polymorphisms, which allowed us to obtain details on the size effect of each variant on the phenotypes studied. The haplotype analysis showed that the association of the SNP rs4845625 with lipid levels is independent from rs4537545 (although the two SNPs are physically relatively close one to the other, having a LD of $r^2 = 0.48$) and confirmed our previous separate SNPs analyses results. The haplotype 2, where the minor rs4845625*T allele is

Table 7. Results of eQTL analysis

Gene	*Probe*	*Tissue*	P-*value*	*FDR*
AQP10	ILMN_2090004	Blood	3.08×10^{-5}	0.01
IL-6R	ENSG00000160712	Blood, testis, transverse colon, tibial artery	1.40×10^{-8}	0.00
	ILMN_1754753	Blood	2.29×10^{-5}	0.00
TDRD10	ENSG00000163239	Visceral adipocytes, liver, blood, lung, testis, atrial appendage, thyroid, visceral adipocytes, subcutaneous adipocytes	8.28×10^{-7}	0.00
	ILMN_1751630	Liver, Blood	1.10×10^{-8}	0.00
UBAP2L	ILMN_1814789	Blood	6.75×10^{-5}	0.03
UBE2Q1	ILMN_1776325	Blood	5.52×10^{-7}	0.00

Abbreviations: eQTL, expression quantitative trait loci; FDR, false discovery rate; IL-6R, interleukin 6 receptor.

Table 8. Previous studies associating the *IL-6R* SNPs rs4845625 (a), rs4537545 (b) and SNPs in strong LD ($r^2>0.8$) with CRP, lipids and CHD traits

(a)				
SNP	*Associated trait*	*Beta*	P-*value*	*Reference*
rs4845625	Triglycerides	−	0.027	PMID: 26238946
rs4845625	CHD	+	3.64×10^{10}	PMID: 23202125
rs4845625	CRP	+		PMID: 23505291
(b)				
rs2228145	Chol/Hdl ratio	+	0.01	PMID: 21835044
rs2228145	LDL/HDL ratio	+	0.02	PMID: 21835044
rs2228145	Triglycerides	+	0.001	PMID:23479153
rs2228145	Triglycerides	−	0.026	PMID: 16817825
rs2228145	Triglycerides	−	0.009	PMID: 20186139
rs2228145	CHD	−	1.53×10^{-5}	PMID: 22421340
rs2228145	CHD	−	4.5×10^{-5}	PMID: 22421339
rs2228145	CRP	−	1.6×10^{-19}	PMID: 22421339
rs4537545	CRP	−	5.1×10^{11}	PMID: 19567438

Abbreviations: CHD, coronary heart disease; CRP, C-reactive protein; HDL, high-density lipoprotein; IL-6R, interleukin 6 receptor; LD, linkage disequilibrium; LDL, low-density lipoprotein; SNP, single nucleotide polymorphism. Rs4537545 is in almost in complete LD with rs2228145 ($r^2=0.97$). The positive/negative values represent the beta effect direction of the minor allele of the SNP.

present, has been associated with increased LDL-C (in discovery population and nominally in the replication population) and ApoB levels (nominal association in the two populations). Even if the association of the haplotype 2 for ApoB levels did not reach significance based on the cut off set for multiple testing control, it has the same direction as in the independent SNPs analysis, thus supporting the identified results. For the CRP levels, the two SNPs have opposite effects. Indeed, the haplotype 1 (CT) is associated with decreased CRP levels ($P=0.01$) while the haplotype 2 (TC) is associated with increased CRP levels ($P=0.007$). So far, this is the first time that an haplotype analysis investigates associations of *IL-6R* gene polymorphisms with lipid traits and that one frequent (41,6%) in the population haplotype (haplotype 2) can be designed as risk factor for simultaneously increasing LDL-C, ApoB and CRP levels.

A series of *in silico* re-sequencing as well as functional analysis helped us to identify potential functional biological processes underlying the associations of the two *IL-6R* SNPs with lipid metabolism.

Our results are of particular importance, especially, given the previously reported involvement of *IL-6R* in the JAK-STAT3 pathway[1] that we confirmed, as we further showed that *IL-6R* rs4845625 might regulate the JAK-STAT3 pathway and, via this action, affect lipid levels. Previous studies showed that the overexpressed STAT3 (activated by the IL-6/sIL-6R complex) reduces acetyl-coA carboxylase, as well as the amount of acyl-Coa oxidase enzymes.[23] Also, mice that are knock out for the *STAT3* gene had higher adipose tissue mass and weight.[24] Thus, the STAT3 pathway could in fact promote lipolysis.

Our results, especially those concerning the haplotype 2 (TC), may have important value in pharmacogenomics studies given the fact that *IL-6R* gene is a target of the pharmacological agent tocilizumab,[25] an immunosuppressive drug widely used for the treatment of rheumatoid arthritis and which competitively inhibits IL-6R, resulting in a reduction of CRP and fibrinogen concentrations.[26] One of the side effects of tocilizumab is the increase of LDL-C levels in patients,[27] which could lead to a risk of suffering from ischaemic heart disease. Moreover, the tocilizumab blocks both classic and trans-signalling pathways and, looking at the complex biology of IL-6, the extended blockage of this cytokine should be extensively studied. So far, it is not clear whether those blood lipids changes reflect mechanism-based effects of IL-6R modification, or tocilizumab-specific effects. Nonetheless, our results could also give interesting clues on the functionality of the drug.

MATERIALS AND METHODS

Ethics statement

The discovery and replication samples are part of a human sample storage platform: the Biological Resources Centre 'Interactions Gène- Environnement en Physiopathologie CardioVasculaire' (BRC IGE-PCV, number BB-0033-00051) in Nancy, France.

A written informed consent was obtained from each participant, and the study protocols were approved by the corresponding ethics committee of the recruitment centres.

Study populations

The discovery population enrolled 368 unrelated adults of French origin, a sub-sample from the longitudinal SFS. This cohort aims mainly to investigate the genetic and environmental factors associated with cardio-vascular diseases traits. The study population was recruited at the Centre for Preventive Medicine (CMP) of Vandoeuvre-lés-Nancy. Participants were Caucasians residents of Vosges and the South of Meurthe-et-Moselle, located in the East of France. They were apparently healthy, not users of lipid-lowering and/or cardio-vascular medication and did not have a diagnosis of other chronic diseases.

The replication cohort included 995 unrelated adults of French origin, who were included in the BRC IGE-PCV. Their inclusion criteria were the same as in the discovery cohort.

An informed consent was obtained from all subjects participating in this study.

Data collection and biological measurements

Blood samples were taken from the individuals after an overnight fast. Body mass index was measured as weight divided by height squared (kg m^{-2}). Serum total cholesterol (TC) and triglycerides were assessed enzymatically with an AU 640 automated device (Olympus, Rungis, France). Low-density lipoprotein cholesterol levels were calculated using the Friedewald equation (LDL-C = TC − high-density lipoprotein cholesterol (HDL-C) − (TG/5)).[28] Serum ApoB and CRP concentrations were measured by immunonephelompetry with a Behring Nephelemeter Analyzer II (Dade Behring, Marburg, Germany). ApoB and CRP levels were not available in the replication population.

Genotyping

The two SNPs were genotyped by Genoscreen, using a Sequenom iPLEX Gold assay-Medium Throughput Genotyping Technology and by using the competitive allele-specific PCR (KASP) chemistry coupled with a FRET-based genotyping system.

Data analyses and statistical modelling

Blood lipids and CRP levels were square root and log transformed and the normality of distribution was tested by Kolmogorov–Smirnov test. Hardy–Weinberg equilibrium was tested using the chi-square test.

The SNP-phenotypes associations were assessed using linear mixed effects model with the GWAF package for R, although the pedigree was not taken into account as the individuals were not related between them.[29] The models were adjusted for age, gender and body mass index under an additive model using the minor allele as the reference allele. Populations' characteristics were determined using the SPSS statistical software version 20.0 (SPSS, Inc, Chicago, IL, USA).

The Bonferroni correction was applied, in order to adjust the type 1 error (a) by the total number of tests (a/n tests). The necessary *P*-value threshold was of $P << 0.008$ for the discovery population, and $P < 0.016$ for the replication population.

Haplotype analysis was performed using the --hap function of the Plink 1.07 software. Haplotypes with frequency $\geqslant 0.01$ were included in the analysis, and level of missingness per individual per haplotype was set at < 0.5. The *P*-value threshold was < 0.05/number of haplotypes.

In silico re-sequencing and functional analysis

We first performed an *in silico* sequencing analysis to identify the genetic variants that are linked with the two *IL-6R* SNPs. We used Arggr browser and targeted a region of 1 Mb at both sides of the SNPs with $r^2 \geqslant 0.2$. Afterwards an expression quantitative trait loci analysis was performed using available data of expression probes from blood, liver and visceral adipocytes to identify potentially functional variants associated with expression levels (eSNPs). All *IL-6R* LD-SNPs and their associated linked SNPs were included in three different expression identified tools, including SNiPA, GTEx and Blood expression quantitative trait loci browser in data sets of blood, liver and visceral adipocytes expression probes to find regulatory variants. We used a cut of $r^2 \geqslant 0.8$ and false discovery rate < 0.05 as linked eSNPs to *IL-6R* variants. We combined SNPs, linked SNPs and linked eSNPs to assemble a query list of potentially related genes found so far, and submit it as an input to Gene Ontology Consortium and to composite network tools implemented in GeneMANIA to predict gene function and potential biological processes in which these genes are involved. We set those GO terms associated with similar biological function in one category as biological function group. Biological function group enrichment was calculated using the following formula:

$$\frac{\left(\frac{\text{Number of genes in each bilogical function group in our network}}{\text{Total genes in our network}}\right)}{\left(\frac{\text{Number of genes in each biological fuction group in entire genome}}{\text{Total genes in entire genome}}\right)}$$

To show that these enriched biological processes within our network are highly unlikely to have occurred by chance, we performed the exact hypergeometric probability test via GeneProf software.[30]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Weblinks
https://phgkb.cdc.gov/PHGKB
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Supplementary Information accompanies this paper on *Genes and Immunity* website (http://www.nature.com/gene)

Supplementary table 1. Identification of the L.D variants associated with the two *IL-6R* SNPs residing in the vicinity of *IL-6R* led to 287 SNPs in LD with the 2 SNPs at $r^2 \geq 0.2$

gSNP	LD-SNPs with $r^2 \geq 0.8$	LD-SNPs with r2≥0.5	LD-SNPs with $r^2 \geq 0.2$
rs4845625	31	33	166
rs4537545	32	36	121
total	63	69	**287**
common	0	1	70
individual	63	68	217

Supplementary table 2. Prediction of possible common biological functions by gene ontology, 24 biological functions were significantly revealed (at FDR<0.05)

Accession	Function	FDR	Genes in network					Genes in genome
GO:0042517	positive regulation of tyrosine phosphorylation of Stat3 protein	0.0002315708196368445	4	IL6R	IL6	IL6ST	CNTF	23
GO:0070851	growth factor receptor binding	0.0002315708196368445	5	IL6R	IL6	IL6ST	CNTF	74
GO:0005126	cytokine receptor binding	0.0002315708196368445	6	IL6R	IL6	IL6ST	CNTF	147
GO:0042516	regulation of tyrosine phosphorylation of Stat3 protein	0.0002446370444666558	4	IL6R	IL6	IL6ST	CNTF	29
GO:0042503	tyrosine phosphorylation of Stat3 protein	0.0002588225872409049	4	IL6R	IL6	IL6ST	CNTF	31
GO:0007259	JAK-STAT cascade	0.0003293847802057481 5	5	IL6R	IL6	IL6ST	CNTF	91
GO:0042531	positive regulation of tyrosine phosphorylation of STAT protein	0.0004310676201459018 5	4	IL6R	IL6	IL6ST	CNTF	38
GO:0046427	positive regulation of JAK-STAT cascade	0.0006726393305278473	4	IL6R	CNTF	IL6	IL6ST	45

GO:0042509	regulation of tyrosine phosphorylation of STAT protein	0.0006726393305278473	4	IL6R	IL6	IL6ST	CNTF	44
GO:0007260	tyrosine phosphorylation of STAT protein	0.0007885284087498134	4	IL6R	IL6	IL6ST	CNTF	48
GO:0071354	cellular response to interleukin-6	0.0012120324108552227	3	IL6R	IL6	IL6ST		14
GO:0046425	regulation of JAK-STAT cascade	0.001742504121301376	4	IL6R	IL6	IL6ST	CNTF	61
GO:0070741	response to interleukin-6	0.0019109881910089224	3	IL6R	IL6ST	IL6		17
GO:0018108	peptidyl-tyrosine phosphorylation	0.008291209509250764	5	IL6R	IL6	IL6ST	CNTF	210
GO:0018212	peptidyl-tyrosine modification	0.008291209509250764	5	IL6R	IL6	IL6ST	CNTF	210
GO:0050731	positive regulation of peptidyl-tyrosine phosphorylation	0.009492111549455031	4	IL6R	IL6	IL6ST	CNTF	100
GO:0045669	positive regulation of osteoblast differentiation	0.011565976111158664	3	IL6R	IL6	IL6ST		33
GO:0006833	water transport	0.016817690559459968	3	AQP10	AQP9	AQP3		38
GO:0042044	fluid transport	0.01862789809438206	3	AQP10	AQP9	AQP3		40
GO:0006954	inflammatory response	0.025806763117896155	5	IL6R	IL6	IL6ST	HCK	282
GO:0050730	regulation of peptidyl-tyrosine phosphorylation	0.025806763117896155	4	IL6R	IL6	IL6ST	CNTF	138
GO:0010035	response to inorganic substance	0.03689675124788528	4	IL6	AQP9	AQP3	NEDD4	153
GO:0045667	regulation of osteoblast differentiation	0.04491618210760434	3	IL6R	IL6	IL6ST		57
GO:0009617	response to bacterium	0.04648394114776275	4	IL6R	IL6	HCK	ERAP1	166

Supplementary table 3. The enrichment scores of each biological function group and their corresponding significance obtained from the exact hypergeometric probability test

Criteria	JAK-STAT Phatway group	Water transport	immunity response
Numer of genes in biological function in our network	6	3	6
Numer of genes in biological function in entire genome	172	307	836
Total genes in our network	25	25	25
Total genes in entire genome	20687	20687	20687
hypergeometric probability	4.5×10^{-8}	0,005	0,0003

Table 4. SNP Analysis. rSNPBase quering results showed that both SNPs analyzed are referenced as regulatory SNPs. Rs4845625 is involved in a proximal transcriptional regulation mechanism in monocytes.

Element	Type	Cell type	Source
2 regulations zones	Promoter/enhancer	Monocyte/neutrophil	Ensembl
Rs4845625 **Rs4537545**	rSNP	-	rSNPBase
Rs4845625	Proximal transcriptional regulation	Monocyte	rSNPBase

Supplementary figure 1. Regulation profile of the *IL6R* gene in different cell types expressing the IL6R protein. The 2 polymorphisms, rs4537545 and rs4845625, are highlighted with vertical red lines. Both SNPs are located in intronic zones. They are positioned near two active regulatory regions (within the red rectangle).

Publication N°3

Pleiotropy of ABO gene; Correlation of rs644234 with E-selectin and lipid levels

Alex-Ander ALDASORO ARGUINANO[1], Ndeye Coumba NDIAYE[1], Christine MASSON[1], Sophie VISVIKIS-SIEST[¥1, 2]

CCLM (Clinical Chemistry and Laboratory Medicine). Accepted with minor changes.

Throughout the years, it has been demonstrated the existence of pleiotropic effects of the ABO locus (241), reinforcing the idea that the ABO gene is affecting CVD in diverse ways. The association between the ABO polymorphisms and soluble E-selectin levels are especially strong, as this locus explains up to 20% of the variance of this adhesion molecule, making the ABO locus the main one affecting the soluble E-selectin levels (242). A recent genome-wide association study associated polymorphisms in ABO with total cholesterol (243) and phytosterol levels (244). Also, genetic variants in ABO have been demonstrated to be associated with HDL and triglyceride levels in a Taiwanese population, and this association was dependent of the blood group of the participants involved (245). Other variants at ABO have been described to be associated with plasma levels of pancreatic lipase and high intestinal cholesterol absorption (246, 247). The apolipoproteins B and E (ApoB and ApoE) are also important phenotypes related with CVD. However, so far, no association has been found between the ABO polymorphisms and these apolipoprotein levels.

In our study, we analysed the SNP rs644234 located within the ABO gene, which tags the blood group O with a r^2 of 0.89, the blood group A with r^2 of 0.58 and the blood group B with r^2 of 0.16 (248). This polymorphism has been already related with higher risk of suffering myocardial infarction (248), showing the importance of this genetic region with the risk of suffering diverse diseases. We analysed the association of the SNP rs6442324 with soluble E-selectin levels and several lipid phenotypes, including the apolipoproteins B and E (ApoB and ApoE), total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) levels.

Objective

The goal of this work was to study the association of the SNP rs644234 located in the ABO gene with different intermediate phenotypes related with diseases were the ABO gene has been involved.

Results

Genetic association of rs644234 with soluble E-selectin levels

After performing conditional analysis by adjusting for the rest of the phenotypes studied to the model, results showed a strong association between the polymorphism rs644234 and the soluble E-selectin levels (p-value= $8.7x10^{-12}$, β= 0.28). The major rs644234*T allele was associated with increased levels of soluble E-selectin.

Genetic association of rs644234 with lipid levels

Although the SNP showed significant associations with ApoB when first linear regressions were made (p-value= 0.018), this association was no more significant after performing conditional analysis (p-value= 0.068). Conversely, ApoE remained significantly associated to rs644234 after performing conditional analyses for the rest of phenotypes studied (p-value= 0.001), making this association independent as the major rs644234*T allele is decreasing the levels of ApoE. The HDL-C was also significant before and after performing conditional analysis (p-value= 0.012 and p-value= 0.013, respectively), where the major rs644234*T allele was associated with increased levels, although this association remained marginal once adjusted for Bonferroni (p-value set at P<0.0071). According to the LDL-C levels, the association with the major rs644234*T allele was the opposite of that found for HDL-C, showing decreased levels with this allele. However, this association remained marginal once adjusted for Bonferroni (p-value= 0.032). The rest of the lipid phenotypes studied, more specifically total cholesterol and triglycerides, were not significant.

Associations of the SNP rs644234 with sex

The same phenotypes were analyzed by separating them by sex (n=173 males and 175 females). In males and females the relationship with sE-selectin was significant and showed

not sexual dimorphism according to the direction of the effect, although a big part of the significance was lost, probably due to the smaller sample sizes. According to the lipid levels, the significance was lost when the entire population was separated by gender, with the exception of the HDL-C levels, which was significant in males (p-value= 0.001) but not in females (p-value= 0.25).

Discussion

We managed to replicate previously found associations of the ABO polymorphisms with soluble E-selectin levels (242), as our results showed that the major rs644234*T allele is highly associated with increased levels of E-selectin. Among the lipid traits studied we found significant associations with ApoE levels, as the major rs644234*T allele is related with decreased levels of ApoE. HDL-C and LDL-C were marginally associated after adjusting the p-value. We found sexual dimorphism according to HDL-levels, were the major rs644234*T allele was significantly increasing the levels of HDL-C only in male individuals. Teng et al. (245) also found significant associations with HDL-C levels and ABO blood groups. Concerning apolipoproteins, important phenotypes related with cardiovascular diseases and closely related with lipoprotein levels, so far, our results are the first ones associating a polymorphism within the ABO gene with ApoE levels. Other studies have found associations between the ABO blood groups and erythrocyte-bound apolipoprotein B levels (249), which is related with atherosclerosis. It is important to mention here that the serum ApoB levels are not associated with erythrocyte-bound ApoB levels (249). By performing conditional analysis, we demonstrated that ABO gene has pleiotropic effects as it increases the E-selectin and HDL levels while decreasing the ApoE levels with effects independent from each other. Thus, the ABO variants could affect these phenotypes through different molecular mechanisms.

It is important to mention that we inferred the main ABO blood group that tags the SNP rs644234 (250) being mostly the O blood group (r^2=0.89) and in a less amount the A and B blood groups (r^2=0.58 and r^2=0.16 respectively). Taking this into account, we could suggest that our results are more likely representative in individuals with the O blood group. Indeed, other authors have already demonstrated that a SNP in strong LD with rs644234 (r^2=0.9) is associated with myocardial infarction, but only in the subgroup of individuals having the O blood group. One study showed that the minor rs644234*G allele was associated with the risk of myocardial infarction (248). Also, the minor alleles of other SNPs in strong LD with our

SNP of interest have been associated with coronary heart disease (241), large artery atherosclerotic stroke (251) and venous thromboembolism (252, 253). Thus, even if the major rs644234*T allele has simultaneously effects on phenotypes that could be considered to be risky/protective for certain diseases, looking to the previous studies performed and our results, we could deduce that the major allele acts with a protective effect when it concerns cardiovascular related diseases, and more specifically on lipid traits, with lower ApoE levels and higher HDL levels.

These genetic associations of the ABO gene with different endophenotypes show how complex are the effects of this gene in different diseases, including CVD, as it affects different pathways. They also show the need for further studies in order to better understand the mechanistic phenomena of these relationships and their potential therapeutic translation.

Pleiotropy of ABO gene; Correlation of rs644234 with E-selectin and lipid levels

Alex-Ander ALDASORO ARGUINANO[1], Ndeye Coumba NDIAYE[1], Christine MASSON[1], Sophie VISVIKIS-SIEST[¥1, 2]

[1]INSERM UMR U1122; IGE-PCV 'Interactions Gène-Environnement en Physiopathologie Cardio-vasculaire', Faculté de Pharmacie – Université de Lorraine, Nancy, F-54000, France
[2] Department of Internal Medicine and Geriatrics, CHU Technopôle Nancy-Brabois, Rue du Morvan, F-54511, Vandoeuvre-lès-Nancy, France

[¥]Corresponding author:
Dr Sophie VISVIKIS-SIEST
INSERM UMR U1122; IGE-PCV, Faculté de Pharmacie – Université de Lorraine
30 rue Lionnois
54000 Nancy, France
Tel: +33.6.07.60.25.69
E-mail address: sophie.visvikis-siest@inserm.fr

ABSTRACT

Background

The ABO gene has been widely studied and associated with many different diseases such as myocardial infarction and diabetes. Pleiotropic effects of the ABO locus have been demonstrated. Indeed it affects different phenotypes such as E and P-selectins, triglycerides and total cholesterol. The goal of this work was to study the SNP rs644234 located in the ABO gene with different phenotypes related with diseases were the ABO gene has been involved.

Methods

We analyzed the SNP rs644234 located in the ABO gene, by performing association studies with different lipid phenotypes as well as with the soluble E-selectin levels in 348 adults from the STANISLAS Family Study.

Results

The major rs644234*T allele was associated with increased levels of soluble E-selectin (p-value= $8.7x10^{-12}$). According to the lipid phenotypes, the major rs644234*T allele was associated with decreased levels of ApoE (p-value= 0.001) and LDL-C (p-value= 0.032), while was associated with increased levels of HDL-C (p-value= 0.013). The association of the HDL-C was especially significant in the male individuals (p-value= 0.001).

Conclusions

We confirmed that ABO is a major locus for serum E-selectin levels variability and we also correlated this gene with different lipid phenotypes. Furthermore, we demonstrated that this pleiotropic effect is independent. This is the first time that a correlation has been made between the ABO gene and ApoE levels. According to these results, the major allele of rs644234 may have a protective effect when it comes to cardiovascular related diseases, and more specifically when it comes to the lipid phenotypes.

KEYWORDS

Cardio-vascular diseases, soluble E-selectin, ApoE, HDL-C

BACKGROUND

The ABO gene encodes enzymatic proteins that are related to the ABO blood group system. The enzymes modify the oligosaccharides of the glycoproteins located on cell surface and variations in the sequence of the ABO gene will determine the type of modifications made (1). The ABO blood groups have been widely studied throughout the years, and different studies have confirmed its relationship with the risk of suffering myocardial infarction (2), thrombosis (3), different types of cancer (4, 5) and diabetes (6).

Throughout the years, it has been demonstrated the existence of pleiotropic effects of the ABO locus (7), reinforcing the idea that the ABO gene is affecting cardio-vascular diseases (CVD) in diverse ways. One of the studied relationships of the ABO gene is with the Von Willebrand factor (VWF) (8), which is related with the platelet adhesion to wound sites, and therefore, is considered a risk factor for thrombosis. Also, the ABO locus has been associated with several markers of endothelial function such as E and P-selectins (9-11) and soluble intercellular adhesion molecule-1 (sICAM-1) (12). The association between the ABO polymorphisms and soluble E-selectin levels are especially strong, as this locus explains up to 20% of the variance of this adhesion molecule, making the ABO locus the main one affecting the soluble E-selectin levels (10). At the same time, the genetic-inferred ABO blood groups have been associated with the levels of soluble E-selectin levels (9, 13). It's important to point out that the adhesion molecules such as P and E-selectins have important roles in the development of CVD events (14).

A recent genome-wide association study associated polymorphisms in ABO with total cholesterol (15) and phytosterol levels (16). Also, genetic variants in ABO have been demonstrated to be associated with HDL and triglyceride levels in a Taiwanese population, and this association was dependent of the blood group of the participants involved (17). Other variants at ABO have been described to be associated with plasma levels of pancreatic lipase and high intestinal cholesterol absorption (18, 19). The apolipoproteins B and E (ApoB and ApoE) are also important phenotypes related with cardiovascular diseases and other diseases, however, so far, no association has been found between the ABO polymorphisms and the apolipoprotein levels. Only one study has found an association between the erythrocyte-bound ApoB in relation to ABO blood groups (20). Although the link between the ABO gene and lipids has been described, the mechanistic basis of these relationships and their possible potential for personalised medicine are not clear.

In our study, we analysed the SNP rs644234 located within the ABO gene, which tags the blood group O with a r^2 of 0.89, the blood group A with r^2 of 0.58 and the blood group B with r^2 of 0.16 (2). This polymorphism has been already related with higher risk of suffering myocardial infarction (2). Other polymorphisms located in the ABO gene and in strong linkage disequilibrium (L.D) with rs644234 have been also associated with pancreatic cancer (21), coronary heart disease (7), duodenal ulcer (22) and venous thromboembolism (23, 24), showing the importance of this genetic region with the risk of suffering diverse diseases.

The goal of this work was to replicate the possible association of the SNP rs644234 with soluble E-selectin levels and to determine if some of the lipid phenotypes are related to the ABO gene. We analysed the apolipoproteins B and E (ApoB and ApoE), as well as total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) levels in apparently healthy individuals of French origin, from the STANISLAS Family Study (SFS) (25).

METHODS

Ethics statement

The samples are part of a human sample storage platform: the Biological Resources Centre 'Interactions Gène- Environnement en Physiopathologie CardioVasculaire' (BRC IGE-PCV - number BB-0033-00051) in Nancy, East of France. All subjects gave written informed consent and the local ethics committees approved the projects' protocols.

Study participants

The individuals are from the STANISLAS Family Study, which is a 15-year longitudinal follow-up study conducted since 1994 on 1006 families (25). Subjects were recruited during a medical examination at the centre for preventive medicine (CMP) of Vandoeuvre-les-Nancy (East of France). Participants were of French origin and were apparently in good health, not under lipid-lowering and free of acute or chronic diseases such as stroke, myocardial infarction or cancer. For this study, a subsample of 348 unrelated adults was used, where the SNP rs644234 was genotyped. In those individuals, the phenotypes were already measured prior to this study. The data concerning smoking habits, alcohol consumption and physical activity were also available in the studied individuals.

Males and females with mean average alcohol consumption lower than 40 and 20 gr/day, respectively, were considered as low risk individuals. Males and females with mean average alcohol consumption between 40-60 gr/day and 20-40 gr/day, respectively, were considered to be in medium risk. Males and females with higher mean average alcohol consumption than 60 and 40 gr/day were considered as high risk individuals.

Data collection and biological measurements

Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. All measurements were made according to the standard procedures established by the CMP of Vandoeuvre-les-Nancy. Blood samples were taken from the individuals after an overnight fast. Plasma and serum samples were frozen at -80°C until analysis. Plasma levels of E-selectin were measured with enzyme-linked immunosorbant assay (R&D Systems, Abington, UK). Serum total cholesterol (TC) and triglycerides (TG) were assessed enzymatically with an AU 640 automated device (Olympus, Rungis, France). HDL-C was measured after precipitation of other lipoproteins with phosphotungstate-magnesium on the COBAS Mira analyser (Roche Diagnostics, Basel, Switzerland). Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald equation (LDL-C= TC – high-density lipoprotein cholesterol [HDL-C] – (TG/5)) (26). Serum apolipoprotein B (ApoB) concentrations were measured by immunophelompetry with a Behring Nephelemeter Analyzer II (Dade Behring, Marburg, Germany). Serum ApoE concentration was measured by electroimmunoassay (Hydragel LpE reagent set; Sebia, Issy-les-Moulineaux, France) with agarose gel foils containing an anti-apoE polyclonal antibody, performed according to the manufacturer's recommendations.

Genotyping

Genomic DNA was extracted from venous blood samples by the salting-out method (27). Genotyping of rs644234 was performed in a subsample of unrelated 348 individuals by using a multilocus assay with an immobilized probe approach designed by Roche Molecular Systems, Pleasanton, California, USA (28).

Statistical analyses

All phenotypes were log transformed and the normality of distribution was tested by Kolmogorov-Smirnov test. Hardy-Weinberg equilibrium was tested for the polymorphism by using the chi-square test. The SNP-phenotypes associations were assessed through a linear

regression under an additive genetic model and using the minor allele as the reference allele. The individuals analyzed were unrelated and association studies were performed using the Plink Software (29). The population's characteristics were determined using the SPSS statistical software version 20.0 (SPSS, Inc, Chicago, Illinois). We fitted multivariable models adjusting for age, sex and BMI. The Bonferroni correction was used, in order to adjust the type 1 error (a) by the total number of tests (a/n tests). The necessary P-value threshold was set at $P<0.0071$. Conditional analyses were also performed by adjusting all the phenotypes studied to the model, in order to test if the associations observed were independent from each other.

RESULTS

The phenotypic characteristics of the studied individuals are shown in Table 1. In total, we included 348 adult individuals from the SFS. Table 2 shows the demographic variables and other risk factors separated by sex and table 3 shows the genotype distribution of the SNP rs644234 in the population. The genotypes of the polymorphism were in agreement with Hardy-Weinberg equilibrium.

Genetic association of rs644234 with soluble E-selectin levels

Association studies were performed between the different genotypes of the SNP rs644234 and the soluble E-selectin levels. After performing conditional analysis by adjusting for the rest of the phenotypes studied to the model, results showed a strong association between the polymorphism and the soluble E-selectin levels (p-value= $8.7x10^{-12}$, β= 0.28). The major rs644234*T allele was associated with increased levels of soluble E-selectin, indeed, individuals being homozygotes for G allele have a mean of 35.93 ng/mL of sE-selectin while individuals homozygote for the major allele T have a concentration of 61.69 ng/mL. The levels in the case of the heterozygous are in between, with a mean value of 50.68 ng/mL. The genetic additive effect explained up to 12.7% of the total variance of its levels. More detailed results are shown in table 4 and figure 1.

Genetic association of rs644234 with lipid levels

Association analyses were then performed between the different lipid phenotypes and the SNP rs644234 genotypes in the 348 individuals. Although the SNP showed significant associations with ApoB when first linear regressions were made (p-value= 0.018, β= -0.04), this association was no more significant after performing conditional analysis (p-value=

0.068, β= -0.03). According to ApoE, without being adjusted for the E-selectin and the rest of lipid phenotypes, the p-value showed to be significant (p-value= 0.028, β= -0.05). When performing conditional analysis by adjusting these phenotypes to the model, we found an even more significant association (p-value= 0.001, β= -0.07), being this association independent. Thus, the major rs644234*T allele is decreasing the levels of ApoE. However, although the differences between the individuals being homozygous for G and for T are significant, we did not found a significant difference among the GT and GG genotypes.

The HDL-C was significant before and after performing conditional analysis (p-value= 0.012, β= 0.05 and p-value= 0.013, β= 0.05 respectively), where the major rs644234*T allele was associated with increased levels, although this association remained marginal once adjusted for Bonferroni (p-value for statistical significance set at P<0.0071). As well as with the sE-selectin levels, the concentration of HDL-C is increasing gradually in the individuals being heterozygotes (GT) and homozygotes (TT).

According to the LDL-C levels, the association with the major rs644234*T allele was the opposite of that found for HDL-C, showing decreased levels with this allele. Although this association was significant before and after performing the conditional analysis (p-value= 0.041, β= -0.04 and p-value= 0.032, β= -0.04 respectively), this association remained marginal once adjusted for Bonferroni.

The genetic additive effect of this SNP is explaining 1.2% of the variance in the case of the ApoE, 1.4% in the case of HDL-C levels and 0.8% in the case of LDL-C. More detailed results are shown in table 4 and figures 2 and 3. The rest of the lipid phenotypes studied, which included total cholesterol and triglycerides, were not significant. More detailed results are shown in table 4.

Associations of the SNP rs644234 with sex

The same phenotypes were analyzed by separating them by sex (n=173 males and 175 females). In males and females the relationship with sE-selectin was significant and showed not sexual dimorphism according to the direction of the effect, although a big part of the significance was lost, probably due to the smaller sample sizes (tables 5 and 6). According to the lipid levels, the significance was lost when the entire population was separated by gender, with the exception of the HDL-C levels, which was significant in males (Table5; p-value= 0.001 and β= 0.11) but not in females (Table 6; p-value= 0.25 β= 0.03).

DISCUSSION

The studies performed in the ABO histo blood groups show consistent associations with many different human diseases, such us different type of cancers (4, 5, 21), coronary heart disease (7), venous thromboembolism (23, 24), duodenal ulcers (22), and diabetes (30, 31). This demonstrates not only the importance of the study of the ABO gene, but also shows its pleiotropic effects, as it affects a wide different variety of diseases.

The aim of this work was to investigate intermediate phenotypes that are known to be related with some of those diseases, and more specifically with cardiovascular diseases. Consequently, we studied the relationships of the polymorphism rs644234 located in the ABO gene with soluble E-selectin levels and different lipid phenotypes. We also investigated the possible sex-specific effects of this polymorphism on the phenotypes studied. We managed to replicate previously found associations of the ABO polymorphisms with soluble E-selectin levels (10). Our results showed that the major rs644234*T allele is highly associated with increased levels of E-selectin (p-value= $8.7x10^{-12}$, β= 0.28). Among the lipid phenotypes studied, we found significant associations with ApoE levels, as the major rs644234*T allele is related with decreased levels of ApoE (p-value= 0.001, β= -0.07). In this case, only the individuals being homozygous for the major allele T have significantly decreased ApoE levels (table 4, figure 2). Total cholesterol was not associated with the polymorphism, while the HDL-C and LDL-C were significantly associated before adjusting for bonferroni. However, after adjustment this association remained marginal (table 4). We didn't find sexual dimorphism according to sE-selectin and lipid levels, with the exception of the HDL-levels, were the major rs644234*T allele was significantly increasing the levels of HDL-C only in male individuals. The other lipid phenotypes lost their significance, probably because of a reduction in the number of individuals when performing the analysis. Teng et al. (17) also found significant associations with HDL-C levels and ABO blood groups. However, they also described significant associations with triglyceride levels which we did not find. Recent genome-wide association studies have also found a significant association between polymorphisms in ABO with total cholesterol (15). Concerning our results, it is the first time that an association has been made between a polymorphism within the ABO gene and ApoE levels. Apolipoproteins are considered important phenotypes, as they are related with cardiovascular diseases. Other studies have found associations between the ABO blood groups and erythrocyte-bound apolipoprotein B levels which is related with atherosclerosis (20). However, we did not find significant association of serum ApoB levels with rs644234. It

is important to mention here that Klop et.al argued that the serum ApoB levels are not associated with erythrocyte-bound ApoB levels (20). Conditional analyses were performed to ensure the independency of the gene-phenotype associations founded. In order to do this, all the phenotypes used in the study were adjusted to the statistical model. It is not the first time that this type of antagonistic pleiotropy effects are shown in the ABO gene, indeed, Qi et al., (9) showed antagonistic and independent associations of ABO variants with sICAM1 and TNFα versus the soluble E-selectin levels, suggesting that the ABO variants affect these markers through different molecular mechanisms. These results show the complexity by which the ABO gene is acting in the different diseases where it is involved.

Unfortunately and as already said in the introduction, the blood groups of the individuals participating in the study were not available. Thus, we inferred the main ABO blood group that tags the SNP rs644234 (9), being mostly the O blood group (r^2=0.89) and in a less amount the A and B blood groups (r^2=0.58 and r^2=0.16 respectively). Taking this into account, we could suggest that our results are more likely representative in individuals with the O blood group. Indeed, other authors have already demonstrated that a SNP in strong L.D with rs644234 (r^2=0.9) is associated with myocardial infarction, but only in the subgroup of individuals having the O blood group (2). Same authors determined that when analyzing individuals with non-O blood groups, the association with myocardial infarction was no longer significant.

Our results showed that the major rs644234*T allele increases soluble E-selectin and HDL-levels, while decreasing the serum ApoE levels. Because of these pleiotropic effects, this SNP could act as a risk or protective factor in diseases where the associated phenotypes are involved. Indeed, increased HDL-C levels are widely considered to be protective against cardiovascular diseases (32), while increased soluble E-selectin levels are considered as risk factor for atherosclerosis (33). Concerning ApoE levels, the studies performed in ApoE knockout mice show that this apolipoprotein plays a protective role in atherosclerosis (34).

One study showed that the minor rs644234*G allele was associated with the risk of myocardial infarction (2). Also, the minor alleles of other SNPs in strong L.D with our SNP of interest have been associated with coronary heart disease (7), with large artery atherosclerotic stroke (35) and venous thromboembolism (23, 24). According to our results, the major rs644234*T allele affects different phenotypes, some considered to be protective (increased HDL levels) while others considered to be a risk factor (increased E-selectin levels). However, considering the previous studies and our results, this allele could be considered to have a protective effect when it comes to cardiovascular diseases. This type of

pleiotropy found in the ABO gene has been described in other alleles of major histocompatibility complex (36) and one of the possible explanations is that because of this antagonistic pleiotropy, the ABO gene is not under strong negative selection, as the different effects of the same gene will be favorable in different environmental situations.

These genetic associations of the ABO gene with different endophenotypes show how complex is the effect of this gene in different diseases, including cardio-vascular diseases, where it affects different pathways. They also show the need for further studies in order to better understand the mechanistic phenomena of these relationships and their potential therapeutic translation.

TABLES AND FIGURES

Table1. Means and confidence intervals of the used variables in the STANISLAS population. N = 348 (50.28% female).

Phenotype	Mean [95% CI]	SD
Age (years) [95% CI]	43.53 [42.97-44.08]	5.25
BMI (kg/m²) [95% CI]	24.78 [24.39-25.16]	3.62
ApoE (mg/dL)	4.20 [4.01-4.39]	1.84
ApoB (mg/dL)	101.08 [98.6-103.6]	23.77
E-selectine (ng/mL)	53.76 [50.95-56.57]	26.68
Total cholesterol (mmol/L)	5.73 [5.62-5.84]	1.02
LDL (mmol/L)	3.84 [3.74-3.94]	0.97
HDL (mmol/L)	1.61 [1.57-1.66]	0.47
TG (mmol/L)	1.34 [1.12-1.56]	2.09

Table 2. Demographic variables and other risk factors in the STANISLAS population. N=348. (50.28% females).

Population Characteristics	Percentages (%)		
	Males N=173	Females N=175	Total Population N=348
Smoker			
Yes	26.6	17.7	22.1
No	37	58.9	48
Ex-Smoker	35.8	22.3	29
Missing info	0.6	1.1	0.9
Alcohol consumption			
Low-risk consumption	80.3	92.6	86.5
Medium-risk consumption	12.7	4.6	8.6
High-risk consumtion	6.4	1.7	4
Missing info	0.6	1.1	0.9
Physical activity			
Low	52.6	80.6	66.7
Moderate	41	15.4	28.2
High	6.4	3.4	4.9
Missing info	-	0.6	0.3

Table3. Genotype distribution of the rs644234 in the studied population. The minor allele frequency of the SNP rs644234 was of 34%.

Genotype	N	%
GG	40	11.5
GT	157	45.1
TT	151	43.4

Table4. Association of the major rs644234*T allele (ABO) with the studied phenotypes. P-value threshold is set at **P<0.0071**

Phenotypes	β	Standard error	P value	Variance explained (%)
ApoE (mg/dL)	-0.07	0.02	**0.001**	1.2%
ApoB (mg/dL)	-0.03	0.01	0.068	-
E-selectine (ng/mL)	0.28	0.04	**$8.7x10^{-12}$**	12.7%
Total cholesterol (mmol/L)	-0.005	0.01	0.68	-
LDL (mmol/L)	-0.044	0.02	**0.032**	0.8%
HDL (mmol/L)	0.05	0.02	**0.013**	1.4%
TG (mmol/L)	0.024	0.03	0.44	-

Table 5. Association of the major rs644234*T allele (ABO) with the studied phenotypes in males. P-value threshold is set at **P<0.0071**

Phenotypes	**Males (N=173)**		
	β	Standard error	P value
ApoE (mg/dL)	-0.03	0.02	0.14
ApoB (mg/dL)	-0.04	0.02	0.07
E-selectine (ng/mL)	0.28	0.05	**4.9x**10^{-8}
Total cholesterol (mmol/L)	0.00	0.02	0.82
LDL (mmol/L)	-0.03	0.03	0.25
TG (mmol/L)	-0.06	0.05	0.21
HDL (mmol/L)	0.11	0.03	**0.001**

Table 6. Association of the major rs644234*T allele (ABO) with the studied phenotypes in females. P-value threshold is set at **P<0.0071**

Phenotypes	**Females (N=175)**		
	β	Standard error	P value
ApoE (mg/dL)	-0.05	0.03	0.12
ApoB (mg/dL)	-0.04	0.02	0.16
E-selectine (ng/mL)	0.28	0.06	**1.2x**10^{-5}
Total cholesterol (mmol/L)	0.00	0.01	0.73
LDL (mmol/L)	-0.03	0.02	0.23
TG (mmol/L)	0.00	0.05	0.88
HDL (mmol/L)	0.03	0.02	0.25

Figure1. Effects of the rs644234 genotypes on serum E-selectin levels.

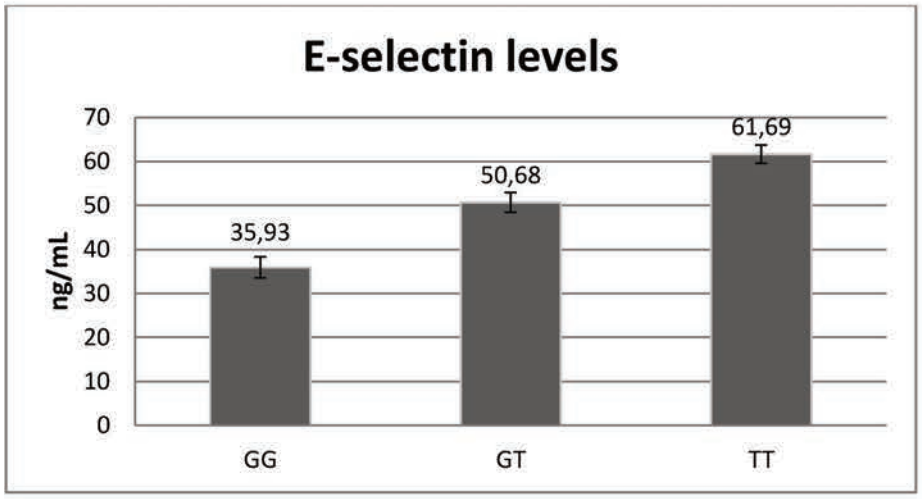


Figure2. Effects of the rs644234 genotypes on serum ApoE levels.

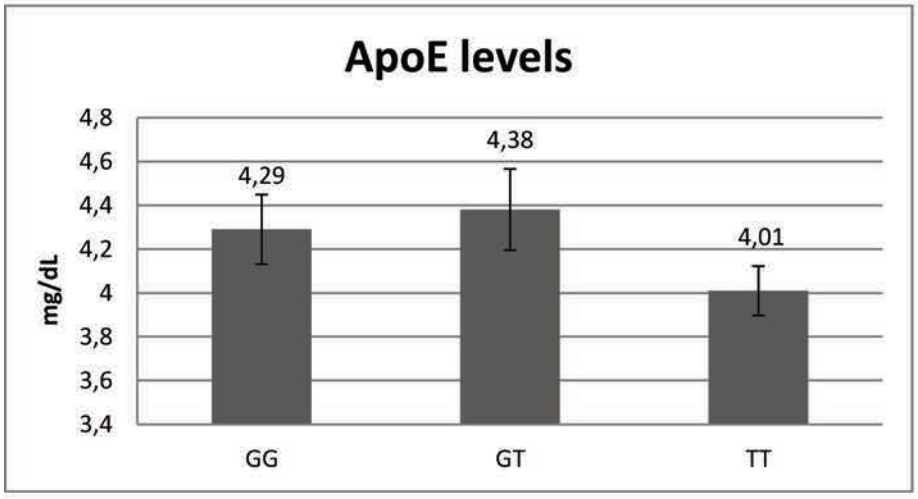
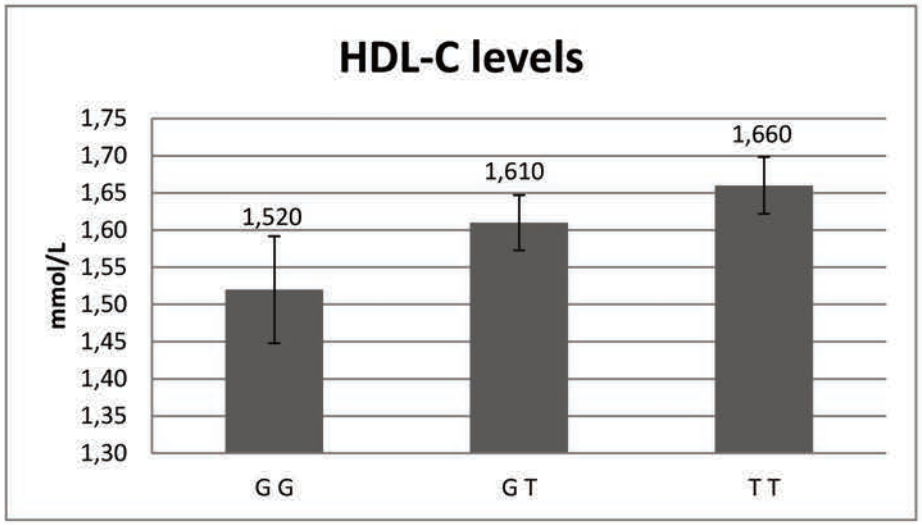


Figure3. Effects of the rs644234 genotypes on serum HDL-C levels.



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Publication N°4

The polymorphism rs6918289 located in the upstream region of the TREM-2 gene is associated with TNF-α levels and intima media thickness of the femoral artery

Alex-Ander Aldasoro Arguinano[1], Vesna Gorenjak[1], Maria Stathopoulou[1], Dwaine R.Vance[3], Christine Masson[1], Sophie VISVIKIS-SIEST[¥1, 2]

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The TREM-2 molecule is primarily expressed on the cell surface of the macrophages and dendritic cells derived from monocytes, as well as in microglia and osteoclast. TREM-2 has anti-inflammatory properties during the immune response (254, 255). It stimulates phagocytosis and suppresses cytokine production, including TNF-α levels (256, 257), which is one of the most important molecules for the regulation of inflammation and reflects the degree of inflammatory response.

These evidences are giving to TREM-2 a role as a protective molecule in chronic inflammatory diseases. Studies have demonstrated that lack of TREM-2 protein may reduce microglial activity and results in neuro-inflammation, which plays a major role in all neurodegenerative diseases (258). Furthermore, TREM-2 has been shown to play an important role in the stability of atherosclerotic plaques (259).

Polymorphisms located in the TREM-2 gene have been correlated with neurodegenerative and chronic inflammatory diseases, such as Alzheimer's disease (260, 261), Parkinson's disease (262, 263), inflammatory bowel disease (264) and stroke (265). However, so far, no genetic determinant has been identified in the TREM-2 locus affecting TNF-α levels.

Objectives

The goal of this study was to investigate polymorphisms located in the TREM2 gene region and their relation with TNF-α levels and the intima media thickness of the femoral artery in two independent populations of French origin.

Results

<u>Genetic association of the SNPs in TREM-2 region with TNF-α levels</u>

In the first step, association analyses were performed with 415 children of the SFS. SNPs that were previously genotyped and were located in the TREM-2 gene region were tested for association with TNF-α levels. Among the 5 SNPs studied, the minor allele of the SNP rs6918289 was significantly associated with increased levels of TNF-α (p-value: 0.0003). The SNP rs6918289 was then genotyped in 393 additional adult relatives from the SFS, and analyses were performed in the combined sample of SFS (N=808). The additive genetic model showed a positive association between the polymorphism rs6918289, located in the TREM-2 gene upstream region, and TNF-α levels (p-value= 0.0026). The recessive model showed an even stronger association with the TNF-α levels (p-value= 0.0017). Association studies were performed in an independent population of 915 individuals, showing a marginal association for the additive model (p-value= 0.073) and significant association for the recessive model (p-value= 0.023). The minor rs6918289*T allele was associated with increased levels of TNF-α in the discovery and replication populations.

Genetic association of rs6918289 with IMT-F

A sub-group of 350 individuals from the SFS population, where IMT-F measurements were available, was used for association studies with rs6918289. The additive genetic model showed significant association (p-value= 0.026). The association was also significant for the dominant model (p-value=0.026). The minor rs6918289*T allele was associated with increased thickness of the femoral artery.

Discussion

The anti-inflammatory effects of TREM-2 have been described in several studies. Indeed, knockdown or silencing of TREM-2 gene results in increased levels of different pro-inflammatory molecules, among them, TNF-α (254, 255, 266). Despite the fact that several polymorphisms within the TREM-2 gene have been related with numerous neurodegenerative and inflammatory diseases, (260, 262, 264, 265) so far, no genetic determinant has been identified in the TREM-2 locus affecting TNF-α levels.

After performing association studies for the SNPs available we found that the minor rs6918289*T allele, located in the upstream region of the TREM-2 gene was associated with increased levels of TNF-α. The minor rs6918289*T allele of the same SNP was also associated with increased intima-media thickness of the femoral artery. Therefore, the minor T allele could be considered as a risk allele for inflammatory diseases and atherosclerosis. The role of both studied phenotypes in the development of atherosclerosis and the prognosis of

atherosclerotic patients is well documented. Changes of TNF-α levels lead to higher inflammation and a subsequent deterioration of the outcome of patients with CVD (267, 268). Also, one of the early processes that lead to atherosclerosis is the arterial remodeling and one effective way that provides information about this process is measuring the IMT. Indeed, IMT is predictive of atherosclerosis in asymptomatic individuals (269, 270) and also provides information about the degree of atherosclerosis (271, 272) as well as of the risk of suffering myocardial infarction (273).

Despite the fact that this polymorphism gives insights about its possible roles in the development of CVD, the exact mechanisms, by which it is orchestrating this process is so far unknown and further studies are needed. Indeed, there exist no previous study that associates this SNP (or SNPs in LD) with TNF-α levels or IMT-F. However, we have reasons to believe that this association is real. Given the IMT-F results, we think that the effect of this SNP could be indirect. Indeed, the remodeling of the artery is highly dependent on the inflammatory state, thus, increased TNF-α levels produced by the minor rs6918289*T allele could be responsible for the increased thickness of the femoral artery.

The polymorphism rs6918289 located in the downstream region of the TREM2 gene is associated with TNF-α levels and IMT-F

Alex-Ander Aldasoro Arguinano[1], Vesna Gorenjak[1], Sébastien Dadé[1], Maria Stathopoulou[1], Dwaine R.Vance[3], Christine Masson[1], Sophie Visvikis-Siest[¥1, 2]

[1]INSERM UMR U1122; IGE-PCV 'Interactions Gène-Environnement en Physiopathologie Cardio-vasculaire', Faculté de Pharmacie – Université de Lorraine, Nancy, F-54000, France

[2] Department of Internal Medicine and Geriatrics, CHU Technopôle Nancy-Brabois, Rue du Morvan, F-54511, Vandoeuvre-lès-Nancy, France

[3] Randox Laboratories Limited, Crumlin, Co. Antrim, Northern Ireland, United Kingdom

[¥]Corresponding author:

Dr Sophie VISVIKIS-SIEST

INSERM UMR U1122; IGE-PCV, Faculté de Pharmacie – Université de Lorraine

30 rue Lionnois

54000 Nancy, France

Tel: +33.6.07.60.25.69

E-mail address: sophie.visvikis-siest@inserm.fr

ABSTRACT

Triggering receptor expressed on myeloid cells 2 (TREM2) is known for its anti-inflammatory properties during the immune response, and influences negatively on TNF-α expression levels. Genetic epidemiology studies have identified polymorphisms located in the TREM2 gene associated with neurodegenerative and chronic inflammatory diseases. TREM2 levels have been observed to affect plasma levels of TNF-α and plaque stability in symptomatic and asymptomatic patients with carotid stenosis. In this study, we investigated polymorphisms located in the TREM2 gene region and association with TNF-α levels and the intima media thickness of the femoral artery. The discovery population, (STANISLAS Family Study) comprised of 808 individuals, whereas the replication population utilized an independent cohort of French origin (n=915). Our results suggest that the minor allele (T, rs6918289) is positively associated with elevated plasma levels of TNF-α in both populations (P=0.0026, SE=0.04 and P=0.023, SE=0.09, respectively), including femoral artery thickness in the discovery cohort (P=0.026, SE=0.009). Results indicate that rs6918289 may be considered as a risk factor for inflammatory diseases and could be used in stratified medicine with patients diagnosed with chronic inflammatory-related conditions, such as atherosclerosis.

INTRODUCTION

The triggering receptors expressed on myeloid cells (TREM) family molecules are members of the immunoglobulin superfamily of receptors. All five genes from the TREM family (Table 1) are situated in the 6p21.1 region of the chromosome [1] and mediate signaling in immune cells, thus playing critical roles in inflammatory responses [2]. The region 6p21.1 is in proximity to the MHC / HLA region of the genome, which is implicated in immune response, autoimmunity and risk of autoimmune diseases [3,4]. The TREM2 molecule is primarily expressed on the cell surface of macrophages and dendritic cells derived from monocytes, as well as in microglia and osteoclast. It binds to the DAP12 trans-membrane molecule and causes a series of tyrosine phosphorylation reactions that regulate various inflammatory responses [2,5]. TREM2 portrays anti-inflammatory properties during the immune response [6,7], including: stimulation of phagocytosis and suppression of cytokine production, e.g. TNF-α [8,9], which is one of the most important molecules for the regulation of inflammation and reflects the degree of inflammatory response.

It is evident that TREM2 acts as a protective molecule in chronic inflammatory diseases. For example, studies in transgenic mice have demonstrated that deficiency of TREM2 protein may accelerate the aging process, reduce microglial activity and result in neuroinflammation, which plays a major role in all neurodegenerative diseases [10]. Furthermore, atherosclerosis is a cardiovascular chronic inflammatory disease, caused by activation of immune system mediating the chronic inflammatory process of the arterial wall. TREM2 has also been shown to play an important role in the stability of atherosclerotic plaques [11].

Polymorphisms located in the TREM2 gene have been correlated with neurodegenerative and chronic inflammatory diseases, such as Alzheimer's disease [12,13], frontotemporal dementia [14,15], Parkinson's disease [16,17], inflammatory bowel disease [18] and stroke [19]. However,, no genetic determinants have been identified in the TREM2 locus affecting plasma levels of TNF-α and/or intima media thickness.

Due to the role of TREM2 in the inflammatory response and stability of atherosclerotic plaques, we hypothesize that polymorphisms in the TREM2 gene region may influence the inflammatory process and subsequent atherosclerotic plaque formation. In this investigation, we have studied the association of variants located in the TREM2 gene region, with plasma levels of TNF-α and intima media thickness of the femoral artery (IMT-F).

RESULTS

The information on the genotyped polymorphisms as well as the demographic and clinical characteristics of the studied populations are shown in Table 2 and 3 respectively. The SNPs analyzed were in agreement with Hardy-Weinberg equilibrium (P>0.001).

Genetic association of SNPs in TREM2 gene region with TNF-α levels

Firstly, an association analysis was performed in 415 children of the SFS. SNPs that were previously genotyped and were located in the TREM2 gene region were tested for association with TNF-α concentration. Among the five SNPs studied, the minor allele of rs6918289 was significantly associated with increased levels of TNF-α (P=0.0003, Table 4).

Secondly, rs6918289 was genotyped in 393 adult relatives from the SFS and analysis was performed in the combined population of SFS (n=808) using three genetic models. The additive genetic model showed a positive association between polymorphism rs6918289, located in the TREM2 gene downstream region and TNF-α plasma levels (P= 0.0026, β= 0.13, Table 5). The recessive model showed stronger genetic association with TNF-α plasma levels (P= 0.0017, β= 0.498, Table 5).

Further association analysis was performed in an independent population of 915 individuals, showing nominal association for the additive model (P=0.073, β=0.042, Table 5). A significant association was observed for the recessive model (P=0.023, β=0.202, Table 5). The minor allele (T, rs6918289) was associated with elevated levels of TNF-α in the discovery and replication populations (Figures 1 and 2 respectively).

Genetic association of rs6918289 with IMT-F

A sub-group of 350 individuals, including adults and children from the SFS population, where IMT-F measurements were available, was used to identify association with rs6918289. The additive genetic model showed significant association (P=0.026, β=0.02, Table 6). The association was also significant for the dominant model (P=0.026, β=0.024). Thus, the minor allele (T, rs6918289) was associated with increased thickness of the femoral artery (Figure 3).

Bioinformatics analyses

<u>Location</u>

The SNP rs6918289 is located on chromosome 6 in p21.1 region. Located at 41 134 089 bp, it is an intron variant of adenylate cyclase 10 pseudogene 1 (ADC10P1). The polymorphism on the forward strand is G>T with a minor allele frequency of 0.07 for thymine in 1000 Genome project (Sup. Figure 2) [20,21].

Phylogenetic context

The guanine polymorphism of rs6918289G>T is evolutionary well-conserved in primates and mammals in general (Sup. Table 1). Further phylogenetic studies performed in 33 mammals and 46 vertebrates (Sup. Figure 1) showed that this polymorphism has a slower evolution rate than the expected one, having a PhyloP score of 0.061 and 0.056 respectively. This strengthens the idea that this variant is involved in important molecular mechanisms and that its preservation has been sustained throughout the natural selection processes.

Genomic context

This rs6918289 variant overlaps 2 transcripts and is located between two terminal exons of ADCY10P1: ADCY10P1-202 (3785 bp - between exon 17 and 18) and ADCY10P1-203 (4569 bp - between exon 18 and 19), both leading to transcripts that are not translated into proteins (Sup. Figure 2). Furthermore, according to the expressed sequence tags database (dbEST) available in Ensembl, the polymorphism rs6918289 is located within an intense transcriptionally active locus (Sup. Figure 3) [20,21].

DISCUSSION

The anti-inflammatory effects of TREM2 have been described in several studies. Indeed, knockdown or silencing of TREM2 gene results in increased levels of different pro-inflammatory molecules, among them TNF-α [6,7,22]. Despite the fact that several polymorphisms within the TREM2 gene have been related with numerous neurodegenerative and inflammatory diseases, [12,16,18,19] to date, no genetic determinants have been identified in the TREM2 locus (6p21) affecting TNF-α levels.

In this study, we evaluated the effects of SNPs located in the TREM2 gene region on TNF-α levels and IMT-F measurements. In 415 children from the discovery population, genotypes for five SNPs (rs7748777, rs6918289, rs7759295, rs9357347 and rs6915083) were readily available for the TREM2 gene region. After conducting association analysis for the five aforementioned SNPs with TNF-α levels, we observed the minor rs6918289*T allele, located

in the downstream region of the TREM2 gene to be associated with elevated levels of TNF-α (P=0.0003). Further analysis in adults of the same population, as well as replication in an independent population confirmed this novel association. Subsequently, we also performed association analysis of rs6918289 with intima-media thickness of the femoral artery in 350 individuals of the discovery population. Results suggest that the minor allele (T, rs6918289) is associated with increasing intima-media thickness of the femoral artery. Therefore, this is evidence that the minor T allele may be considered as a risk allele for inflammatory diseases and atherosclerosis.

The role of both studied phenotypes in the development of atherosclerosis and the prognosis of atherosclerotic patients is well documented. TNF-α is a key regulator of immune response and changes of its levels lead to higher inflammation and a subsequent deterioration of the outcome of patients with cardiovascular diseases [23,24]. Also, one of the early processes that lead to atherosclerosis is the arterial remodeling and one effective way that provides information about this process is measuring the intima-media thickness. Indeed, IMT is predictive of atherosclerosis in asymptomatic individuals [25,26], and also provides information about the degree of atherosclerosis [27,28] as well as of the risk of suffering myocardial infarction [29].

The 6p21.1 genetic region, which is in proximity to the MHC and HLA regions, has been highly studied, and associated with autoimmune diseases [3,4]. Interestingly, previous GWAS studies have associated polymorphisms located within this genetic region with atherosclerotic stroke [30]. Although the rs6918289 is not in linkage disequilibrium with the variants associated with atherosclerotic stroke (rs556621 and rs556512), our results strengthen the idea that the 6p21.1 region could, indeed, be correlated with atherosclerosis risk. Our results are providing new insights on possible genetic regulations of pathological pathways that could lead to increased risk of atherosclerosis.

Despite the fact that this polymorphisms contributes novel insights about its potential role in the development of cardiovascular diseases, the exact mechanisms by which this process is orchestrated is so far unknown and further studies are needed. Indeed, there are no previous studies that associate this SNP or SNPs in linkage disequilibrium with TNF-α levels or IMT-F. However, we have reasons to believe that this association is real. In fact, previous studies have showed that TREM2 is affecting the levels of TNF-α [7-9,22]. Also, our bioinformatics analyses indicate that rs6918289 is located in a transcriptional region of ADC10P1 gene that could affect TREM2 transcription levels [31]. Indeed, rs6918289 is referred by rSNPBASE as a post-transcriptional regulatory element and, in lymphocyte B cells, rs6918289 is associated

with the PABPC1 protein. The PABPC1 protein, binds to the 3' poly(A) region of the mRNAs. Although the binding of this protein is necessary for the translation initiation, it is also required for poly(A) shortening, which is the first step in mRNA decay [32]. Thus, the minor rs6918289*T allele could affect the PABPC1 protein, which would at the same time affect the stability of the TREM2 mRNA, consequently contributing to raised TNF-α levels.

An alternative or synergistic mechanism is also conceivable. As shown by several studies, many RNAs bind to CTCF to modulate its regulatory functions [33]. The rs6918289 is located between two CTCF genomic sequences that are "together" (Sup. Figures 2 and 3), meaning that they are forming a chromatin loop leading to a TAD (topologically associating domain). Thus, we could hypothesize that CTCF, in association with the non-coding RNAs of ADCY10P1, could affect TREM2 expression levels. The minor rs6918289*T allele could promote the binding of CTCF and consequently trigger an insulation mechanism for TREM2 gene.

Concerning the IMT-F results, we think that the effect of this SNP could be indirect. Indeed, the remodeling of the artery is highly dependent on the inflammatory state, thus, increased TNF-α levels produced by the minor rs6918289*T allele could be responsible for the increased thickness of the femoral artery. Further studies will be necessary in order to know if this polymorphism or polymorphisms in linkage disequilibrium are capable of modulating the TREM2 protein levels and which are the mechanisms behind this observation. However, important applications could be found as these findings could be used for personalised treatments in patients with chronic inflammatory diseases [34].

In summary, our study indicates variant rs6918289 located in the downstream region of the TREM2 gene as candidate risk factor for inflammatory diseases because of its tight association with plasma levels of TNF-α. These findings are supporting the results of previous studies relating variants in 6p21.1 loci to atherosclerosis and thus raising the awareness to consider the 6p21.1 as candidate susceptibility loci for atherosclerosis.

METHODS

Ethics statement

The samples are part of a human sample storage platform: BRC IGE-PCV number BB-0033-00051 in Nancy, East of France. All participants gave a written informed consent. All the populations involved in this study were recruited in accordance with the latest version of the

Declaration of Helsinki for Ethical Principles for Medical Research Involving Human Subjects. All the protocols were approved by the local ethics committees for the protection of subjects for biomedical research: the Comité Consultatif de Protection des Persones dans la Recherche Biomédicale (CCPPRB).

Study participants

The discovery and replication populations are part of the Biological Resources Center 'Interactions Gène-Environnement en Physiopathologie CardioVasculaire' (BRC-IGE-PCV, number BB-0033-00051) in Nancy, France.

Individuals, comprising children and adults from the STANISLAS Family Study (SFS) [35]were used as the discovery population. The SFS include more than 1,000 nuclear families, each composed of at least 4 individuals (two parents and two children). All families are of French origin and were recruited at the Centre for Preventive Medicine of Vandoeuvre-lés-Nancy (East France). All individuals were free of chronic diseases. Firstly, we used samples from 415 children of the SFS, where genotype information was readily available for the TREM2 gene region, to conduct association studies with plasma levels of TNF-α. Subsequently, 393 adult samples from the corresponding families were included, reaching a total of 808 participants.

The replication cohort included 915 unrelated adults of French origin. Their inclusion criteria were the same as in the discovery cohort.

Blood samples and biological measurements

Blood samples were taken from individuals after an overnight fast (>8 hours). The plasma concentrations of TNF-α were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, UK) according to manufacturer instructions. Body mass index (BMI) was calculated as weight divided by height squared (Kg/m^2). IMT-F was measured in 350 SFS individuals (including children and adults) using B-mode ultrasound methods [36]. The right and left femoral arteries were examined with a 7.5 MHz probe, according to a protocol already described [37,38]. For each individual, two IMT-F measurements were obtained and right and left measurements were used to calculate the mean IMT-F (in mm). IMT-F data were not available in the replication population.

Genotyping

Genome-wide genotypes were readily available for all children samples from the SFS (n=415). Genotyping was performed using Illumina® human CNV370-Duo array [39]. The Illumina® protocol for the BeadStation genotyping platform was used, followed by GenCall® software analysis to automatically collect, call genotypes, and designate confidence scores using the GenTrain clustering algorithm. The first step was to extract the SNPs available that were located in the TREM2 gene locus. We employed PLINK software [40] to conduct analysis and we chose the TREM2 gene region to check the SNP availability in that locus, totaling five SNPs.

After performing initial association analysis in the child cohort of the SFS, polymorphism rs6918289 was de novo genotyped in the adult population of SFS (n=393) and the replication population (n=915). Genotyping of rs6918289 in the replication population was conducted by Laboratory of the Government Chemist (LGC), using a PCR-based KASP assay [41].

Statistical analysis

Normal distribution was tested by Kolmogorov-Smirnov test. If phenotypes did not conform to normal distribution, data were log transformed in order to reach normality. The Hardy-Weinberg Equilibrium (HWE) was tested using the chi-square test ($P>0.001$). The SNP effects on the studied phenotypes were tested through linear regression adjusted for age, gender and BMI under three inheritance models (additive, dominant and recessive) and using the minor allele as the reference allele. Analyses were performed using the R package GWAF (Genome-Wide Association/Interaction Analysis and Rare Variant analysis with Family Data) [42] taking into account familial resemblance. Alternatively, population characteristics were determined using SPSS statistical software version 20.0 (SPSS, Inc., Chicago, Illinois). Firstly, we tested the association of five SNPs located in the TREM2 gene region that were extracted from the GWAS available in children of the SFS (n=415) with plasma TNF-α. Secondly, we tested the significance of rs6918289 with TNF-α plasma levels in all available individuals of SFS (children and adults combined; n=808) and in the replication population (n=915). Thirdly, in 350 individuals of the SFS, association was tested for association with IMT-F. Bonferroni correction was applied in order to adjust for multiple testing. The P-value threshold was set at $P<0.01$ for the first analysis made in children, $P<0.05$ for the analysis made in all individuals of the discovery population (children and adults) and $P<0.05$ in the replication population.

Bioinformatics analysis

Location, genomic and phylogenetic context of rs6918289 were determined on the Human genome (GRCh38.p10) using Ensembl browser [20]. The putative regulatory role of rs6918289 was established using rSNPBASE [21], and the PhyloP score was obtained using PhyloP software [43].

COMPETING INTERESTS STATEMENT

The authors declare that they have no significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

AUTHOR CONTRIBUTIONS

Conceptualization: A.A.A and S.V.S; Formal analysis: A.A.A and S.D; Investigation: A.A.A, V.G, S.D, C.M; Writing – original draft: A.A.A, V.G, S.D; Writing – review & editing: A.A.A, V.G, S.D, S.V.S, M.S, D.R.V.

COMPETING FINANCIAL INTERESTS STATEMENT

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ADDITIONAL INFORMATION

Extensive data is provided with this article and further information is available from the authors on request.

TABLES

Table 1: TREM family of genes

Gene	Gene name	Chromosome
TREML1	triggering receptor expressed on myeloid cells like 1	6p21.1
TREML2	triggering receptor expressed on myeloid cells like 2	6p21.1
TREML4	triggering receptor expressed on myeloid cells like 4	6p21.1
TREM1	triggering receptor expressed on myeloid cells 1	6p21.1
TREM2	triggering receptor expressed on myeloid cells 2	6p21.1

Table 2: Characteristics of the genotyped polymorphisms in TREM 2 region. With the exception of the SNP rs6918289, the rest were only genotyped in children.

	Children SFS				
SNP	**Gene**	**Minor allele**	**MAF**	**Chromosome**	**HWE P**
rs7748777	LOC105375056	A	0.43	6p21	0.88
rs6918289	ADCY10P1	T	0.14	6p21	0.97
rs7759295	LOC105375056	T	0.11	6p21	0.25
rs9357347	LOC107986595	C	0.29	6p21	0.12
rs6915083	TREML2	G	0.37	6p21	0.33
	Adults SFS				
rs6918289	ADCY10P1	T	0.13	6p21	0.73
	Replication population (Adults)				
rs6918289	ADCY10P1	T	0.12	6p21	0.89

Table 3: Demographic and clinical characteristics of studied populations.

	SFS Children	SFS Adults	SFS Total	Replication Population
Sample size [% female]	415 [49%]	393 [49%]	808 [49%]	915 [51%]
Age (years) [S.D]	17.14 [3.6]	44.18 [4.4]	30.28 [14.1]	55.5 [11.2]
BMI (kg/m^2) [S.D]	20.94 [3.01]	24.97 [3.85]	22.9 [3.99]	26.58 [3.47]
TNF-α (pg/ml) [S.D]	3.51 [2.38]	2.98 [1.77]	3.26 [2.1]	2.65 [0.9]
IMT-F (mm) [S.D]	0.46 [0.036]	0.53 [0.06]	0.495 [0.056]	-

Table 4: Genetic association of the SNPs in TREM2 region with TNF-α levels in children of the SFS (n=415). P-value threshold is **P<0.01.** Significant p-values are highlighted in bold.

Polymorphism	Model	P-value	Beta	S.E
rs7748777	Additive	0.05	0.11	0.05
rs6918289	Additive	**0.0003**	0.28	0.07
rs7759295	Additive	0.92	0.01	0.09
rs9357347	Additive	0.04	0.13	0.06
rs6915083	Additive	0.10	0.09	0.06

Table 5: Association analysis of the polymorphism rs6918289 with TNF-α levels in the discovery and replication populations. P-value threshold is **P<0.05.** Significant p-values are highlighted in bold.

Population	Genetic model	N	Beta	SE	P-value
STANISLAS Family Study	**Additive**	808	0.13	0.04	**0.0026**
	Dominant	808	0.11	0.05	**0.022**
	Recessive	808	0.49	0.15	**0.0017**
Replication population	**Additive**	915	0.042	0.02	0.073
	Dominant	915	0.03	0.03	0.189
	Recessive	915	0.20	0.09	**0.023**

Table 6. Association analysis of the polymorphism rs6918289 with IMT-F in the discovery population (not available in the replication population). P-value threshold is **P<0.05.** Significant p-values are highlighted in bold.

Population	Genetic model	N	Beta	SE	P-value
STANISLAS Family Study	**Additive**	350	0.02	0.009	**0.026**
	Dominant	350	0.024	0.01	**0.026**
	Recessive	350	0.024	0.025	0.34

FIGURES

Figure 1: Mean values of TNF-α levels according to the different genotypes of rs6918289 (GG vs TG vs TT) in the STANISLAS population. Thin bars show standard errors.

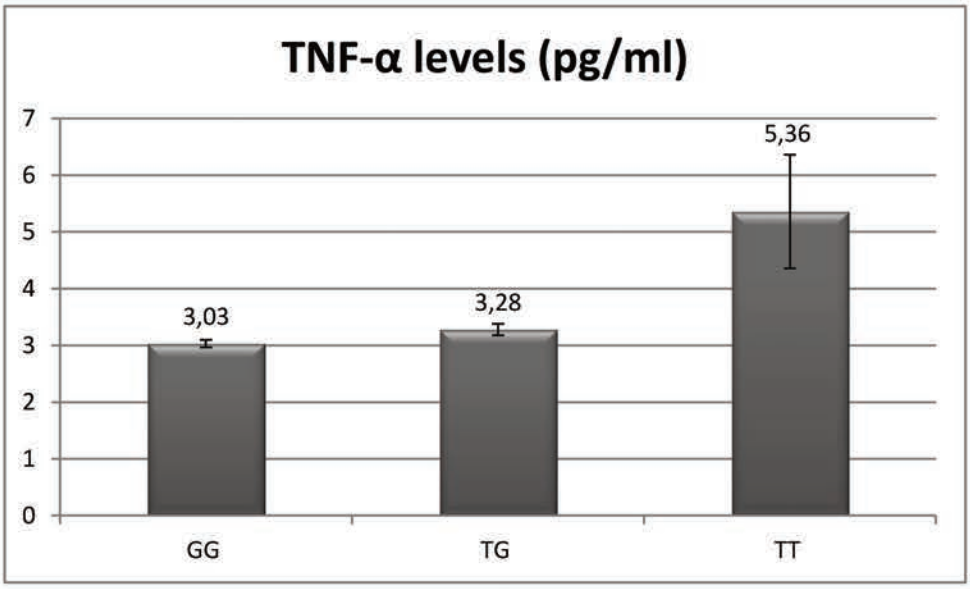


Figure 2: Mean values of TNF-α levels according to the different genotypes of rs6918289 (GG vs TG vs TT) in the replication population. Thin bars show standard errors.

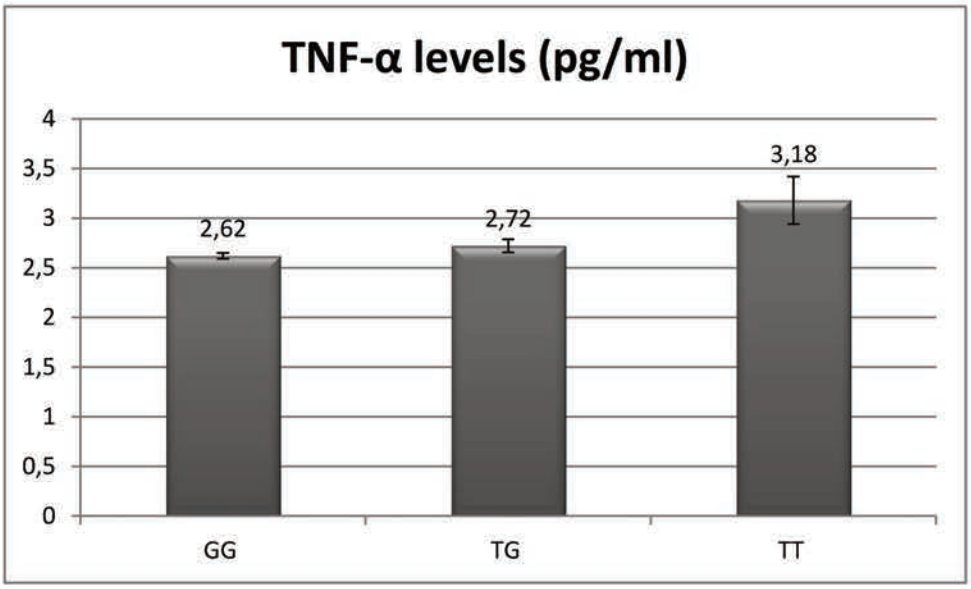
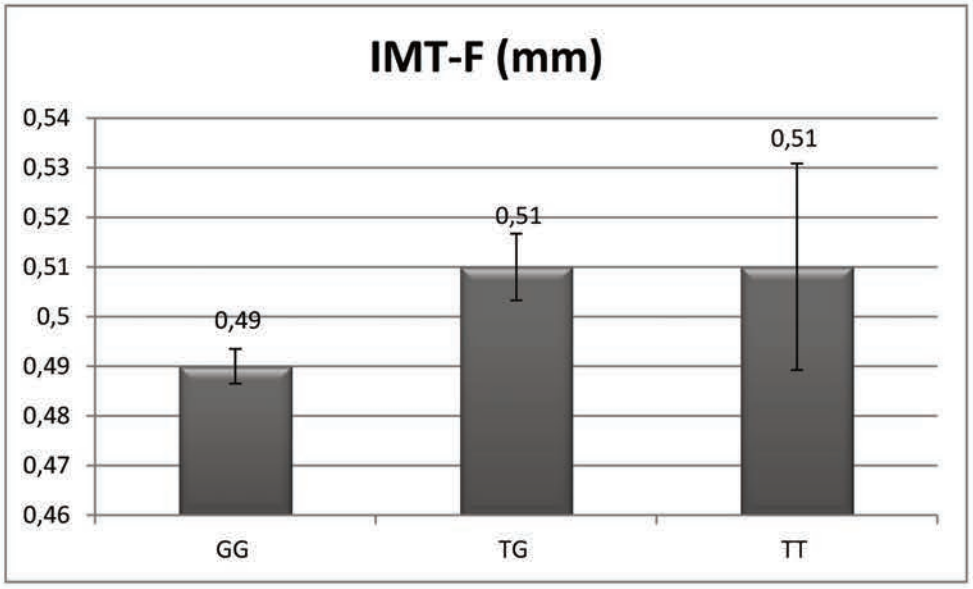


Figure 3: Mean values of intima media thickness of IMT-F according to the different genotypes of rs6918289 (GG vs TG vs TT) in the STANISLAS population. Thin bars show standard errors.



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SUPPLEMENTARY DATA

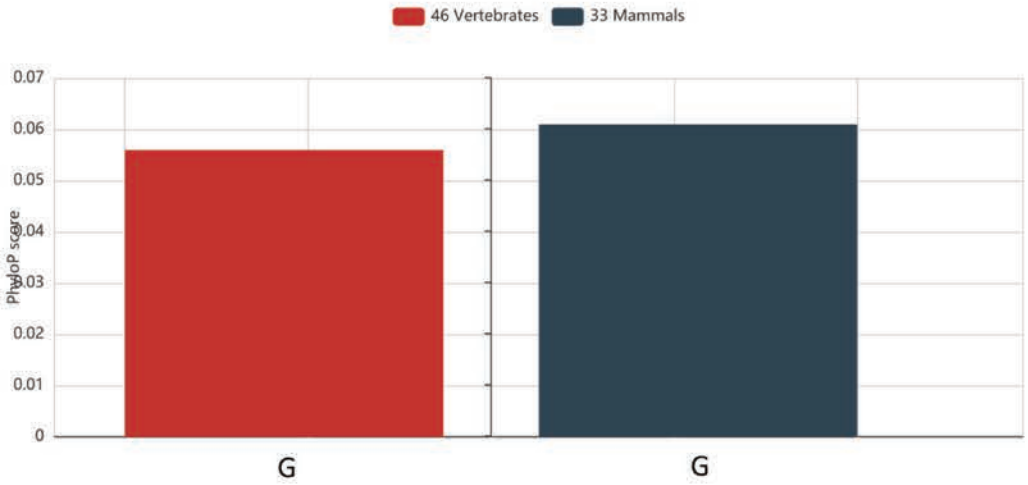
Sup. Table 1: Guanine conservation of rs6918289 through primates and one eutherian mammal

Specie	Sequence	Location
Human ›	TCACTTTCTTGAGGGATTCAG	chromosome:GRCh38:6:41134079:41134099:1
Chimpanzee ›	TCACTTTCTTGAGGGATTCAG	chromosome:CHIMP2.1.4:6:41696034:41696054:1
Gorilla ›	TCACTTTCTTGAGGGATTCAG	chromosome:gorGor3.1:6:42328798:42328818:1
Orangutan ›	TCACTTTCTTGAGGGATTCAG	chromosome:PPYG2:6:41219530:41219550:1
Vervet-AGM ›	TCACTTTCTTGAGGGATTCAG	chromosome:ChlSab1.1:17:31023954:31023974:-1
Macaque ›	TCACTTTCTTGAGGGATTCAG	chromosome:Mmul_8.0.1:4:42085379:42085399:1
Olive baboon ›	TCACTTTCTTGAGGGATTCAG	chromosome:PapAnu2.0:4:40443409:40443429:1
Marmoset ›	TTACTTTCTTGAGGGATTCAG	chromosome:C_jacchus3.2.1:4:42316089:42316109:1
Rabbit ›	TCACTTTCTTGGTGGGTCTGT	chromosome:OryCun2.0:12:30888427:30888447:1

Variants Focus variant

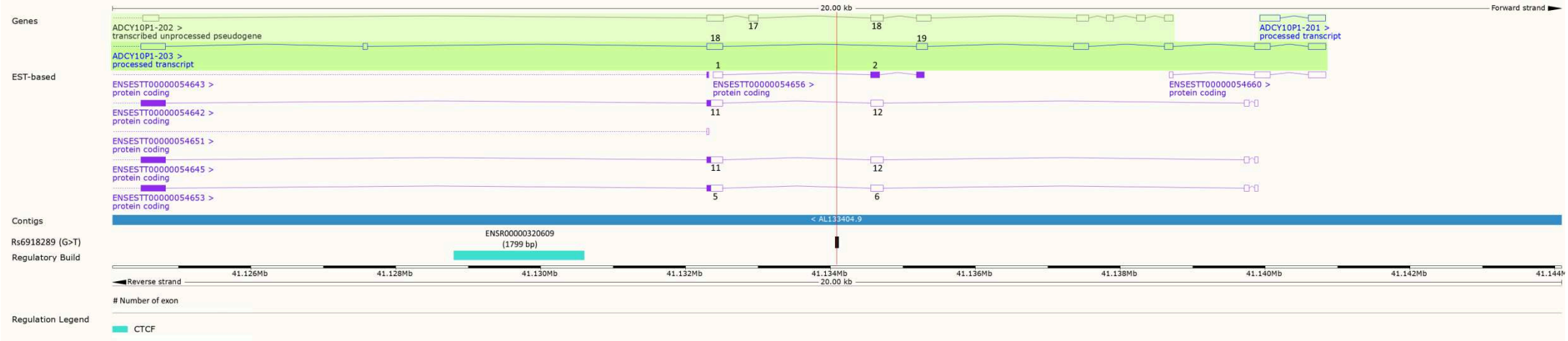
Other Differs from primary species

Sup. Figure 1: PhyloP scores of 46 vertebrates and 33 mammals for the guanine of rs6918289



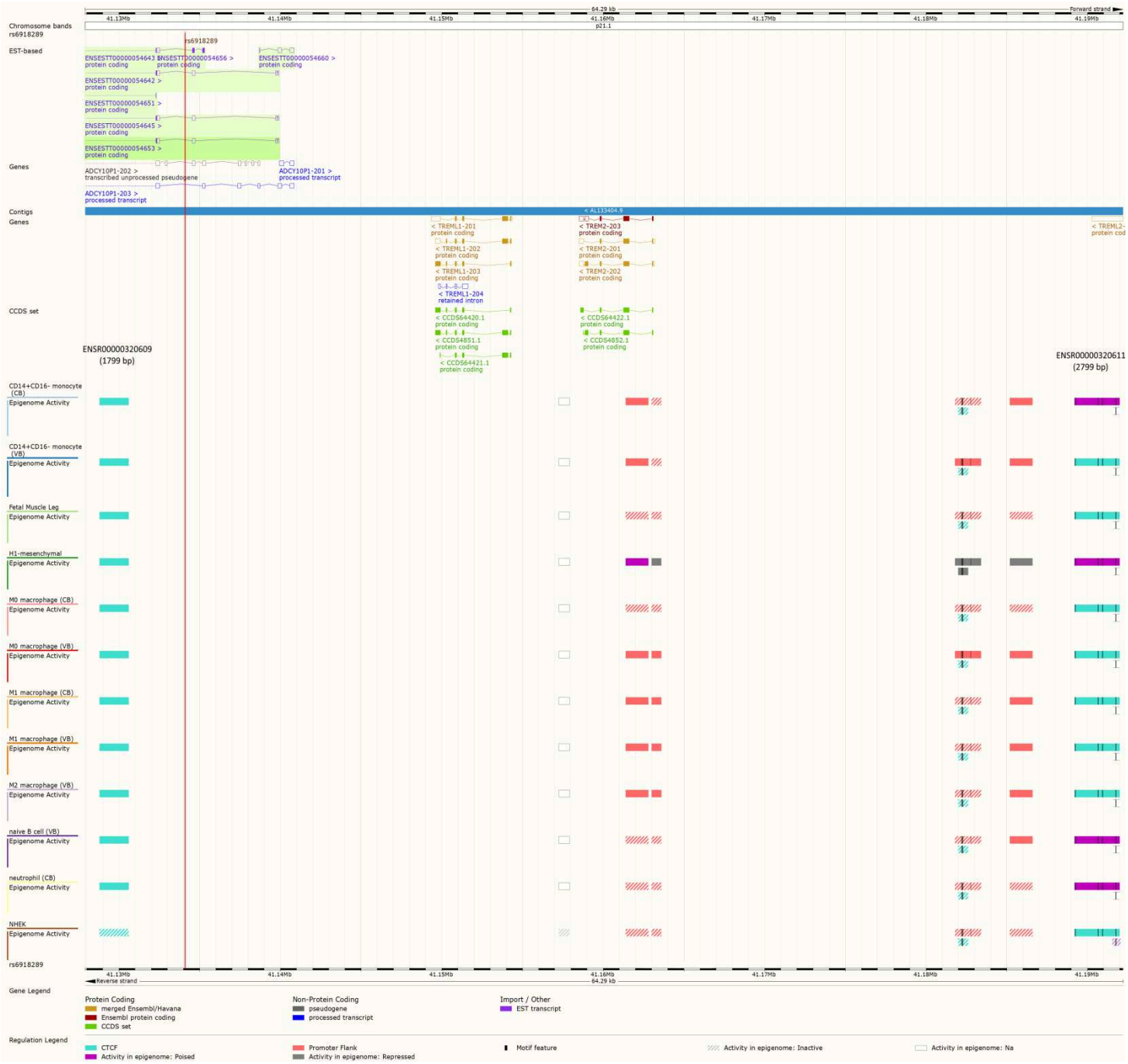
Sup. Figure 2: Location and genomic context of rs6918289 on chromosome 6 in p21.1 region (41 134 089 bp)

Furthermore, according to the expressed sequence tags database (dbEST) available in Ensembl, the polymorphism rs6918289 is located within an intense transcriptionally active locus. Apart of the gene ADY10P1, it also belongs to ENSESTG00000024983 gene. This gene has 7 transcripts and among them, ENSESTT00000054656 which is translated into a 77 amino acids protein, contains rs6918289 between exon 1 and 2, the ENSESTT00000054642, which is translated into a 262 amino acids protein, contains rs6918289 between exon 11 and 12, the ENSESTT00000054645 which is translated into a 262 amino acids protein, contains rs6918289 between exon 11 and 12, and the ENSESTT00000054653 translated into a 262 amino acids protein, contains rs6918289 between exon 5 and 6. A CTCF binding site, the ENSR00000320609, is located at 3489 bp in the 5' direction from rs6918289. Another CTCF binding site, the ENSR00000320611 is also present, located at 26015 bp from TREM2 gene, in the 5' direction.



Sup. Figure 3: Regulatory environment of rs6918289 and TREM2 gene.

These two CTCF binding sites are together active (In turquoise) or poised (in purple) in only 6 cell types over the 23 studied: monocyte (CB and VB), neutrophil (CB), fetal muscle leg, H1-mesenchymal, macrophage M0, M1 (CB and VB) and M2 (VB), and naïve B cell (VB).



Publication N°5

Cardio-vascular disease related GNB3 C825T polymorphism has significant sex-specific effect on serum soluble E-selectin levels

Kokoè Mélinda GBADOE, Nazha BERDOUZI, Alex-Ander ALDASORO AGUIÑANO, Ndeye Coumba NDIAYE[§], Sophie VISVIKIS-SIEST[§]

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The polymorphism rs5443, located within the GNB3 gene has been involved in cardiovascular pathophysiological processes and CVD. Indeed, it has been associated with obesity (274), higher blood pressure (275), hypertension (276-278), carotid atherosclerosis (279), incident cerebrovascular events (280) and coronary diseases (281), even though these effects have also been controverted in the literature (282, 283).

Some of these studies showed a sex-specific effect of this SNP Brand et al. observed a higher blood pressure in T homozygotes males than in C allele male carriers, while no significant difference was observed in females (275). Hengstenberg et al. also observed a significantly higher prevalence of arterial hypertension in TT genotype subjects as compared to the other genotypes, but this association was predominantly found in men (276). Frey et al. observed that the TT genotype was a significant risk factor for fatal and non-fatal myocardial infarction, independently of other established cardiovascular risk factors at a population level, but only in males, while in females no significant association was observed (281). All these elements encourage further investigations of a sex-specific effect of this polymorphism.

Objective

The aim of our study was then to investigate a possible sex-specific effect of the GNB3 rs5443 polymorphism on serum soluble cell adhesion molecules such as sE, sP and sL-selectins.

Results

No significant association of rs5443 was observed in the whole population with serum sE, sP and sL-selectins after adjusting for age, sex, body mass index, systolic blood pressure, anti-inflammatory drug and hormonal contraceptives consumption (respective p-values=0.876,

0.158 and 0.770). However, when taking into account the gender of the participants, a significant interaction of rs5443 was observed for sE-selectin ($p<0.001$), but not for sP and sL-selectins ($p=0.079$ and $p=0.607$ respectively). After adjusting for covariables, the T allele was significantly associated with an additive increase effect on serum sE-selectin levels in males ($p=0.020$), while a significant additive decrease effect was observed in females ($p=0.030$). These associations stayed significant after correction for multiple tests ($p=0.045$ in males and in females).

Discussion

The levels of E-selectin are increased during endothelial dysfunction and vascular remodelling (284, 285). Increased levels have been also associated with atherosclerosis and coronary artery diseases (286, 287). The GNB3 C825T polymorphism has been associated with these same CVD, as mentioned before (274-281). A link between the GNB3 C825T polymorphism and E-selectin is then consistent with previous findings, even if current knowledge does not show a direct involvement of the GNB3 gene in the production of E-selectin molecule. The GNB3 gene encodes a beta subunit of the G proteins, which transmit signals from the cell surface to intracellular signal cascades (288). It is established that G proteins play important roles in regulating the cardio-vascular system [25]. Indeed, studies about the involvement of G proteins in mechanochemical signal transduction show that endothelial alignment, atherosclerotic lesion formation, vascular remodelling, and vasoactive molecule release are all mediated in part by hemodynamic forces, with G-protein activation (289). It has been also argued that G proteins are associated with the flow-stimulated mechanochemical transducer within endothelial cells and fluid mechanical forces directly influence endothelial cell structure and function (290). In this perspective, Chappel et al. showed that prolonged oscillatory fluid shear stress induces expression of endothelial cell leukocyte adhesion molecules, including E-selectin (291). Thus, we suggest that the link between the GNB3 C825T polymorphism and E-selectin found in this study is possible through G proteins' effect within endothelial cells, by a mechanotransduction process. In this SNP carriers, G proteins may therefore have enhanced activity within endothelial cells, and consequently generate high levels of E-selectin.

In our study, increased levels of sE-selectin have been observed in males but not in females. We observed that the strength of the SNP effect on sE-selectin was approximately similar in males and in females, while the direction of the effect was opposite in these two sex groups.

We can then conclude on a significant qualitative interaction of this SNP with sex on sE-selectin levels ($p<0.001$). This sex-specificity explains why we were not able to detect this SNP's effect in the whole population, as the addition of the opposite effects resulted in an almost null global effect. These results demonstrate the importance of looking for a sex-specific effect of one or more variants that can constitute a study in itself. Indeed, sex-specific studies can reveal interesting associations that would be hidden in classical genetic association studies. In our study, we also observed that the additive variance explained by the SNP rs5443 was more than 20% in males while this proportion was ten times less in females. Genetic factors seem to have a more pronounced effect on E-selectin phenotype in males, while in females, other elements as constitutional or environmental factors and their interactions might be more implicated. The SNP interaction with sex in our study could be due to hormonal influence in females.

Our results help to better understand in males, the deleterious effect of the GNB3 gene on CVD, as hypertension. Because of this, we suggest that the GNB3 C825T polymorphism may improve significantly endothelial cells activities in inflammatory pathways, by an enhanced mechanotransduction signalling in males carriers of this variant and consequently induces susceptibility to CVD.

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RESEARCH Open Access

Cardiovascular diseases-related *GNB3* C825T polymorphism has a significant sex-specific effect on serum soluble E-selectin levels

Kokoè Mélinda Gbadoe, Nazha Berdouzi, Alex-Ander Aldasoro Aguiñano, Ndeye Coumba Ndiaye* and Sophie Visvikis-Siest*

Abstract

Background: The C825T polymorphism (rs5443) of the Guanine Nucleotide-Binding protein subunit β3 (*GNB3*) gene has been associated with obesity, essential hypertension, atherosclerosis, coronary diseases, and cerebrovascular events, but with some sex-specific effects. Its association with inflammatory mediators such as cell adhesion molecules has not been studied, although they are heavily involved in cardiovascular diseases' (CVDs) processes. The aim of our study was then to investigate a possible sex-specific effect of the *GNB3* C825T polymorphism on serum soluble cell adhesion molecules such as E, P and L-selectins (sE, sP and sL-selectins).

Results: Participants were from the STANISLAS Family Study and were free of chronic disease as CVDs or cancer. We included in total 771 subjects aged 6 to 58 years (391 males (50.71%) and 380 females (49.29%)). No significant association of rs5443 was observed in the whole population with serum sE, sP and sL-selectins after adjusting for age, sex, body mass index, systolic blood pressure, anti-inflammatory drugs and hormonal drugs consumption. A significant interaction of rs5443 was observed with sex for sE-selectin ($p < 0.001$), but not for sP and sL-selectins. After adjusting for covariables, the T allele was significantly associated with an additive increase effect on serum sE-selectin levels in males ($\beta = 5.03 \pm 2.18$; $p = 0.020$), while a significant additive decrease effect was observed in females ($\beta = -4.46 \pm 2.06$; $p = 0.030$). These associations stayed significant after correction for multiple tests ($p = 0.045$ in males and in females). The additive phenotypic variance was 21.54% in males versus 1.91% in females.

Conclusions: In our Caucasian population, the *GNB3* C825T polymorphism showed a significant sex-specific effect on serum sE-selectin levels, with a disadvantage for males, as increased sE-selectin levels has been associated with CVDs outcomes. The T allele has been previously associated with the same CVDs as increased sE-selectin, but more often in males. The link we observed between this polymorphism and E-selectin is then consistent with previous findings, and helps to better understand the deleterious effect of the *GNB3* 825 T allele on CVDs outcomes in males. We revealed in this study an important pathway through which the *GNB3* gene induces CVDs' outcomes.

Keywords: Sex-specific effect, Cell adhesion molecules, Selectins, *GNB3* gene, Cardiovascular diseases

Background

The Guanine Nucleotide-Binding protein subunit β3 (*GNB3*) gene is present on chromosome 12 at the location 12p13 and presents a polymorphism called rs5443, resulting from a cytosine (C) to a thymine (T) substitution at position 825 (C825T) located in exon 10 of the gene [1]. This polymorphism is involved in cardiovascular pathophysiological processes and cardiovascular diseases (CVDs). Indeed, it has been associated with obesity [2], higher blood pressure [3], hypertension [1, 4, 5], carotid atherosclerosis [6], incident cerebrovascular events [7] and coronary diseases [8], even though these effects have also been controverted in the literature [9, 10].

Some of these studies showed a sex-specific effect of this single nucleotide polymorphism (SNP). Brand et al. observed a higher blood pressure in T homozygotes

* Correspondence: ndeye-coumba.ndiaye@univ-lorraine.fr; sophie.visvikis-siest@inserm.fr
UMR INSERM U1122; IGE-PCV "Interaction Gène-Environnement en Physiopathologie CardioVasculaire", Faculté de Pharmacie, Université de Lorraine, Nancy F-54000, France

males than in C allele male carriers, while no significant difference was observed in females [3]. Hengstenberg et al. also observed a significantly higher prevalence of arterial hypertension in TT genotype subjects as compared to the other genotypes, but this association was predominantly found in men [4]. Frey et al. observed that the TT genotype was a significant risk factor for fatal and non-fatal myocardial infarction, independently of other established cardiovascular risk factors at a population level, but only in males, while in females no significant association was observed [8]. All these elements encourage further investigations of a sex-specific effect of this polymorphism.

Furthermore, the association of the *GNB3* 825 T allele with lipids, body mass index (BMI) [11], and blood pressure [1, 3–5] have been studied but not with molecules involved in inflammatory pathways such as cell adhesion molecules (CAM), while their importance in cardiovascular processes is well known [11, 12].

In this perspective, the aim of our present study was to investigate a possible sex-specific effect of the *GNB3* C825T polymorphism on the serum soluble cell adhesion molecules E, P and L-selectins (sE, sP and sL-selectins).

Methods

Study participants

Participants are from the STANISLAS (Suivi Temporaire annuel Non Invasif de la Santé des Lorrains Assurés sociaux) Family Study (SFS), a 15-year longitudinal monocentric family survey. Individuals were invited to three quinquennial medical check-ups that were held in 1994–1995, 1998–2000, and 2000–2003 [13, 14]. The SFS includes Caucasian volunteers, frequenting the Centre for Preventive Medicine of Vandoeuvre-lès-Nancy (East of France). All the subjects recruited were free of chronic diseases as CVDs or Cancer. Data on sE, sP and sL-selectins have been collected for subjects present during the second quinquennial medical check-up which occurred in 1998–2000. Among the 2,532 subjects (754 families) present at this second recruitment, we included those who were genotyped for the SNP rs5443, and for which data on the following factors were available: age, sex, BMI, systolic blood pressure (SBP), and anti-inflammatory drugs consumption (nonsteroidal drugs and corticosteroids). In females, we added a supplementary criterion: the availability of data about hormonal drugs consumption including oral contraceptives and hormonal replacement therapy containing oestrogen or progesterone).

Blood samples and data collection

Serum concentrations of sE, sP and sL-selectins were measured using commercially available enzyme-linked immunosorbent essay according to the manufacturer's specifications (ELISA kits, R&D Systems, Abingdon, Oxon, UK). The intra- and inter-assay coefficients of variation were as follows: sE-selectin, 9.4 and 14.9% (sensitivity: 0.027 ng/mL, assay range: 0.1–8 ng/mL); sP-selectin, 5.8 and 7.0% (sensitivity: 0.5 ng/mL, assay range: 0.8–46 ng/mL); sL-selectin, 8.9 and 11.7% (sensitivity: 0.3 ng/mL, assay range: 1.0–58 ng/mL), respectively.

Frozen aliquots of serum were stored in the Biological Resources Centre (BRC) "Interactions Gène-Environnement en Physiopathologie CardioVasculaire" (IGE-PCV).

Information about drug consumption and personal medical history was collected using relevant questionnaires and procedures under the supervision of trained nurses.

Weight and height were measured while the participants were standing in light clothing without shoes and BMI was calculated as weight in kilograms divided by height in meters squared.

Blood pressure was measured under constant temperature (19 °C-21 °C) and standardized conditions (supine position) using a manual sphygmomanometer. The recorded values were the means of 3 readings on 20 min intervals.

Genotyping

Genomic DNA was extracted from venous blood samples by the salting-out method [15]. Genotyping of the SNP rs5443 was part of a multilocus assay performed with an immobilized probe approach designed by Roche Molecular Systems, Pleasanton, California, USA [16]. Genotyping was validated by classical Polymerase Chain Reaction methodology [17] in 50 individuals.

Statistical analysis

The Hardy-Weinberg Equilibrium (HWE) of our study population was verified by a Chi square test. For statistical analyses, we used parametric method. The normality of the distribution was tested by Kolmogorov-Smirnov test. We controlled variances homogeneity of the compared groups, by the test of Levene.

The three phenotypes (sE, sP and sL-selectins) were introduced in a genetic additive model as quantitative variables, in an observational transversal and analytical design. The SNP effect was tested by a linear regression model, using an ANalysis Of VAriance (ANOVA). Covariates introduced in the model for adjustment are age, sex, BMI, SBP, and anti-inflammatory drug consumption. Age, BMI and SBP were considered in the model in their quantitative form. In the model testing the SNP effect in females, we added "hormonal drug consumption" as a supplementary qualitative covariate. Familial correlations in our study population were accounted for by using a linear mixed effects model that uses a relationship coefficient matrix as within pedigree correlation structure.

We performed analyses with the R package GWAF (Genome-Wide Association/Interaction Analysis and

Rare Variant analysis with Family Data) [18]. The type I error, alpha was fixed at 5%. The power of our study has been calculated *a posteriori* with the Quanto software (http://biostats.usc.edu/Quanto.html).

Firstly we tested the association of the SNP rs5443 with phenotypes in the whole population, and secondly we tested the SNP interaction with sex. If a significant interaction of the SNP with sex was observed for a phenotype, we then tested a new association of the SNP with this phenotype separately in males and in females. A False Discovery Rate (FDR) correction was done in this case, for the SNP associations' *p*-values.

Results and discussion

We included in total 771 subjects aged 6 to 58 years in our study, involving 391 males (50.7%) and 380 females (49.3%) (Fig. 1). Table 1 shows the characteristics of the study population, and Table 2, the genotypes distribution of the SNP rs5443 in the studied population. The total population showed no significant deviation from the HWE ($p = 0.409$). Table 3 shows the means and confidence intervals at 95% of quantitative variables introduced in the linear regression model testing the SNP effect on selectins.

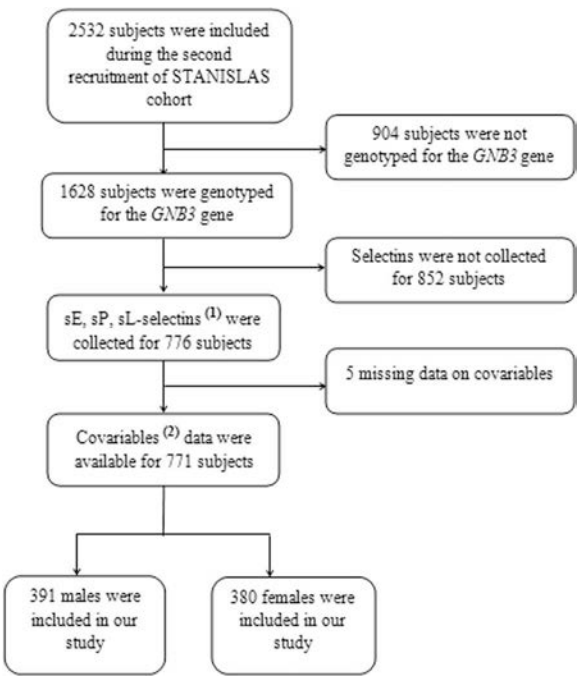

Fig. 1 Flow chart of the selected population. 1. Serum Soluble E, P and L-selectins. 2. Age, sex, body mass index, systolic blood pressure, anti-inflammatory drugs, and hormonal drugs consumption (oral contraceptives and hormonal replacement therapy containing oestrogen or progesterone)

Table 1 Characteristics of the study population

Population characteristics	Percentages (%)		
	Males $n = 391$	Females $n = 380$	Total population $N = 771$
BMI(kg/m^2)			
< 18.5	11.5	13.7	12.6
18.5–25	61.6	66.3	63.9
25–30	22.5	15.5	19.1
> =30	4.3	17	4.4
Age (years)			
< 15	15.9	17.4	16.6
15–24	37.6	34.7	36.2
25–34	2.0	1.3	1.7
35–44	21.5	32.1	26.7
45–54	22.5	14.2	18.4
55–64	0.5	0.3	0.4
SBP(mmHg)			
< 120	41.7	62.6	52.0
120–139	50.9	31.6	41.4
140–159	7.4	5.0	6.2
> =160	0.0	0.8	0.4
Anti-inflammatory drug consumption			
Yes	3.8	5.0	4.4
No	96.2	95.0	95.6
Hormonal drug consumption			
Yes	0	31.6	15.6
No	100	68.4	84.4

BMI Body Mass Index, *SBP* Systolic Blood Pressure

In the whole population

The additive model showed no significant result for sP, sL, and sE-selectins (respective *p*-values = 0.876, 0.158 and 0.770) after adjusting for covariables (sex, age, SBP, BMI, hormonal drugs, or anti-inflammatory drug consumption).

Interaction of the SNP with sex

After adjusting for covariables, the interaction of rs5443 with sex was significant with sE-selectin ($p < 0.001$) while

Table 2 Genotype distribution and allele frequencies in the studied population

Genotypes	Males		Females		Total Population	
	n	%	n	%	n	%
CC	176	45.0	158	41.6	334	43.3
CT	171	43.7	168	44.2	339	44.0
TT	44	11.3	54	14.2	98	12.7
Total	391	100.0	380	100.0	771	100.0

Minor Allele Frequencies in males, Females and total population were respectively 33.5, 37.0, 35.7%
p-value of Hardy-Weinberg Equilibrium test was 0.4

Table 3 Means and confidence intervals of quantitative variables introduced in the linear regression model, studying the SNP rs5443 effect on serum soluble selectins in the STANISLAS population

Means CI (95%)			
Variables	Total population	Males	Females
sE-selectin (mg/l)	56.0 [54.0;58.0]	60.0 [57.1; 62.9]	51.8 [49.0; 54.5]
sP-selectin (mg/l)	132.5 [129.7; 135.3]	140.5 [136.4; 144.5]	124.4 [120.8; 128.0]
sL-Selectin (g/l)	1227.7 [1191.0; 1264.3]	1221.4 [1165.5; 1275.5]	1234.1 [1185.4; 1282.04]
Age (years)	29.4 [28.4; 30.4]	29.5 [28.1; 31.0]	29.3 [27.9; 30.7]
BMI (kg/m^2)	22.5 [22.2; 22.8]	22.8 [22.4; 23.2]	22.2 [21.8; 22.6]
SBP (mmHg)	120.0 [119.1; 120.9]	122.4 [121.2; 123.6]	117.5 [116.3; 118.8]

CI(95%): Confidence Interval at 95%
Dependent variables: sE, sP, and sL-selectins (serum soluble E, P and L-selectins), Covariables: BMI, SBP, Age
BMI body mass index, *SBP* systolic blood pressure

the results with sP and sL-selectins were not significant ($p = 0.079$ and $p = 0.607$ respectively). Indeed, in the multivariate analysis, the T allele was significantly associated with an increased additive effect on serum sE-selectin levels in males ($p = 0.020$), while in females we observed a significantly decreased additive effect ($p = 0.030$). After correction by the FDR method, p-values remained statistically significant in males ($p = 0.045$) and females ($p = 0.045$). The effects of the T allele on sE-selectin in males, females and the whole population are illustrated in Fig. 2.

Additive genetic variances

The proportion of sE-selectin variance explained by the genetic additive effect in males was 21.54% versus only 1.91% in females.

Association of the C825T polymorphism with E-selectin

This study revealed a significant additive effect of the *GNB3* C825T polymorphism on serum sE-selectin levels, in males and females populations.

E-selectin also called Endothelial-Leucocyte Adhesion Molecule 1 (ELAM 1) is a cell surface glycoprotein expressed by endothelial cells (ECs) and is involved in leukocyte adhesion [19]. Its levels are increased during endothelial dysfunction and vascular remodelling [20, 21]. Increased levels of sE-selectin have been associated with atherosclerosis [22, 23], essential hypertension [24, 25], cerebrovascular diseases [26] and coronary artery diseases [27, 28]. The *GNB3* C825T polymorphism has been associated with these same CVDs, as aforementioned [1–8]. A link between the *GNB3* C825T polymorphism and E-selectin is then consistent with previous findings, even if

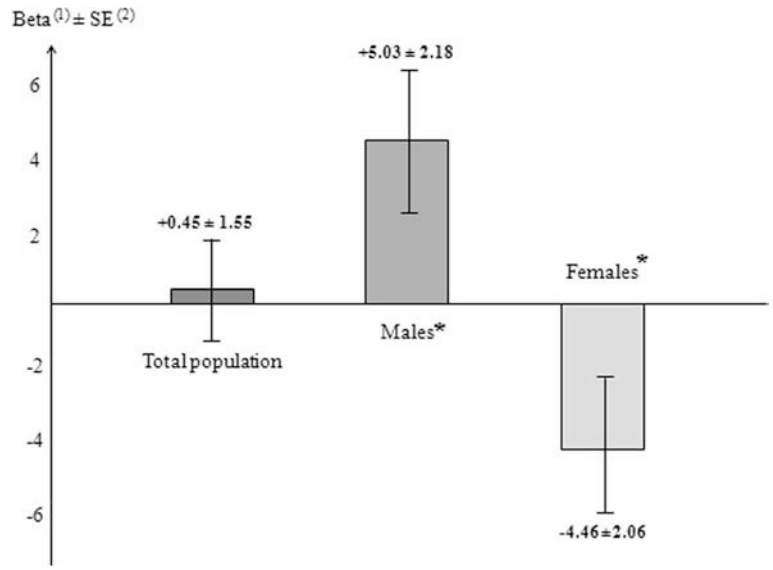

Fig. 2 Effects of the *GNB3* 825 T allele on serum soluble E-selectin levels in the STANISLAS population. 1. The additive genetic effects of the *GNB3* 825 T allele on serum soluble E-selectins levels (mg/l), in the total population ($N = 771$), and separetly in males ($n = 391$) and females ($n = 380$). 2. Standard error of the effects. *P-value ≤ 0.05 (Significance level) after false discovery rate correction: The *GNB3* 825 T allele significantly increased serum soluble E-selectin levels in males and significantly decreased these levels in females, while in the total population (males + females), no significant effect was observed

current knowledge does not show a direct involvement of the *GNB3* gene in the production of E-selectin molecule. This molecule is rather encoded by the *SELE* (Selectin E) gene [29]. However, the *GNB3* gene may be involved in a mechanism regulating levels of E-selectin.

The *GNB3* gene encodes a beta subunit of the heterotrimeric guanine nucleotide-binding proteins also called G proteins which transmit signals from the cell surface to intracellular signal cascades [30]. It is established that G proteins play important roles in regulating the cardiovascular system [25]. Gudi et al. have studied the involvement of G proteins in mechanochemical signal transduction and reported that within the circulation, endothelial alignment, atherosclerotic lesion formation, vascular remodelling, and vasoactive molecule release are all mediated in part by hemodynamic forces, with G-protein activation [31]. Authors also concluded that G proteins are closely associated with the flow-stimulated mechanochemical transducer within endothelial cells. It has been demonstrated that fluid mechanical forces directly influence endothelial cell structure and function [32]. In this perspective, Chappel et al. showed that prolonged oscillatory fluid shear stress induces expression of endothelial cell leukocyte adhesion molecules, including E-selectin [33]. We suggest that the link between the *GNB3* C825T polymorphism and E-selectin found in this study is possible through G proteins' effect within endothelial cells, by a mechanotransduction process. Otherwise, the *GNB3* C825T polymorphism is associated with increased transmembrane signal transduction [1]. In this SNP' carriers, G proteins may therefore have enhanced activity within endothelial cells, and consequently generates high levels of E-selectin.

A likely association of the *GNB3* C825T polymorphism with P-selectin

P-selectin is expressed in activated endothelial cells as well as E-selectin, while L-selectin is expressed on leukocytes [34]. Knowing the effect of G proteins within endothelial cells, we can assume that the *GNB3* C825T polymorphism association would also be positive for P-selectin, but not with L-selectin. Our results seem to follow this trend. Indeed, we found that the sex-specific effect of the SNP was likely with P-selectin (p-value = 0.08), but unlikely with L-selectin (p-value = 0.61). We think that in a larger population, the sex-specific effect will also be positive for P-selectin at a significance level of 5%. We specify that confounding factors as antiplatelet medications or anticoagulants can hide this association. In our study population, none of the subjects reported taking these drugs.

Interaction of the SNP rs5443 with sex, on sE-selectin

In our study, increased levels of sE-selectin have been observed in males but not in females. We observed that the strength of the SNP effect on sE-selectin was approximately similar in males and in females (varying between 2.85–7.21 for males and 2.40–6.52 for females) while the direction of the effect was opposite in these two sex groups. We can then conclude on a significant qualitative interaction of this SNP with sex on sE-selectin levels ($p < 0.001$). This sex-specificity explains why we were not able to detect this SNP's effect in the whole population, as the addition of the opposite effects resulted in an almost null global effect.

These results demonstrate the importance of looking for a sex-specific effect of one or more variants that can constitute a study in itself. Indeed, sex-specific studies can reveal interesting associations that would be hidden in classical genetic association studies.

In our study, we also observed that the additive variance explained by the SNP rs5443 was more than 20% in males while this proportion was ten times less in females. Genetic factors seem to have a more pronounced effect on E-selectin phenotype in males, while in females, other elements as constitutional or environmental factors and their interactions might be more implicated. We could explain this sex-specificity by (inter alia) hormonal interactions. Indeed, Caulin-Glaser et al., in their study on subjects with coronary artery disease, observed a statistically significant increase in sE-selectin levels in men and postmenopausal women not receiving oestrogen replacement therapy (ERT) compared with women receiving ERT [35]. Others studies revealed that, a decrease in the number of inflammatory cells is observed, in follicular phase, in cyclic women, when oestrogen level is high, compared to an increase in males and postmenopausal females [36, 37]. Then, the SNP interaction with sex in our study could be due to hormonal influence in females. Indeed, our study focused on a Caucasian population from an industrilized country and it has been reported that the age at menopause among women from this population is around midlife. [38]. The proportion of women over 50 years in our study is only 0.04% (17 women). Our results in females may therefore be influenced by this selection bias about hormonal status.

In males, a case-control study of Wang et al. showed a significant interaction between the *SELE* gene and sex on essential hypertension [39]. The *SELE* gene was not associated with essential hypertension in females. Knowing the effect of the *GNB3* 825 T allele on essential hypertension often found in males, the sex-specific effect of this polymorphism on E-selectin observed in our study is consistent with this finding.

Our results help to better understand in males, the deleterious effect of the *GNB3* gene on CVDs, as hypertension. We suggest that the *GNB3* C825T polymorphism may improve significantly endothelial cells activities in inflammatory pathways, by an enhanced mechanotransduction

signalling in males carriers of this variant, and consequently induces susceptibility to CVDs.

Limitations and perpectives

In our study, the significance regarding the effects of the 825 T allele on sE-selectin in males and females is marginal after FDR correction ($p = 0.045$). We acknowledge that a replication in a larger study population would ameliorate the data dispersion and significantly improve the precision of the estimates.

It would be interesting that replication studies test hormonal interaction with the *GNB3* C825T polymorphism on E-selectin. We were not able to test this interaction in our study, as the proportion of women over 50 years was low.

Although we have demonstrated in this study an effect of the *GNB3* C825T polymorphism on sE-selectin, the precise mechanism of this gene on E-selectin molecule need to be clarified by further investigations.

The results of our study support an involvement of the SNP rs5443 in CVDs, particularly in men. A question arising from this observation is whether special monitoring in males carrying this variant is needed, especially when the mean age of cardiovascular risk is reached. Controversial results of some studies on this issue did not allow authors to decide clearly about it. Otherwise, the impact of this variant on CVDs' outcomes was investigated but it would be also interesting to focus on its impact as cardiovascular prognostic factor to determine whether the evolution of CVD in carriers of this polymorphism is adverse or not. This could lead to consider therapeutic targets fitting this profile of patients. In this context, knowledge about the mechanism of this variant in the cardiovascular pathophysiological process would be of great use.

Conclusions

In this present study, we were able to highlight, in Caucasians, a sex-specific association between the *GNB3* 825 T allele and sE-selectin, with however a disadvantage for males concerning the known impact of increased E-selectin on CVDs' outcomes. This association might be hidden in classical studies, because of the opposite effect observed in males as compared to females. Our findings revealed an important pathway through which the *GNB3* gene induces CVDs' outcomes and help to better understand the deleterious effect of the 825 T allele on CVDs outcomes in males.

Abbreviations

BMI: Body mass index; BRC IGE-PCV: Biological Resources Centre "Interactions Gène-Environnement en Physiopathologie CardioVasculaire"; CAM: Cell adhesion molecules; CVDs: Cardiovascular diseases; ERT: Oestrogen replacement therapy; FDR: False discovery rate; GNB3: Guanine nucleotide-binding protein subunit β3; INSERM: Institut National de la Santé et de la Recherche Médicale; SBP: Systolic blood pressure; SFS: STANISLAS (Suivi Temporaire Annuel Non-Invasif de la Santé des Lorrains Assurés Sociaux) Family Study; SNP: Single nucleotide polymorphism

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Availability of data and materials

Datasets on which the conclusions of the manuscript rely will be provided.

Authors' contributions

Conception and design: KMG, NB, NCN, SVS. Acquisition of Data: SVS. Analysis of Data: KMG, NCN. Interpretation of Data: KMG, NB, AAAA, NCN, SVS. Drafting of the manuscript: KMG, NB, NCN. Revision of the manuscript for important intellectual content: KMG, NB, NCN, AAAA, SVS. All authors read and approved the final manuscript.

Authors' information

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The population involved in the present study was recruited in accordance with the latest version of the Declaration of Helsinki for Ethical Principles for Medical Research Involving Human Subjects and all participants, or legal representative for children, gave written informed consent. Studies protocols were approved by the local ethics committees for the protection of subjects for biomedical research: the "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB) de Lorraine". The samples are part of a human sample storage platform: the Biological Resources Centre (BRC) "Interactions Gène-Environnement en Physiopathologie CardioVasculaire" (IGE-PCV) in Nancy, East of France.

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CHAPTER IV

GENERAL DISCUSSION

The present thesis had as principal objective to study the effects of genes of interest, taken each one individually, on a number of different intermediate phenotypes that are known to be involved in CVD as well as in other complex diseases. We also focused our efforts on the possible translation of the results obtained into clinical application. We believe that genome-based personalized medicine is an important way of reducing the deaths caused by CVD, which are the main causes of worldwide mortality (1).

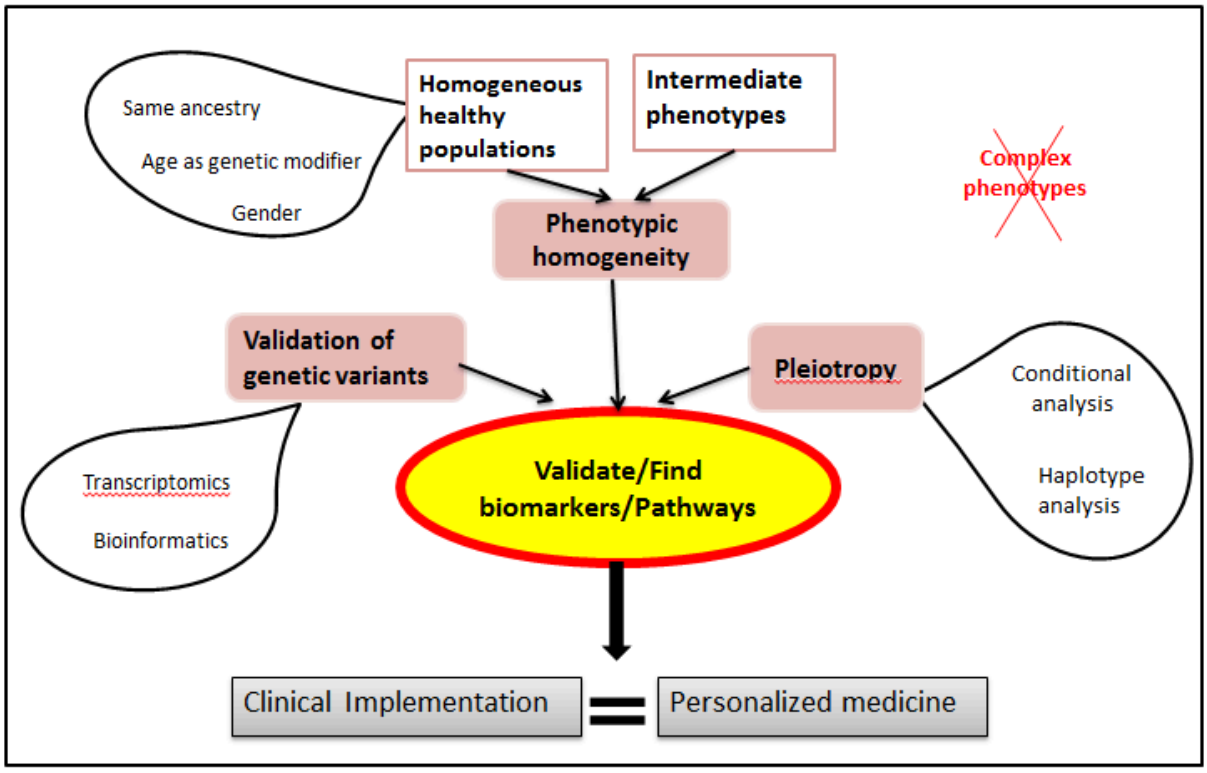
Complex diseases, and among them CVD, are defined as diseases that are determined by diverse interacting genetic and environmental factors. The genetic epidemiology of these diseases has been a subject of a revolution during the last decade, thanks to novel approaches such as GWAS, PheWAS, QTL and expression QTL (eQTL) (12-14). Although these novel exploratory approaches give many insights about the complex diseases genetic background, more than ten years after the first published GWAS (15, 16), the general feeling among the experts in the field is currently bittersweet.

The multifactorial diseases that those novel approaches (GWAS, PheWAS, eQTL…) were supposed to solve have proven to be even more complex than previously thought. Indeed, some of the systems of the human body that are involved in these diseases are astonishingly complex. As an example, the immune system regulation is under the control of many different genes, and needs a big number of biochemical and physiological mechanisms investigations in order to be fully apprehended (292). At the same time, in order to maintain homeostasis, it is constantly responding via feedback mechanisms to the environmental changes. Thanks to this complex regulatory system, and the compensatory mechanisms within, even if one or several elements of the system fails, other components can emulate their role, thus maintaining homeostasis.

Previous GWAS showed thousands of polymorphisms and genes associated with different phenotypes related to CVD. However, taken together, all these genes explain only a fraction of CVD heritability (17). These unsatisfactory results raised the issue nowadays known as the "missing heritability" (18). At the same time, the results obtained from GWAS have limited clinical applicability so far, as it is difficult to elucidate the real biological effect of the identified polymorphisms. Hence, unfortunately, many of the molecular mechanisms by which these polymorphisms act in the complex diseases, are still largely unknown.

During this thesis, we used alternative methods beyond the classical GWAS to uncover the mechanisms that underlie CVD and other complex diseases (Figure 29). First of all, we choose a homogeneous population coming from the region of Lorraine in France as a discovery population. By doing so, the goal was to reduce the enormous cardiovascular-related heterogeneity of the population, thus, limiting the potential confounders. Also, we investigated the so-called "novel" risk biomarkers of CVD (293, 294), putting a special focus on the inflammatory genes. We worked on linking genes of interest (TREM-1, IL6-R and TREM-2, among others) with intermediate phenotypes (endophenotypes) representing metabolic pathways instead of linking them directly with the disease. This approach can represent better the real biological pathways, where the gene of interest is involved.

Figure 29: Summary of the framework during the thesis.



Together with homogeneous populations and novel intermediate phenotypes, we studied inflammatory genes (among others) that participate in complex systems of the human body, and we studied their pleiotropic associations with other intermediate phenotypes. We also investigated gender as a possible genetic effect modifier (gene-gender interactions). Furthermore, we performed conditional analysis and haplotype analysis in order to ensure that the gene-phenotype associations founded were independent from each other. Also, we tried to

validate the genetic variants detected by performing transcriptomic analyses and by using bioinformatic tools. Last but not least, during this thesis we have always been searching for the clinical applicability of our results, with a strong focus in genetics of personalized medicine.

Following the goals and approaches synthetized above, during the last three years, we discovered new associations between genes of interest and intermediate phenotypes that are involved in CVD and other complex diseases (23, 24). Our results helped to better understand how the studied genes are exerting their effects at the molecular level, ultimately affecting the outcome of the individuals suffering from CVD. Our results will hopefully be taken into account in future personalized treatments.

More specifically we obtained these results:

A- TREM-1 SNP rs2234246 regulates TREM-1 protein and mRNA levels and is associated with plasma levels of L-selectin

This study was performed in the SFS population, in collaboration with the Regional University Hospital of Nancy and INOTREM SA. We choose to study the TREM-1 gene because recent studies about this recently discovered inflammatory gene showed that it is closely associated with the development of atherosclerosis (142). The goal was to investigate whether polymorphisms within TREM-1 are regulating serum TREM-1 levels, the expression levels of their mRNA as well as the possible associations with blood levels of selectins L, P and E.

We first genotyped the minimum set of SNPs that was capturing the majority of genetic variation at the TREM-1 gene. After performing association studies, the SNP rs2234246 was highly associated with soluble TREM-1 protein levels. We replicated this result in an independent population and further conferred functional properties, as this polymorphism was also associated with TREM-1 mRNA levels. Interestingly, rs2234246 was related with the mRNA that codes the membrane bound TREM-1 and not with the mRNA coding the soluble TREM-1 protein. These results support the hypothesis that the levels of sTREM-1 are controlled post transcriptionally by metalloproteases, rather than by alternatively spliced

forms of RNA (215, 221). We also strengthen our results by performing bioinformatic-epigenetic approaches. We showed that the rs2234246 has a specific H3K36me3 methylation epigenetic pattern, which is especially interesting as it is considered to be a hallmark of actively transcribed gene bodies (295). Also, we studied the potential transcription factors binding the locus of the SNP, which could be different depending on the allele according to the TRANSFAC R.3.4 database. Finally we associated this polymorphism with soluble L-selectin, but not with E and P-selectin levels. This strengthens the hypothesis that the TREM-1 gene acts in the trans-epithelial migration of monocytes and neutrophils to sites of acute and chronic inflammation, such as atherosclerosis plaque sites (140, 142, 149).

In this work we extended the knowledge about the genetic background of the TREM-1 gene and we elucidated some aspects of its biological background. This polymorphism could be used in further personalized medicine, and the first clinical trials to test its implication in sepsis are being organized (296). Due to the highly interesting results, a patent has been filed in Europe. The applicants are INSERM, University of Lorraine, Regional University Hospital of Nancy and INOTREM SA.

B- IL6R haplotype rs4845625*T/rs4537545*C is a risk factor for simultaneously high CRP, LDL and ApoB levels

In this work we choose the IL6R gene, which is mediating IL-6's biological functions (297), playing an important role in different diseases, including CVD (225). The goal was to investigate two polymorphisms located within the IL6R gene that have been previously associated with CRP levels, and with controversial effects on lipid traits: SNPs rs4845625 and rs4537545.

The analysis were performed in the SFS, and replicated in an independent population. The results showed that both investigated SNPs were antagonistically related with CRP levels. Concerning the lipid traits, for the first time, the SNP rs4845625 was associated with higher LDL-C and ApoB levels. Thus, we enlarged the already known pleiotropy of the IL6R gene. In addition, we performed haplotype analysis, which confirmed that the association of the SNP rs4845625 with lipid levels is independent from rs4537545. The haplotype analysis showed that the TC haplotype, having the minor rs4845625*T allele, was related simultaneously with increased CRP, LDL-C and ApoB levels, thus could be considered as a

risk factor. A series of in silico re-sequencing as well as functional analysis helped us to identify potential functional biological processes underlying the associations of the two IL6R SNPs with lipid metabolism.

Our results may have important value in pharmacogenomics studies, given the fact that IL6R gene is a target of the pharmacological agent tocilizumab, an immunosuppressive drug widely used for the treatment of rheumatoid arthritis and which inhibits IL6R, resulting in a reduction of CRP concentrations (240). One of the side effects of tocilizumab is the increase of LDL-C levels in patients, leading to an increased risk of suffering from ischemic heart disease (22). Our discoveries could give interesting clues on the functionality of the drug.

In this study we used a post-GWAS approach, as we prioritized previous GWAS hits by selecting these two polymorphisms as the starting point of the investigation. Then we deepened the knowledge about these two polymorphisms and their interactions with different intermediate phenotypes involved in CVD. This alternative approach helped us to uncover previously unknown molecular mechanisms of the IL6R gene, proving to be a powerful method of investigation. Further, we showed that even though two SNPs are relatively close to each other, they might have very different effects.

C- Pleiotropy of ABO gene; Effects of rs644234 on E-selectin and lipid traits

The polymorphism rs644264, located within the ABO gene has already been associated with risk of suffering various diseases, including myocardial infarction (241, 248, 252, 253). The ABO histo blood group gene is already known to be pleiotropic, as it has been associated with many different diseases. The aim of this work was to investigate intermediate phenotypes that are known to be related with CVD, such as myocardial infarction. Consequently, we studied the relationships of the polymorphism rs644234 located in the ABO gene with soluble E-selectin levels and different lipid traits. We also investigated the possible sex-specific effects of this polymorphism on the phenotypes studied.

We managed to replicate previously found associations of the ABO polymorphisms with soluble E-selectin levels, as our results showed that the major rs644264*T allele was associated with increased levels of E-selectin. Our results also showed a significant association of the major allele with decreased levels of ApoE and LDL-C, while being

associated with increased HDL-C levels. So far, our results are the first ones associating a polymorphism within the ABO gene with ApoE levels. We didn't find sexual dimorphism according to sE-selectin and lipid levels, with the exception of the HDL-levels, were the major rs644234*T allele was significantly increasing the levels of HDL-C only in male individuals. We inferred the main ABO blood group that tags the SNP rs644234 (250), being mostly the O blood group (r^2=0.89) and in a less amount the A and B blood groups (r^2=0.58 and r^2=0.16 respectively). Taking this into account, we could suggest that our results are more likely representative in individuals with the O blood group. Indeed, other authors have already demonstrated that a SNP in strong LD with rs644234 (r^2=0.9) is associated with myocardial infarction, but only in the subgroup of individuals having the O blood group (248).

These results show the complexity by which the ABO gene is acting in the different diseases where it is involved, and despite those known pleiotropic effects of the ABO gene, no mechanistic study has been able to explain the real functional basis for those wide ranges of associations. One of the possible explanations is that because of this antagonistic pleiotropy, the ABO gene is not under strong negative selection, as the different effects of the same gene will be favorable in different environmental situations. Even if the major rs644234*T allele has simultaneous effects on phenotypes that could be considered to be risky/protective for certain diseases, looking to the previous studies performed and our results, we could deduce that the major allele acts with a protective effect when it concerns cardiovascular related diseases, and more specifically on lipid traits, with lower ApoE levels and higher HDL levels.

D- The polymorphism rs6918289 located in the upstream region of the TREM-2 gene is associated with TNF-α levels and intima media thickness of the femoral artery

TREM-2 is known for its anti-inflammatory properties during the immune response (254, 255), and is known to influence negatively the TNF-α expression levels (256, 257). TREM-2 protein levels have been shown to affect also the plaque stability in symptomatic and asymptomatic patients with carotid stenosis (259). We analyzed different polymorphisms located in the TREM-2 gene region and their possible associations with TNFα levels and the intima media thickness of the femoral artery (IMT-F).

Our results showed that the minor rs6918289*T allele is increasing TNFα levels in both populations studied. Despite the fact that several polymorphisms within the TREM-2 gene

have been related with numerous inflammatory diseases so far, no genetic determinants have been identified in the TREM-2 locus affecting TNF-α levels. The same polymorphism is also increasing the IMT-F in the discovery population. One of the early processes that lead to atherosclerosis is the arterial remodeling, and one effective way that provides information about this process is measuring the intima-media thickness. Indeed, IMT is predictive of atherosclerosis in asymptomatic individuals (269, 270), and also provides information about the degree of atherosclerosis (271, 272) as well as of the risk of suffering myocardial infarction (273). Those results suggest that the minor rs6918289*T allele could be considered as a risk factor for different chronic inflammatory diseases such as atherosclerosis.

Despite the fact that this polymorphism gives insights about its possible roles in the development of CVD, the exact mechanisms by which it is orchestrating this process is so far unknown and further studies are needed. According to the IMT-F, we think that the effect of this SNP could be indirect. Indeed, the remodeling of the artery is highly dependent on the inflammatory state, thus, increased TNF-α levels produced by the minor rs6918289*T allele could be responsible for the increased thickness of the femoral artery. Further studies will be necessary in order to know if this polymorphism or polymorphisms in LD are capable of modulating the TREM-2 protein levels and which are the mechanisms behind this observation. However, important applications could be found as these findings could be used for specific treatments in patients with chronic inflammatory diseases (298).

E- Cardiovascular disease related GNB3 C825T polymorphism has significant sex-specific effect on serum soluble E-selectin levels

The C825T polymorphism (rs5443) of the GNB3 gene has been associated with atherosclerosis and coronary diseases among others (279, 281). The goal of this work was to assess the possible sex-specific effect of this polymorphism on serum soluble selectin molecules (sE, sP and sL-selectins).

When performing analysis with all the participants, no significant association of rs5443 was observed with the three phenotypes studied. However, when performing the association by gender, the T allele of the rs5443 was significantly associated with increased levels of sE-selectin in males, while being associated with decreased levels in females. Even if our results are consistent with previous findings, current knowledge does not show a direct involvement

of the GNB3 gene in the production of E-selectin molecule. However, these findings could bring some insights about the involvement of GNB3 in pathways were the E-selectin level production might be regulated. It would be of high interest to check the impact of this polymorphism as cardiovascular prognostic factor to determine whether the evolution of CVD in carriers of this polymorphism is adverse or not. This could lead to consider therapeutic targets fitting this profile of patients.

This antagonistic gender-specific effect is interesting, and shows the importance of taking into account the gender-gene interactions when studying the populations. Using all the individuals, we were not able to detect this antagonistic effect, as the real effect is hidden because of the heterogeneity of the population (in this case when it comes to gender). Many of the classical approaches does not take into account the different subgroups involved in their studies, and many of the actual existent effects are ignored because they do not appear to be significant in the whole population. This approach shows the importance of considering factors that could make the subgroups even more homogeneous when it comes to detecting biomarkers involved in complex diseases, such as in CVD.

CHAPTER V

CONCLUSION AND PERSPECTIVES

As mentioned before, the detection of associations between genes and phenotypes influencing complex diseases such as CVD is a complicated task. In order to achieve this, it is necessary to reduce the "noise" that is hiding the real effect of a gene in specific intermediate phenotypes. The human physiology is highly complex, and different phenotypic endpoints can be reached by the same polymorphism, or inversely, different polymorphisms can end to the same clinical endpoint.

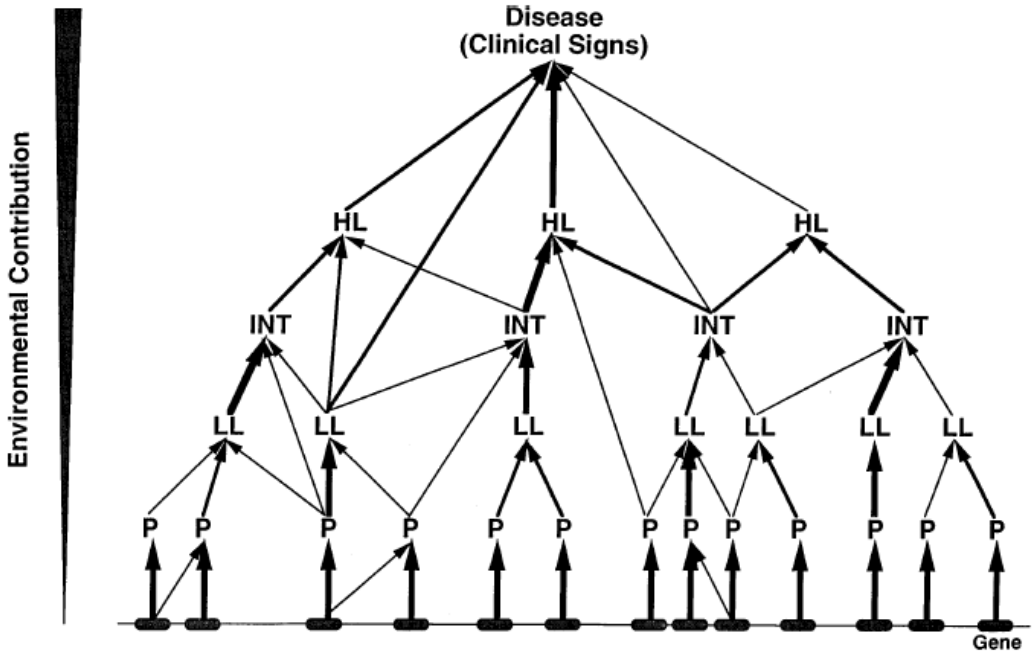
We used complementary approaches in order to achieve our goals: we chose GWAS hits through bibliographical searches and by using databases such as the HuGE navigator and the NCBI dbSNP database. In addition, we studied intermediate phenotypes instead of more broad general phenotypes/diseases, we used as much higher homogeneous populations as possible and we centered our efforts in studying the pleiotropic and gene-environment interactions effects of the genes selected. By using these approaches, our results showed new associations between genes and intermediate phenotypes that are related with complex diseases. However, future studies will be needed to strengthen and deepen the results and hypotheses found in this work.

The SFS was the principal discovery population used. All the individuals are coming from the region of the Northeast France and have European descent. Also, the SFS individuals are considered to be free of any chronic disease. Thanks to this, we ensured the homogeneity of the population, not only at the genetic level but also at the environmental level. By using homogeneous populations, we obtain higher statistical power advantages (299) compared to other populations having higher allelic heterogeneity (300). Homogeneous and genetically isolated populations have proved to be a useful in the identification of genes associated with diseases (301). Indeed, the use of several isolated and thus homogeneous populations such as the Kainuu population from Finland (302), populations from Scotland (303), the Hutterite population in Canada (304) or the Basques in Spain (305) have helped to uncover genetic variants associated with complex diseases. Also, the environmental and phenotypic heterogeneity is lower in homogeneous populations. This is partly because they will have more uniform patterns of nutrition, exposure to pathogens or homogeneous diagnosis standards. All this will facilitate the detection of disease-gene associations by reducing variance caused by environmental effects. However, although these populations may facilitate the detection of genetic variants associated with disease intermediate phenotypes, not all variants found may have effects in other genetically and environmentally different populations. Despite this apparent disadvantage, our results help to find the biological

pathways and pathophysiological mechanisms where the gene studied is involved, and this information could be used in other populations or investigation fields (19). It would be of high interest to specifically use the young individuals of the SFS population as already done as the first step of the TREM-2 study. Among the younger individuals, the effect that the environment exerts on the phenotypes and hence the disease itself is lower as they have been exposed to the environment less time than the adults. Because of this, the genetic components could have a greater contribution to disease risk in younger individuals, which may make the study of CVD more precise (306).

Also, using intermediate phenotypes to link the effects of the polymorphisms helped us to uncover novel associations that are involved in CVD. The path from the genes until the disease related clinical traits is complex (Figure 30). It is much easier to connect the gene and the lower part of the pyramid's intermediate phenotypes (showed as P, LL and INT in the figure 1). Indeed, the closer the phenotype is to the gene, the fewer will be the number of genes and other factors influencing it. Also, the phenotypes located at the lower levels of the biochemical and physiological hierarchy will be less prone to environmental influences than the upper part's more general phenotypes (20). This approach makes easier to uncover the real gene-phenotype associations, and the pathways involved. Some GWAS ignore the intermediate phenotypes approach and try to directly link genes with the actual disease. Although it may be a useful approach in some cases, in complex diseases like CVD, the too small genes effects obtained are much more difficult to be evaluated.

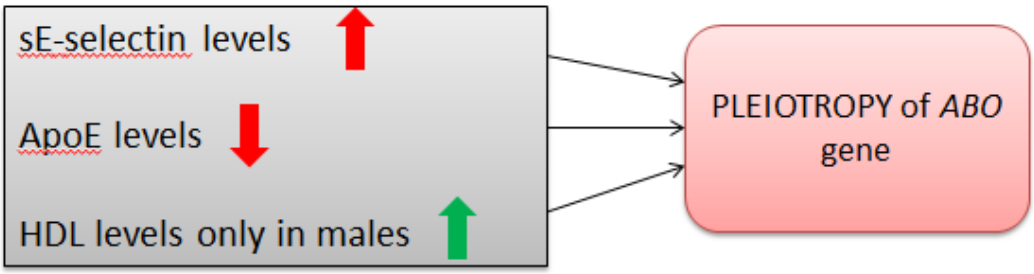
Figure 30: Schematic diagram of the relationship of genes and their phenotypes, until the more obvious clinical manifestations of a disease.



Source: Genetics of Complex Disease; Schork.

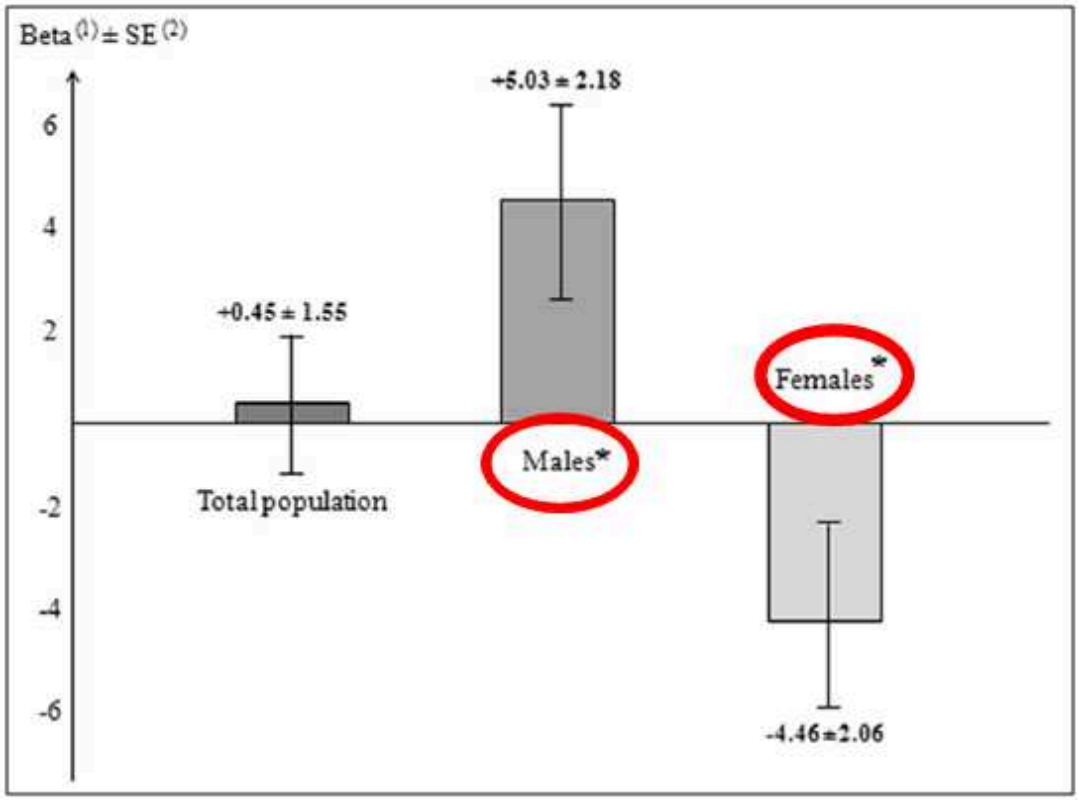
During this thesis we also detected pleiotropic effects in several genes (IL-6R and ABO genes). We found an antagonistic pleiotropy within the IL-6R gene on CRP and lipid trait levels. We also found new pleiotropic effects within the ABO gene, by independently associating the major rs644234*T allele with different traits (Figure 31). The detection of pleiotropy in genes indirectly shows that they are involved in various biological processes, by having their protein products present in various cellular components and by protein-protein interactions (21).

Figure 31: Pleiotropy of the major rs644234*T allele located within the ABO gene.



In addition, we studied the gender-gene interactions by using the gender as a possible genetic modifier. We found some interesting sex-specific associations in two of the genes studied (ABO and GNB3). The polymorphism studied within the ABO gene was associated with increased HDL levels in males but not in females. On the other hand, the polymorphism rs5443 within the GNB3 gene was specifically associated in both genders with soluble E-selectin levels, but with an antagonistic effect (Figure 32). It is interesting to note that in the whole population the association of this polymorphism with the sE-selectin levels was not significant, which points out the importance of taking into account the gender for the detection of novel risk biomarkers involved in CVD.

Figure 32: Beta effect of the GNB3 rs5443 T allele on sE-selectin levels in the STANISLAS population. *P-value ≤ 0.05 (Significance level)



By detecting pleiotropy and sex-specific effects we understand better the biological pathways where the gene is involved. These investigations are source of new hypotheses and make easier the development of new drugs, hence improving prevention and personalized medicine of CVD.

Further, we centered our efforts in implementing the knowledge obtained the last three years at the clinical level, with the aim of ameliorating the genetic inputs of personalized medicine. We obtained two results that could be implemented in clinics. First example of clinical application concerns the polymorphism rs2234246 within the TREM-1 gene. This genetic variant, which is increasing the soluble levels of the TREM-1 protein, could be used as a predictor or risk biomarker for different diseases (including CVD). The second result obtained that could be used in genetics of personalized medicine is concerning the IL-6R haplotype rs4845628*T/rs4537545*C. This haplotype may have important value in pharmacogenomic studies given the fact that the IL-6R gene is a target of the monoclonal antibody tocilizumab.

PERSPECTIVES OF OUR RESULTS

A) TREM-1

It would be interesting to use other intermediate phenotypes to clarify some aspects of our results. Firstly, it would be necessary to study if the SNP rs2234246 is affecting in a different way in the different subtypes of monocytes (CD14++CD16-, CD14+CD16+,CD14+CD16++). Also, it would be interesting to analyze if this SNP is affecting to the mRNA levels of the neutrophils, and not only the monocytes, as described previously. Further, ELISA analysis should be performed in L-selectin levels located in the membrane of lymphocytes and monocytes in order to study if the SNP rs2234246 is also affecting the membrane bound L-selectin levels, and not only the soluble levels as we already demonstrated. This would give an even stronger functional value to this SNP and would also strengthen the idea that TREM-1 is acting in the trans-epithelial migration of inflammatory cells to the intima, which would increase its importance in CVD.

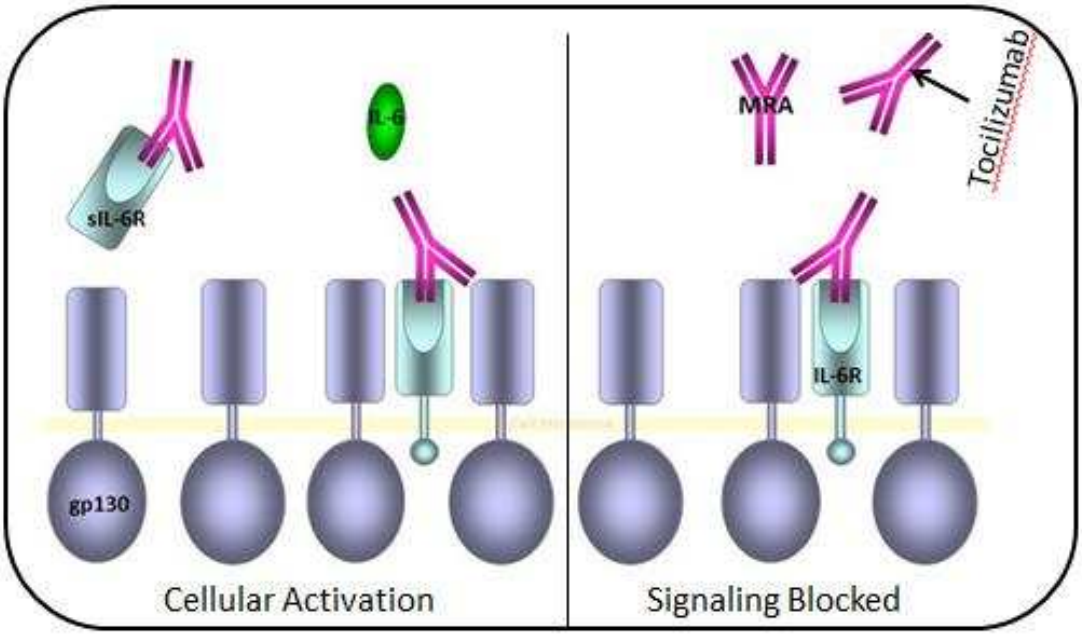
The SNP rs2234246 could be used in personalized medicine. This genetic variant, which is increasing the soluble levels of the TREM-1 protein, could be used as a predictor or risk biomarker for different diseases (including CVD). Indeed, and due to the high potential of this polymorphism, we applied a European patent together with the INOTREM Company, INSERM and University, and we are planning to start Phase I clinical trials in patients suffering from Septic Shock and Acute Myocardial Infarction.

B) IL-6R

The results obtained after studying the IL-6R gene may have important value in pharmacogenomic studies and could be used in genetics of personalized medicine. Indeed, the IL-6R haplotype rs4845628*T/rs4537545*C, is simultaneously increasing the CRP, LDL-C and ApoB levels. The IL-6R gene is a target of the monoclonal antibody tocilizumab (Figure 33), an immunosuppressive drug widely used for the treatment of rheumatoid arthritis, resulting in a reduction of CRP concentrations. One of the side effects of tocilizumab is the increase of LDL-C levels in patients, which could lead to a risk of suffering from ischemic heart disease (22). In order to prevent these side effects, patients carriers of the haplotype

rs4845628*T/rs4537545*C could be treated by analogue drugs or different doses of tocilizumab, which could make the treatment more personalized and hence, more secure and precise.

Figure 33: Blocking of the IL-6R signalling pathway by Tocilizumab



C) ABO

The ABO gene has been associated with a wide variety of human diseases, including CVD. This shows how complex is the effect of this gene and its involvement in different biological pathways. Further studies in bioinformatics and transcriptomics would be needed in order to better understand the mechanistic phenomena of the pleiotropic relationships described and their potential therapeutic translation.

D) TREM-2

It would be necessary to investigate if the SNP rs6918289 located in the TREM-2 gene region is actually explaining a percentage of the variability of the protein levels of TREM-2. Also,

bioinformatic and transcriptomic analyses would help to explain the functionality of the detected SNP in order to strengthen and confirm our results. Also we would have more detailed information about the exact process by which the detected SNP affects the TNF-α levels and the intima media thickness of the femoral artery.

E) GNB3

A replication in an independent study population would strengthen the results obtained. Also it would be interesting to see if there is any hormonal interaction with the GNB3 rs5443 polymorphism on E-selectin. Although a sex-specific effect between the GNB3 rs5443 and sE-selectin levels have been observed in this work, the precise mechanism of this gene on E-selectin molecule need to be clarified by further investigations.

PRESPECTIVES IN GENETICS OF COMPLEX DISEASE

Although huge advancements have been made during the recent years (307-309), the scientific community is far from completely understanding the genetics of complex disease. One of the biggest hopes to undertake this difficult task was the GWAS. However they showed that the reality is even more complicated than previously thought. The famous "missing heritability" problem present in human complex diseases shows the deep complexity of the relationship between genotype and phenotype and the work that still needs to be done to undertake this issue.

Among the experts in the field, several possible solutions have been proposed in order to solve the "missing heritability" problem to clarify the genetics of complex diseases:

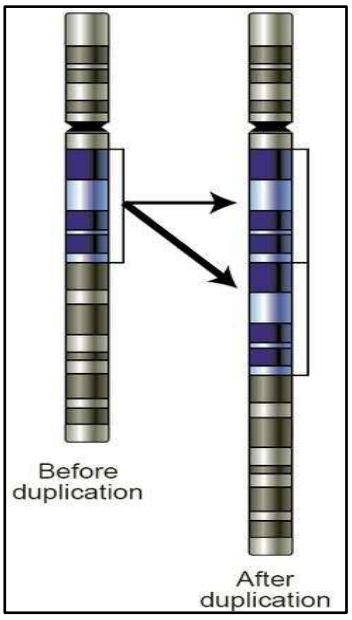
A) We still need to take into account recently discovered "extra layers of genetic regulation" such as the non-coding microRNAs. These mechanisms add a new form of translational regulation to the already complex and highly interconnected genetic architecture of biological and clinical traits (310, 311). To solve part of the missing heritability problem, it will be necessary to understand the genetic variation influencing the expression of these non-coding microRNAs.

B) A source of important discoveries could be achieved by identifying the genetic regions that are not tagged by the typical SNPs. However, in order to detect such variants, it is necessary to perform full-genome sequencing of large samples.

C) One of the fields within genetics that remain poorly studied is the effect of large variants in the genome, such as deletions, duplications and inversions. These variants affect transcription (312) and thus, contribute to several different diseases (313). Although individually rare, they are considered to be common at the population level (314, 315). The study of this landscape could help to improve the comprehension of the human genetic variation.

D) Another field in the human genome that is considered to be understudied is the copy-number polymorphic duplications (CNP) (316) (Figure 34). These regions are composed by hundreds of genes, and recent research indicates that between 4.8-9.5% of the human genome can be classified as copy number variations. These genes are highly variable among individuals and populations, and this variability is increased in genes associated with immunity, environmental interaction and drug detoxification (317). Nevertheless, these genomic regions are considered difficult to study by the current genotyping and sequencing technologies because of their repetitive nature.

Figure 34: Representation of a genomic copy-number polymorphic duplications (CNP) region



E) Transgenerational genetics is, according to some experts, where the real missing heritability is hiding. Some studies in mice proved evidences that genetic variations in actual generations results in phenotypic variations in the incoming generations (318). The already known transgenerational epigenetic inheritance involves histone modifications and DNA methylation (319). Still, novel possibilities are arising, which includes small RNAs (320, 321), DNA methylation mediated by RNA editing enzymes (322), RNA-binding proteins involved in RNA editing (323) as well as RNA-binding proteins involved in microRNA access to their target mRNAs (324). All these mentioned factors are able to control the translation of RNA and are abundant in the gametes (325). Although these novel transgenerational effects have been described in plants, flies and mice (318), it is still unknown if it is functional in humans.

Probably, the answer to missing heritability and hence, to the complex disease genetics will be a mixture of the previously presented ideas. What is clear is that the human genome, and genetics in general is complicated, and we will need a big amount of collaborations and efforts to completely uncover the genotype-phenotype correlations present in complex diseases.

PERSPECTIVES OF PERSONALIZED MEDICINE

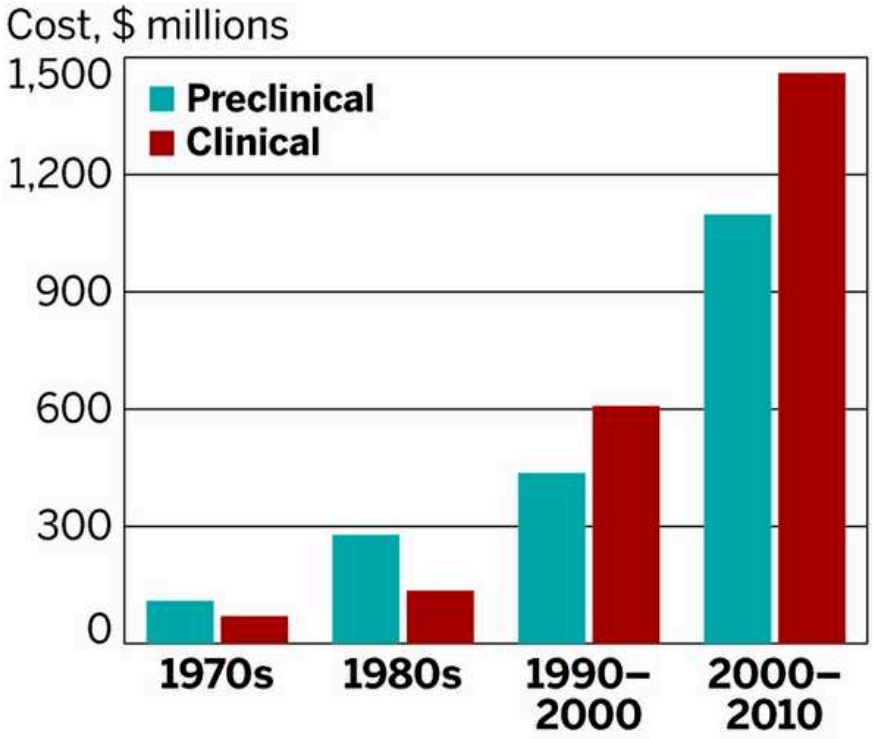
Every year, a higher number of researchers and health clinicians are aware about the importance of a more personalized approach when it comes to complex diseases. Within the personalized medicine, genome-based personalized medicine plays a vital role. Indeed, the risk that each individual have for different diseases is unique and different, and these predispositions are based on the lifestyle and environment as well as on the genome.

Almost half of the times, patients suffering from complex diseases such as asthma, type 2 diabetes and arthritis do not respond to the most commonly prescribed drugs. This shows the heterogeneity in the response to the drugs among different patients, and also makes evident the necessary improvements that still need to be done when it comes to personalized medicine. Thus, it is important to encourage innovative applications of those genomic markers that could improve personalized medicine.

Nowadays we have an enormous amount of data associating genes and polymorphisms to diseases. However, in order to use these markers for the benefit of the patient, many gaps will need to be filled. Unfortunately, the proof of clinical utility of these genetic markers is not enough for pharmacologic targeting and drug development, and more exhaustive investigations need to be done in order to understand the pathways and biochemical processes where these markers are involved.

Another big problem of the personalized genetics is the cost of developing drugs that target genes. During the last years the price of developing new drugs has increased exponentially (Figure 35) and the last estimations show that the cost has more than doubled in the last decade, reaching an astonishing price of 2.5 billion per new drug developed (326). At the same time, genetically targeted therapy is not the most profitable approach for the pharmaceutical companies. On one hand, it would narrow the target population because a percentage of them would be non-responding patients. On the other hand, if the ratio of these non-responding patients reaches a certain limit, many of the new developing drugs would fail to advance phase III trials.

Figure 35: The cost of developing a new drug has exponentially increased the last decades



Source: Tufts Center for the Study of Drug Development

Despite the already explained difficulties, the field of personalized medicine and especially genome-based personalized medicine is evolving rapidly towards the clinical implementation. Publicly available data sets such as pharmGKB (www.pharmgkb.org) are providing the needed updated and reliable source of information that concerns human genetic variation and its impact in drug response. This type of resources are making easier for clinicians and researchers to implement pharmacogenomics knowledge in actual personalized medicine.

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OTHER PUBLICATIONS

Pharmacogenomic challenges in cardiovascular diseases: examples of drugs and considerations for future integration in clinical practice

Jérôme CHATELIN[*, 1,2], Maria G. STATHOPOULOU[*, 1], Alex-Ander ALDASORO ARGUINANO [*, 1], Ting XIE[*, 1], Sophie VISVIKIS-SIEST[*, 1,3]

1. UMR INSERM U1122; Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire (IGE-PCV), Université de Lorraine, Nancy, F-54500, FRANCE.

2. Service Endocrinologie CHU Brabois, Nancy, FRANCE

3. Department of Internal Medicine and Geriatrics, CHU Nancy-Brabois, FRANCE

* Equally contributed authors

Corresponding author:

Dr. Sophie VISVIKIS-SIEST

Director of Research in INSERM

Director of UMR INSERM U1122; IGE-PCV «Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire»

Director of BRC "IGE-PCV"

Université de Lorraine - Faculté de Pharmacie

30, rue Lionnois - 54000 NANCY

Telephone: 00 33 (0)6.07.60.25.69

Email: Sophie.Visvikis-Siest@inserm.fr

Abstract

Introduction

Even if cardiovascular disease (CVD) drugs are supported by high level proofs, the results of CVD treatment present great disparities: there are still patients dying with supposed optimal treatment, patients facing adverse events and CVD remain the primary cause of death in the world. Pharmacogenomics is the basis of personalisation of the treatment able to allow higher medication success rates. In this review, we will present detailed examples of CVD drugs to highlight the complexity of this challenging field and we will discuss novel concepts that should be considered for a fastest integration of pharmacogenomics in clinical practice of CVD.

Areas covered

The complexity of pharmacogenetics and pharmacogenomics of CVD drugs are presented though examples of medications such as statins, with a focus on their effectiveness and adverse effects.

Expert opinion

The application of personalised medicine in the CVD medical practice requires the study of human genome in regards to drugs pharmacokinetics, pharmacodynamics, interactions and tolerance profile. The existing state –of–the–art of CVD drugs gives hopes for a future revolution in the drug development that will maximise cardiovascular patients benefit while decreasing their risks for adverse effects.

Article Highlights box

Coronary heart disease (CHD) remains the first cause of death worldwide

- Cardiovascular treatment has a significant percentage of insufficient efficacy, poor tolerance and compliance
- Predicting the response to therapy while diminishing the side effects is the basis of personalised medicine; pharmacogenomics is leading towards this direction
- The response to CVD therapy and side effects are in the heart of CVD pharmacogenomics and significant progress has been noted.
- The application of pharmacogenomics in the CVD medical practice is facing many methodological, technical, ethical, behavioral and financial issues, while cost-effectiveness is the main prerequisite.
- The consideration of gene × gene × environment interactions and the inclusion of "omics" data in pharmacogenomic studies of CVD drugs will facilitate the generation of reliable results and will promote tailored treatments and new strategies of drug research and development.

Key Words: Cardiovascular diseases, drugs, genome, gene × environment interactions, gene × gene interactions, "omics", personalised medicine, pharmacogenomics

1. Introduction-Existing problems in the efficiency and safety of cardiovascular drugs

Despite primary and secondary cardiovascular prevention and even after an unprecedented fall in cardiac mortality, according to WHO data, coronary heart disease (CHD) remains the first cause of death worldwide, killing 7.2 million subjects per year [1] with an associated cost approximating $865 billion annually [2, 3]. The treatment of cardio-vascular diseases (CVD) includes more than 10 different classes of drugs at the disposition of the clinician in addition to commonly used anti-inflammatory drugs, hormone therapy and antismoking drugs. This large variety of drugs, often used together, is very challenging for the clinical practice, as a wide variability in response variability and eventual toxicity exists along with numerous interactions between drugs. Cardiovascular prevention and treatment are still facing issues of insufficient efficacy, poor tolerance and compliance.

Statins, for example, which are one of the most commonly used class of cholesterol lowering drugs, have multiple actions. They act mainly through 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibition lowering cholesterol synthesis and per se lowering low-density lipoprotein (LDL) [4]. They have also modest actions in lowering triglycerides and raising high-density lipoprotein (HDL) via, respectively, a decrease in triglycerides synthesis in hepatocytes, an enhancement of lipoprotein lipase in adipocytes and an activation of PPAR (Peroxisome Proliferator-Activated Receptor) with a subsequent Apolipoprotein (Apo)-A1 gene induction [5]. Pleiotropic effects of statins have also been associated with cardiovascular protective action as they modulate inflammation, endothelial function and inhibit coagulation [6]. Statins result in a cardiovascular risk reduction close to 25% (in The Long-term Intervention with Pravastatin in Ischaemic Disease study, in the Cholesterol And Recurrent Events study and in Heart Protection Study)[7-9]. In diabetic populations, where atherosclerosis accounts for approximately 80% of deaths, simvastatin was responsible for a 22% risk reduction (in Heart Protection Study), and atorvastatin induced a 32% risk reduction

in primary prevention in Collaborative Atorvastatine Diabetes Study study [10, 11]. However, a large proportion of residual risk remains unexplained from statins. Moreover, statins have adverse effects on muscles: muscle pain, fatigue and weakness [12]. The same patient using statins, may be also using fibrates which, even though they have a triglycerides lowering effect, they have failed in demonstrating a benefic action on cardiovascular risk. [13]. If this patient has also diabetes, he will likely be treated also with metformin, which is the only anti-diabetic drug that meets the severe criteria for cardiovascular prevention [14]. Thus, this patient will be receiving three medications (statins, fibrates and metformin) while there is no way to identify the responsibility of each of them in the demonstration of CVD risk reduction effect or adverse effects. Therefore, the prediction of side effects and the selection of patients who will benefit more from multiple treatments is challenging. This challenge is one of the major subjects of personalised medicine and pharmacogenomics is the research field dealing with this issue.

The European Medicines Agency (EMA) established at an international level, in collaboration with the authorities from USA (Food and Drug Administration), Japan (Pharmaceuticals and Medical Devices Agency), Canada (Health Canada), and EFTA (European Free Trade Association), a definition for pharmacogenomics: the study of variations of DNA and RNA characteristics as related to drug response [15]. Linked with this definition of pharmacogenomics is the definition of pharmacogenetics: a subset of pharmacogenomics defined as the study of variations in DNA sequence as related to drug response [15].

The groups of genes usually involved in pharmacogenomics individual response are [16]:

1) The group of genes involved in the drug pharmacokinetics (absorption, distribution, metabolism, elimination). Drug metabolizing enzymes are at the front of this variability, particularly cytochromes P450 and several transporters. As they are also

metabolizing endogenous substrates, such as steroid hormones or arachidonic derivatives, endobiotic drug interactions should be considered. On the other hand, the metabolites could have interesting properties for CVD. The main cytochromes P450 enzymes involved in the metabolism of CVD drugs are CYP2D6, CYP3A3/A5 and CYP2C. As an example, a list of CVD drugs metabolized by the CYP2C family is presented in table 1. In this list, we can find many sartans, antihypertensive and anti-diabetic drugs, but also clopidogrel. The importance of transporters has been also described and these enzymes are now considered as being active at many cellular surfaces for excreting drugs and metabolites from different classes of CVD drugs. Examples of transporter genes include those encoding for the multi-drug and toxin extrusion proteins 1 and 2 (SLC47A1 and SLC47A2 genes) which are involved in metformin metabolism, the SLCO1B1 gene encoding the Organic Anion Transporting Polypeptide 1B1 (OAT1B1), which regulates hepatocyte uptake of statins, and ABCB1 gene, encoding P-glycoprotein involved in and the metabolism of digoxin [16].

2) The group of genes involved in pharmacodynamic response, essentially those implied in drugs targeting tissues, the blood circulating cells, such as platelet receptors, and cellular enzymes of critical pathways. This last represents a new area for treating hypercholesterolemia patients with antibodies produced against PCSK9, the Proprotein Convertase Subtilisin/kexin type 9, a chaperone protein promoting LDL receptor catabolism. This treatment is an interesting evolution in the CVD treatment; however the side effects need to be studied in details [17, 18].

Exhaustive reviews of studies on pharmacogenomics of CVD drugs have been recently published and contain a large amount of available information concerning drugs and genetic variations in details [16, 17, 19, 20]. In this current work, we aim to present the complexity of

pharmacogenomics of CVD drugs in terms of response to therapy, CVD risk reduction and adverse effects. We are using thus specific examples of drugs that demonstrate multiple sources of considerations from a pharmacogenomics perspective ranging from pleiotropic effects to gene × gene and gene × environment interactions such as statins and clopidogrel. Our search method was based on search in available databases such as Medline using key words such as pharmacogenetics, pharmacogenomics, CVD drugs, adverse effects, CVD adverse effects, statins, clopidogrel. All authors added their findings together and confronted them with these of the others.

2. Pharmacogenetics of CVD drugs

We know that a standard dose of treatment may provoke different consequences: an individual may face a diminished efficacy or even a lack of efficacy but also side effects or toxicity. Significant is also the role of polymorphisms on genes that encode molecules that are targets of drugs, such as the vitamin K epoxide reductase complex subunit 1 (VKORC1 gene) which is the target of warfarin [21]. Many polymorphisms can affect the effect of enzymes responsible for example of drug functionalization (phase I), glucuronidation (phase II) and elimination of the product and its metabolites (phase III). Variability may be also due to a particular physiological state (new born, pregnant women, old age), co-morbidities such as kidney or liver insufficiency and environmental factors such as smoking, nutrition and physical activity. Furthermore, the genetic variations may interact between them (gene × gene) and with the environmental factors mentioned before to further modulate the metabolism and efficiency of a drug. The identification of the specific genetic variants and the validation of their effect and the effect of their interaction will facilitate their use in clinical

practice and the applications of personalised therapies based on specific genetic and clinical background [22].

Examples of clinically relevant polymorphisms in cytochrome P450, transporters genes and phramacogenetic examples of statins are presented below.

2.1. Cytochromes P450

The polymorphisms of the genes of the cytochromes P450, in particular CYP3A4, CYP3A5, CYP2D6, CYP2C9 and CYP2C19 are the most known examples of pharmacogenetic studies in CVD. Among them, CYP2C19 polymorphisms have an important effect on drug activity. CYP2C19 activates clopidogrel by converting it into its active thiol metabolite. CYP2C19*2 and *3 polymorphisms are common loss-of-function variants present in 30% of Caucasian population and 50% of Asian population [23-25]. They are associated with significantly diminished levels of active clopidogrel metabolite, thus a high remaining platelet activity. This finding is clinically relevant and carriers (poor metabolizers of clopidogrel) compared to non-carriers face a higher risk of cardiac events, mainly after percutaneous coronary intervention [26-29]. However, this knowledge hasn't simplified the clinician's prescription. Although there has been a warning from the FDA [30], the lack of proof for alternative drugs superiority has not allowed clear and strong recommendations for genetic screening of the CYP2C19 loss-of-function polymorphisms in CVD patients in the latest guidelines from societies of Cardiology [31, 32]. So, even if clopidogrel/CYP2C19 interaction has reached clinical validity, it has not reached yet clinical utility [33]. Tailored Antiplatelet Therapy Following PCI (TAILOR-PCI) study [34], aims to answer this important question and to determine if genetic testing can identify the best antiplatelet-therapy for poor metabolizers after coronary stenting. This large prospective clinical trial (5270 patients) aims to compare

the risk for major cardiac events, at one year, between a group of patients receiving clopidogrel according to the standard practice (75 mg daily) with retrospective genotyping and two groups of patients with prospective genotyping in which wild type CYP2C19 carriers will receive clopidogrel and hetezygous or homozygous carriers will be assigned to ticagrelor 90 mg twice a day (ticagrelor is a new generation antiplatelet active drug not influenced by CYP2C19 activity). The second endpoint of this study will be to compare the incidence of minor and major bleeding in poor clopidogrel metabolizers under clopidogrel or ticagrelor. Another upcoming study is the non-inferiority trial POPular Genetic Study [35], which aims to compare a genotype-based strategy (ticagrelor in CYP2C19 loss-of-function carriers and clopidogrel in others) against generalized use of ticagrelor or prasugrel in a non-genotyped group in a population of 2700 ST elevation myocardial infarction patients. This study should not only inform about the antithrombotic effect of these alternative evoked treatments in poor metabolizers but also on the associated risk of bleeding. In another study [36], a retrospective comparison of personalised anti-platelet therapy (CYP2C19*2 carriers receiving prasugrel and non-carriers receiving clopidogrel) against empiric ticagrelor therapy has shown a proportion of patients in a validated therapeutic window (after a steady state treatment (≥48h) of antiplatelet therapy as measured by a P2Y12 reaction unit (PRU) >85 and <208) significantly superior in personalised anti-platelet therapy group (50.0% vs 4.1%, $p<0.0001$). This was mainly linked to an increase in low on-treatment reactivity with ticagrelor (95.9% vs 37.3%, $p<0.0001$). According to multivariate analysis, personalised anti-platelet therapy group was the major predictor of achieving PRU values within therapeutic window (odds ratio 20.27; 95% CI: 4.33-94.82, $p=0.0001$)[36].

2.2. Transporters (P-glycoprotein 1)

Digoxin is used in the treatment of tachycardia and atrial fibrillation and has a narrow therapeutic index. Digoxin's absorption, distribution, and elimination are under transporters-mediated processes. P-glycoprotein 1 (permeability glycoprotein, abbreviated as P-gp or Pgp), also known as multidrug resistance protein 1 (MDRD1) or ATP-binding cassette sub-family B member 1 (ABCB1) or cluster of differentiation 243 (CD243), attenuates intestinal absorption and central nervous system distribution of digoxin and participates in its elimination from hepatocytes to bile ducts and from proximal tubule cells to urine [37-39]. The clinically relevant effects of digoxin exposure on safety and efficacy related to P-gp inhibition have resulted in the FDA recommendation to evaluate systematically this transporter mediated non-competitive drug-drug interaction during new drugs developments [40] . For example, due to P-gp non-competitive interaction, verapamil, which may be associated in some circumstances with the treatment of atrial fibrillation, decreases renal tubular elimination of digoxin [37]. A post-mortem analysis of ABCB1 single nucleotide polymorphism (SNP) has revealed that three SNPs (3435C>T, 1236C>T, and 2677G>T) were all positively associated with higher post-mortem levels of digoxin. A gene × gender interaction has been also demonstrated. Female subjects had a more emphatic pattern, suggesting a higher risk of intoxication. These findings are in favor of considering individualized genotyping before digoxin prescription and justify the interest for gender-segregated studies in drugs safety evaluation [41].

2.3 Statins pharmacogenomics and drug efficacy

Pharmacogenomic studies on statins have focused on the efficacy in terms of LDL reduction. The initial efficacy studies were mostly focused on candidate genes involved in lipids abnormalities. Chasman et al [42], assessed 1536 individuals treated with pravastatin 40mg/day for the associations between 148 SNPs within 10 genes related to lipid metabolism

and drug efficacy. Two common and tightly linked SNPs were associated with reduced 24 weeks delivery efficacy of pravastatin. They coded for HMG-CoA reductase. Individuals with a single copy of the minor allele had 22% smaller reduction in total cholesterol and 19% smaller LDL reduction with no difference on HDL levels. Another study from Donnelly et al. [43] identified an effect of a relatively rare SNP in HMGCR gene (gene coding for HMG-CoA reductase) in reducing lipid lowering response in 1601 diabetics patients. Only 3% of these patients carried one minor allele and among them, 51% didn't reach cholesterol target compared to 28% in the major allele homozygous group.

The variation in the apoplipoprotein E gene (APOE) is considered the most reliable genetic determinant for the response to statins and has been widely studied. Ordovas et al.[44] demonstrated that E4 allele was associated with a poorer response to pravastatin and E2 with a greater response compared to E3, in terms of LDL reduction. Same results were found with other statins like simvastatin, fluvastatin and atorvastatin but they were not confirmed in some studies [45-47] . In diabetic populations Donnelly et al.[48] in the Go-DARTS (Genetics of Diabetes and Audit Research Tayside Study) study found an association of APOE genotypes with both baseline and treatment responses. E2 homozygotes achieved lower LDL levels than E4 homozygotes. Minimum LDL was associated linearly with genotype and this association remained even after adjustment for baseline LDL, adherence to treatment, duration, dose, and age. Of importance is the fact that none of the E2/E2 subjects failed to achieve LDL levels goals while only 32% of the E4/E4 reached the therapeutic target. More recently, in a Chilean population it was confirmed that carriers of the E3/E4 genotype presented a lower cholesterol reduction (-18% vs – 29%) compared to genotype E3/E3 in response to atorvastatin therapy [49].

In an analysis of 125 polymorphisms in 61 candidate genes in 386 Chinese patients, the SNP 421C>A in the ATP-binding cassette G2 (ABCG2) gene was highly associated with LDL

response to rosuvastatin ($P=9.2x10^{-7}$), followed by the 18281G>A (V257M) SNP in the flavin-containing monooxygenase 3 (FMO3) gene (P=0.0002), the 1421C>G SNP in the lipoprotein lipase (LPL) gene (P=0.002) and the SNP rs4420638 in the apolipoprotein E/C-I/C-IV/C-II gene cluster (P=0.004). This study also demonstrated a -2.6% LDL lowering answer to rosuvastatin in patients with familial hypercholesterolemia [50]. Replications in other populations are needed to confirm these observations. The analysis of three combined genome-wide association studies (GWAS) in 3932 subjects treated with simvastatin, atorvastatin or pravastatin suggested that a variant in the calmin gene (CLMN) could modulate lipid lowering answer. Although the function of calmin remains unknown, it was demonstrated that CLMN expression was induced by all-trans retinoic acid (atRA), a vitamin A metabolite [51]. This suggests a possible influence of vitamin A status in the answer to statins and a possible gene× environment × treatment interaction. A variant in APOC1, near APOE, was also identified and was associated with variation in the magnitude of LDL lowering effect of the statins [52]. More recently POR*28 SNP (P450 oxidoreductase gene) of the POR enzyme (which transfers electrons from NADPH to CYP450 enzymes including CYP3A which metabolizes atorvastatin) was linked to a reduced effect of atorvastatin in children from families with hypercholesterolemia. The variability induced by this SNP was estimated to cause 8.3% and 7.3% diminished reductions of total cholesterol and LDL [53]. In 2015, a GWAS (1868 individuals of European ancestry from the Pharmacogenomics and Risk of Cardiovascular Disease study) [54] examined 7 subfractions of LDLs and 3 subfractions of IDLs and their response to statin treatment. Among results, four already known loci (SORT1, APOE, LPA and CETP) were strongly associated with lipoprotein subfractions and CETP variants were associated with LDL subfractions but not with total LDL suggesting a potential interest in determining more detailed phenotypes[54].

The gene SLCO1B1 has also some polymorphisms with interest to statin response. In a six years follow-up study, carriers of the rs4149056 C allele had 6.2±1.7mg/dl higher LDL per C allele but were not at higher risk for death/myocardial infarction, while no association was found between rs2306283 and LDL or death/ myocardial infarction [55].

The above-described examples of population pharmacogenetic of CVD support the importance of clinical implementation of pharmacogenetics in the CVD drug development field for personalisation of their efficiency and tolerance.

3. Pharmacogenomics

As already mentioned, statins are very commonly used drugs in CVD. They represent also an excellent example to demonstrate the range of pharmacogenomics considerations in CVD.

3.1 Statins and LDL receptor gene (LDL-R)

Classical Familial hypercholesterolemia (FH) results from gene mutation of LDL-R that decrease by approximately 50% the number of functional LDL receptors in heterozygotes while further decrease is observed in homozygotes. LDL receptors mediate 70% of hepatic LDL uptake. Statins acting through up-regulation of LDL-R expression are consequently relatively ineffective in FH: this is a critical example of genetic influence on a treatment efficacy [56]. While heterozygote FH is not rare (1/500 to 1/200) it is too much under-diagnosed, while the homozygote form remains exceptional, affecting 200 to 400 persons in France [57, 58]. Homozygous Familial Hypercholesterolemia (HoFH) is classified as receptor

negative with 0-2% receptor activity or receptor defective with 2-25% receptor function [59]. In Greek and Italian surveys of HoFH individuals, 71% and 55% were respectively LDL receptor negative [60, 61]. In the HoFH receptor positive Italian subjects, up to 30% residual activity was found, suggesting an increased residual receptor function compensatory mechanism [61]. The standard treatment for homozygote form is LDL apheresis associated with maximum tolerated potent statins, supposing that a minimal LDL receptor function exists, ezetimib and bile acid sequestrants.

3.2 Statins and APOB and PCSK9 genes

Two other types of autosomal dominant FH are due to mutations in APOB and PCSK9 genes. In familial defective APOB FH, the mutated ApoB has a decreased affinity for the LDL receptor with a subsequent LDL-diminished ApoB-mediated hepatic uptake [62]. PCSK9 is synthesized as a soluble zymogene and acts as a proprotein convertase by binding to the epidermal growth-factor-like repeat A domain of the LDL receptor, inducing the destruction of this receptor and the subsequent diminution of LDL hepatic uptake. The first mutation of PCSK9 gene, identified in 2003 in French families, was responsible for an increased PCSK9 activity and hypercholesterolemia [63]. Since then, additional gain-of-function mutations have been identified [64]. Two non-sense (T142X and C679X), loss-of-function mutations, discovered in 2% of African Americans from the Dallas Heart Study, have been associated with a reduction of plasma LDL. These two mutations were rare in European Americans (<0.1%) but associated with 40% lower LDL [65]. In the Atherosclerosis Risk In Communities (ARIC) study, a 15 years follow-up demonstrated a 28% reduction of LDL and 88% reduction of coronary disease in African Americans with non-sense, loss-of-function PCSK9 mutation. In this study, a missense loss-of-function mutation of PCSK9, more

predominant in European Americans, was associated with 15% and 47% reduction of LDL and coronary heart disease. The cardiovascular risk diminution in loss-of-function PCSK9 mutation was more important than attended with LDL lowering per se [66]. This suggests a longtime benefice of low LDL from this PCSK9 mutation. This is of great importance because statins are associated with elevation of PCSK9. The inhibition of HMG-CoA decreases hepatic intracellular cholesterol resulting in increased activity/nuclear translocation of SREBP-2 (sterol regulatory element-binding protein-2), a transcription factor, from the endoplasmic reticulum. Translocation of SREBP-2 into the nucleus not only induces transcription of LDL-R gene with diminution of LDL, IDL (intermediate density lipoprotein) and VLDL (very low density lipoprotein) levels but also PCSK9 mRNA with the opposite effect [67-69]. A meta-analysis from Sahebkar A. et al. [70] revealed a significant increase in plasma PCSK9 concentrations following statin therapy (weighted mean difference: 40.72 ng/ml, 95% CI: 34.79, 46.65, $p<0.001$; I^2: 96.83%). In this meta-analysis, when the studies were stratified according to different types of statins, each of them was associated with PCSK9 elevation: atorvastatin (weighted mean difference: 47.55 ng/ml, 95% CI: 24.87,70.23, $p<0.001$; I^2: 81.68%), simvastatin (weighted mean difference: 58.92 ng/ml, 95% CI: 53.76, 64.09, $p<0.001$; I^2: 0%) and rosuvastatin (weighted mean difference: 26.44 ng/ml, 95% CI: 19.55,33.33, $p<0.001$; I^2: 99.10%). According to lipophilicity, this PCSK9 elevation was verified in both types of statins but was greater with lipophilic (atorvastatin, simvastatin and pitavastatin: weighted mean difference: 50.02 ng/ml, 95% CI: 32.31, 67.73, $p<0.001$; I^2: 88.40%) compared to hydrophilic statins (rosuvastatin and pravastatin: weighted mean difference: 29.15 ng/ml, 95% CI: 22.44, 35.87, $p<0.001$; I^2: 98.72%). These results were persistent along treatment duration. When considering the effect of statin monotherapy versus statin/ezetimibe in the meta-analysis of data from 7 treatments arms, there was no significant difference in terms of PCSK9 level alteration. However, Lakowski et al.[71] showed

significant elevation of plasma PCSK9 after addition of ezetimibe to statin therapy (weighted mean difference: 31.41 ng/ml, 95% CI: 7.86, 54.97, p=0.009; I^2: 50.73%). The interaction between PCSK9 and plasma lipoprotein levels is complex with parallel and reciprocal regulation of the surface LDL receptor and PCSK9 expression, correlation between LDL and PCSK9 levels (since 40% of PCSK9 is bound to LDL) and an association between plasma PCSK9 production and assembly and secretion of triglycerides rich lipoproteins [72]. So a major part of LDL clearance belongs to hepatic cells and is mediated by LDL receptor whose high recycling rate (up to 150 times) suggests that slight changes in LDL receptor availability may provoke important variations in LDL uptake by the liver. The pejorative elevation of PCSK9 with statins could partially the variability of their action in CVD prevention and part of the fact that doubling statin doses only procures 6% LDL lowering effect [73]. The quest for a synergistic action of anti-PCSK9 monoclonal antibodies and statins requires further investigation [69, 74, 75]. Moreover, contrary to the study of Lakowski et al. [71] , one meta-analysis of the effect of ezetimibe addition to statins showed increased PCSK9 levels, which could explain part of the efficacy of this association [70]. This observation is of importance when considering the interest of ezetimibe addition as demonstrated by the IMPROVE-IT study (Improved Reduction of Outcomes: Vytorin Efficacy International Trial) [76] for diminished heart attacks (-13%) and strokes rates (-20%). However, no significant difference in terms of death from CVD was observed [76]. These results opened again the debate on the interest of ezetimib addition to statins, the Higgs particle of lipidologists as named by Einecke D [77]. These possible interactions have to be taken into account when envisaging treating African Americans. Their reported “LDL lower answer” to statins [78]could be accompanied by distinct “PCSK9 answer” when considering their inter relationship. However, the higher frequency of mutations in Niemann-Pick C1-like 1 (NPC1L1) gene, which is the target of

ezetimibe, in this population is causing a higher rate of non-response to ezetimibe therapy [79].

Sterol-regulatory element-binding protein (SREBP)-2 promotes transcription of LDL-R and PCSK9 and women appear protected from hyperlipidemia until menopause. Sterol depletion induces SREBP-2 transcription. Little is known about the role of SREBP-2 in this observation but levels of SREBP-2 were higher in males compared to females in an animal study in rats, and higher in males compared to males treated with oestrogen, suggesting a predominant role of oestrogen [80]. Therefore, gene × environment interactions may explain the different effects of statins, according to sex, gender and menopausal status in women. Also, SREBP-2 interacts with both LDL-R and PCSK9 with opposite effects on LDL uptake underlining the importance of gene × gene interactions, while, hormonal environment with estrogen participates (underlining also the importance of gene × environment) in this complex regulation [80].

These examples of pharmacogenomics of statins indicate that in order to explain the complexity of response to drugs, multiple evidences should be gathered focusing on drugs interactions with genes, drug × drug interactions mediated by genes, gene × gene and gene × environment interactions, which all are related with the resulted response to a specific drug. The same problematic exists for other CVD drugs. The challenging investigation of all these factors is probably the reason why pharmacogenomics results for CVD drugs are still not reliable enough to be integrated into the clinical practice. Nevertheless, the existing results improve the pathophysiological knowledge concerning CVD and promote the development of novel therapeutic targets.

4. Pharmacogenetics and adverse effects

Due to long-term treatments, tolerance and adherence are also very important in CVD treatment. Concerning statins, up to 50% of patients with CVD discontinue statins within one year [81]. One main reason is the development of induced musculo-skeletal side-effects [82]. In clinical practice, as estimated by observational studies, myopathy occurs more often (9-20%) compared to the frequency described in randomized trials (1-5%) [4, 83, 84] . The risk of statin myopathy occurrence depends on the type of statin [85], its dosage and patients-related risk factors (age, co-mormidities, gender, genetics and ethnicity) [86, 87]. Several genetic polymorphisms in various drug transporters, autophagy clearance pathways and enzymes involved in creatine synthesis were associated with statin induced muscular pain [88-92]. The genetic variation in SLCO1B1 (C allele of the rs4149056 SNP), referred to as the *5 variant, causes a V174A substitution in the hepatic transporter protein OATP1B1. It constitutes a risk factor for statin-induced side effects and is responsible for elevated plasma concentrations of statins in a statin specific manner (simvastatin and atorvastatin are most affected, followed by fluvastatin, pravastatin and rosuvastatin) and for parallel clinical muscular effects. Variant *5 is associated with mild reduction of LDL lowering effect. The Clinical Pharmacogenetics Implementation Consortium Guideline for SLCO1B1 and Simvastatin-Induced Myopathy recommend genotyping for this SNP in cases of simvastatin treatment, and selection of other medications in case of adverse effects or low response to treatment (not optimal LDL lowering efficacy) [93]. The G allele of the rs2306283 SNP, referred to as *1B variant, is responsible for a N130D substitution that may increase OATP1B1 function, reduce systemic statin concentrations and the risk of induced myopathy [55].

Apart from CVD medications, there are many drugs with cardiovascular side effects. Among them, anti-VEGF (Vascular Endothelial Growth Factor) therapies are of particular interest. They aim to counteract the complex neo-angiogenesis and inhibit the tumor growth [94]. VEGF and its receptors VEGFR-1, VEGFR-2 and VEGFR-3 are essential survival factors for endothelial cells involved in tumor neo-angiogenesis. Bevacizumab, sunitinib and sorafenib were the first anti-VEGF therapies used in advanced stages of colon, kidney, liver, breast and lung cancers [95-101]. All anti VEGF drugs, whatever their class, are characterized by the same tolerance profile with hypertension and proteinuria issues [95-101]. In clinical trials, criteria for definition of hypertension and proteinuria are based on the NCI-CTCAE classification (Common Terminology Criteria for Adverse Events of the National Cancer Institute) and don't consider international guidelines for the diagnosis and care of hypertension [102]. Therefore, these criteria are used for comparison of adverse events incidence in cancer trials with anti-VEGF therapies. Hypertension is the more frequent adverse event in clinical trials on anti VEGF therapies but its real incidence remains unknown due to the choice of diagnostic criteria. Elevation of blood pressure is constant in the first weeks of treatment regardless of hypertension history of the patient. This elevation of blood pressure often reaches hypertension level or makes blood pressure control more difficult in hypertensive patients. This iatrogenic hypertension is generally controlled with anti-hypertensive treatments and is rarely responsible for drug discontinuation. Its long term consequences haven't been evaluated yet. Rarely this may lead to acute events: malignant hypertension, severe refractory hypertension, reversible posterior leukoencephalopathy. This hypertension is dose dependent [103].

Another frequent complication of anti-VEGF treatment is proteinuria. It may appear with a variable delay and is quasi constantly associated with hypertension. It is also dose-dependent and generally reversible with the interruption of the treatment. According to clinical trials,

proteinuria incidence with bevacizumab varies from 21% to 64%. Generally, it has no consequence on kidney function and on possibility to continue the treatment [104].

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A GWAS study has identified 4 SNPs that explain up to 50% of the inter-individual variability of circulating VEGF levels [105]. These SNPs have been associated with multiple CVD traits including adhesion and inflammation molecules and blood lipids in healthy conditions [106, 107]. Ongoing pharmagenomic studies investigating the role of these polymorphisms in the effectiveness of anti-VEGF treatments and the presence of cardiovascular side effects will allow the personalization of treatment for cancer patients.

These examples demonstrate that polymorphisms may also be associated with adverse effects of CVD drugs directly or through interactions. Apart from that, pharmacogenomics of drugs with CVD cardiovascular should not be neglected.

5. Expert Opinion

Although there are many pharmacogenenetics and pharmacogenomics concerns in CVD treatment and cardiovascular side effects of drugs, they rarely have an application in the clinical practice. Multiple reasons exist for this observation and efforts are needed in order to overcome current obstacles.

First of all, there are few evidences suggesting a significant benefit of the use of stratified therapy based on genome. The notion of personalised medicine must evolve with the evolution of the "-omics" methodologies. The consideration of the important progress in the field of genetics, the integration of gene × gene × environment interactions in the genetic effects analyses, and the complementary and well developed fields of transcriptomics,

proteomics and metabolomics will allow the better understanding of the complex molecular profile of CVD, the multiple interactions that participate in the drugs' metabolism, the medications side effects, thus leading to an efficient tailored therapy. These "omics" research data should be replicated and validated so that they can be reliable enough to justify clinical use.

There is also a need for well-designed large-scale pharmacogenomic studies, taking into account the above, allowing the development of decision-making strategies for personalised treatments. The study of cost-effectiveness of personalised medicine should be demonstrated in order to allow the use of personalised treatments in medical practice. Their acceptance and inclusion in the health care systems is vital. A promising step towards this direction was the Precision Medicine Initiative as promoted by U.S President Barak Obama, himself, in his 2015 speech for the State of the Union. This initiative is focused on personalised medicine and its development in the USA. The Implementing GeNomics In PracTicE (IGNITE) Network had been established aiming to integrate personalised medicine into clinical practice [108]. Implementation of pharmacogenomics has started to take place in several facilities such as the University of Florida Personalized Medicine Program, who is already implementing CYP2C19 and clopidogrel genotyping and the preliminary result was presented at the 2015 American Heart Association Annual meeting.

Furthermore, the application of personalised therapy in clinical practice depends also on the acceptance of their efficacy by the medical doctors and the patients. Pharmacogenetics and Pharmacogenomics should be part of professional education and continuous formation to help clinicians in understanding what it is about, to give them objective evaluation of the state of the art and intellectual tools to consider future development and rise their interest for the necessary clinical implementation trials. Communication strategies like those delivered by scientific societies as ESPT (European Society of Pharmacogenomics and Personalised

Therapy, www.esptnet.eu) [109] should be applied in order to increase the knowledge of the patients and to raise the awareness of the medical staff.

There is also a concern about genetic testing and health privacy rights that puts Pharmacogenetics and Pharmacogenomics in the center of a conflict between very different interests with important ethical and economic consequences.

The cost of the genetic diagnosis might not be a limitation, as new methods faster and cheaper could replace large scale genotyping. Furthermore, the emerging bedside point-of-care genetic assays are expected to reduce the cost of pharamcogenomic testing and facilitate their everyday use in clinical practice [110]. Koelsch et al.[111] have introduced a new "balanced value" business model for personalised medicine, leveraging the emerging opportunities to reduce drug development cost and time for targeted therapies. This model would allow pharmaceutical companies to charge prices for targeted therapy below the likely future thresholds for payers' willingness to pay, at the same time preserving attractive margins for the drug developers. Of course, as previously mentioned, these tests should be analytically and clinically validated before approval [112].

In conclusion, the shift from evidence-based medicine to personalised medicine requires a novel approach taking into account all above mentioned challenges that could influence drugs pharmacokinetics, pharmacodynamics, interactions and tolerance profile. The current progresses on all those fields make us optimists for a near future revolution in the drug-patient maximal benefit goals and achievements. We must be conscious that attempting to efficiently take in charge the care of every patient by a personalised treatment is going to complicate the study of new drugs. The increased number of polymorphisms implied in drugs metabolism and the underlying interactions will inevitably multiply the subgroups of patients to consider (in broad outline up to nearly a patient per group) rendering statistical analysis of efficacy and

tolerance very difficult. We are thus confronted with a revolution in the development of medications.

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Table 1: Cardiovascular drugs metabolized through CYP2C sub-families

Warfarin	Glimepiride	Naproxen
Aceclofenac	Glipizide	Nateglinide
Acenocoumarol	Glyburide	Phenylbutazone
Bupropion	Ibuprofen	Piroxicam
Candesartan	Indapamide	Rosiglitazone
Carvedilol	Indometacine	Suprofen
Celecoxib	Irbesartan	Tenoxicam
Clopidogrel	Irbesartan	Tienilic acid
Diclofenac	Lornoxicam	Tolbutamide
Flurbiprofen	Losartan	Torasemide
Fluvastatin	Mefenamic acide	Valsartan
Glibenclamide	Meloxicam	

Sophie Visvikis-Siest*, Alex-Ander Aldasoro Arguinano, Maria Stathopoulou, Ting Xie, Alexandros Petrelis, Georges Weryha, Philippe Froguel, Peter Meier-Abt, Urs A. Meyer, Vid Mlakar, Marc Ansari, Andreas Papassotiropoulos, Georges Dedoussis, Baishen Pan, Roland P. Bühlmann, Mario Noyer-Weidner, Pierre-Yves Dietrich, Ron Van Schaik, Federico Innocenti, Winfried März, Lynn M. Bekris and Panos Deloukas

8th Santorini Conference: Systems medicine and personalized health and therapy, Santorini, Greece, 3–5 October 2016

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The 8th Santorini Conference was held in Santorini, Greece between the 3rd and 5th October 2016. It was realized in honor of Gérard Siest (link to video) (INSERM U1122; IGE-PCV, University of Lorraine), the President of the seven previous Santorini Conferences, who passed away on 9 April 2016. As in the previous years, it was organized by the INSERM U1122; IGE-PCV (www.u1122.inserm.fr), University of Lorraine research group led by Sophie Visvikis-Siest (Sofia Siest – President of the current and future Santorini Conferences).

***Corresponding author: Dr. Sophie Visvikis-Siest,** INSERM UMR U1122; IGE-PCV, University of Lorraine, 30, rue Lionnois, 54000 Nancy, France, E-mail: sophie.visvikis-siest@inserm.fr
Alex-Ander Aldasoro Arguinano, Maria Stathopoulou, Ting Xie, Alexandros Petrelis and Georges Weryha: INSERM UMR U1122; IGE-PCV, University of Lorraine, Nancy, France
Philippe Froguel: Institut de Biologie de Lille, Lille, France
Peter Meier-Abt, Urs A. Meyer and Andreas Papassotiropoulos: Biozentrum University, Basel, Switzerland
Vid Mlakar, Marc Ansari and Pierre-Yves Dietrich: Hôpital Universitaire, Genève, Switzerland
Georges Dedoussis: Harokopio University, Athens, Greece
Baishen Pan: Medicine Zhongshan Hospital Fudan University, Shanghia, P.R. China
Roland P. Bühlmann: Bühlmann Laboratories AG, Schonenbuch, Switzerland
Mario Noyer-Weidner: Max Planck Institute for Molecular Genetics, Berlin, Germany
Ron Van Schaik: Erasmus MC Rotterdam, Rotterdam, The Netherlands
Federico Innocenti: University of North Carolina, Chapel Hill, NC, USA
Winfried März: SYNLAB Holding Deutschland GmbH, Augsburg, Germany
Lynn M. Bekris: Cleveland Clinic, Lerner Research Institute, Genomic Medicine, Cleveland, USA
Panos Deloukas: Queen Mary University of London, London, UK

The conference was held under the sponsorship of different international organizations (Gold: Bühlmann laboratories, Randox, Roche Diagnostics, ThermoFisher Scientific, Zinfandel Pharmaceutical – Silver: Siemens, Servier, Agena Bioscience, Synlab Akademie, DiaSys – Bronze: Metabolon, AB SCIEX–Others: Opusthree, Mastiha Growers Association), the BANQUE POPULAIRE and with the participation of: The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), the University of Lorraine, the Institut National de la Santé et de la Recherche Médicale (INSERM) and the European Commission. It was under the hospices of IFCC, European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) and European Society of Pharmacogenomics and Theranostics (ESPT).

The scientific program consisted of nine sessions, one round table and five satellite meetings, realized in close collaboration with the ESPT and the H2020 Marie Skłodowska-Curie Actions (MSCA) – Research and Innovation Staff Exchange (RISE) – 2015 MAST4HEALTH Project (4 from ESPT working groups and 1 from MAST4HEALTH Project). In 5 specific poster sessions, 43 posters grouped in 2 thematics have been presented: Group A: Omics, environment and chronic diseases and Group B: Pharmacogenomics. Two 'Gérard Siest' awards have been given to the two best posters, one granted by the University of Lorraine and one by the IFCC. The conference also hosted the 3rd meeting of the VEGF Consortium (www.vegfconsortium.org), which aims to promote the research projects dedicated to the use of vascular endothelial growth factor (VEGF) '-omics' as stratified medicine's biomarkers of chronic diseases. Some 140 participants, coming from 33 countries from all around the world, had the opportunity to attend the presentations of 34 speakers on the following topics:

– First day: Systems Medicine and three specific pharmacogenomics and clinical prospective sessions

– Second day: Phenotypes and environment effects, particularly nutrition, important for the study of physiological variations and chronic diseases
– Third day: Personalized therapy and the use of the new genomic biomarkers. The recent introduction of new drugs and development of biological pathways.

Here we present a summary of the presentations and the key messages of the 3 days of the conference.

From systems biology to systems medicine

Chairs: Sofia Siest, Nancy, France/Philippe Froguel, Lille, France

Sofia Siest (Nancy, France) had the first word to give an introduction to the 8th Santorini Conference. She gave a brief summary about how this colloquium has evolved in the last years, reminding that all these processes would not have been possible without the talent and hard work of Pr. Gerard Siest. Throughout 14 years, 8 "Santorini Conferences" welcomed a world of passion for science, specifically Personalized Medicine sessions on Genetics and Pharmacogenomics of risk factors and chronic diseases [1–4]. Over the years, the Santorini Conferences became one of the most important conferences on genetic predisposition to health, diseases, response to drugs and environment.

The debate about how important it is to develop a personalized treatment in which therapy is optimized and costs controlled was held by **Faiez Zannad** (Nancy, France) using heart failure as an example of diseases. The standard medical treatment for heart failure is complex and there is an increasing interest in detecting cardiovascular biomarkers for a better clinical application. He described the HOMAGE project, an EU FP7 program (http://www.homage-hf.eu/) [5] where the goal is to identify omics-based biomarkers that can detect pathological processes predictive of the development of heart failure to allow early mechanistically driven therapeutic interventions for preventing heart failure.

Finishing with the systems biology to systems medicine session, we had **Andreas Papassotiropoulos** (Basel, Switzerland). He introduced us into the wide field of epigenetic modifications and more specifically, into the DNA methylation undergoing with age [6]. Methylation regulates imprinting, chromosomal inactivation and gene expression. The existing results show that age is the most potent factor correlated with the global DNA methylation. These data can be used to identify the hidden molecular mechanisms of age-related traits relevant to health and disease. Interestingly, age-related methylation loci are within regulatory regions of genes closely related with Alzheimer's disease (AD) and cancer. Thus, the decomposition of blood methylome-wide patterns bears considerable potential for the study of brain-related physiological and pathological traits.

Systems pharmacogenomics and mechanisms of drug action

Chairs: Peter Meier-Abt, Basel, Switzerland/Urs A. Meyer, Basel, Switzerland

John Ryals (Metabolon, Raleigh, USA) reminded us that to be able to advance in precision medicine, it is essential to identify response biomarkers and to precisely define drug action. Although gene sequencing has yielded massive quantities of data, which have resulted in many important insights, it has been difficult to discern actionable signals from it. One way to go through the complexity of the data is by using metabolomics, able to precisely measure 2000 molecules in plasma. Metabolomics provides a real-time assessment of the phenotype, helps to unravel complex traits and to detect meaningful signals within the genome [7]. Novel technological innovations enable metabolomics to deliver signatures of disease and provide an integral tool for assessing mechanisms of drug action, thus expanding the boundaries of precision medicine and systems pharmacogenomics.

Pablo Villoslada (Barcelona, Spain) followed with a presentation about multiple sclerosis (MS), a complex disease where the effect that the combined therapies will have at the clinical level is difficult to predict [8]. In order to predict the effect of combination therapies, the group of Pablo Villoslada has developed the CombiMS EU project, a systems medicine approach to (i) characterize the signaling pathways that mediate MS and (ii) predict new combination therapies based on logic networks simulations. Measurements of the phosphorylation of key proteins involved in MS in 150 patients and 50 controls were analyzed. These phospho-proteins were used to identify the single active network that best fitted the data for each patient. Finally, an approach was proposed for predicting combination therapy based on network topology. All this work can be used for developing combination therapies for other complex diseases.

Continuing within the drug actions field, **Federico Innocenti** (Chapel Hill, NC, USA) discussed the efficacy and toxicity of VEGF inhibitors in cancer patients [9].

Approved in 2004, the first anti-VEGF drug, bevacizumab, is widely used in combination with chemotherapy in a large variety of tumor types. Despite the survival advantage, its use still faces a number of barriers, including significant toxic side effects. The failure to identify clinically useful biomarkers that can consistently predict clinical efficacy of this agent and its safety profile has been a significant hurdle for the use of bevacizumab. It is important to make predictive tools available to physicians to identify those patients who are unlikely to benefit from bevacizumab so that the associated toxicities could be also avoided. Dr. Innocenti has presented studies using germline genomics in a large series of cancer patients to discover the genetic determinants of efficacy and toxicity of VEGF inhibitors.

Pharmacogenomics, immunotherapy and onco-hematology

Chairs: Marc Ansari, Geneva, Switzerland/Maja Krajinovic, Montreal, Canada

Marc Ansari (Geneva, Switzerland) opened the session with the talk on Hematopoietic Stem Cell Transplantation (HSCT) for pediatric patients. He began the lecture by introducing basic data and overview of transplantation. Next, basic decision-making process before transplantation and presentation of data on survival were discussed in order to present the problematics of side-effects such as veno-oclusive disease, graft versus host disease, hemorrhagic cystitis and infection associated with the drugs (busulfan and cyclophosphamide) used for conditioning regimen. The lecture continued with the presentation of busulfan and clinical data on mortality and frequency of adverse side-effects followed by presentation of biochemistry and usage of candidate gene approach to identify association between glutathione transferases genes (*GST*s) and toxicity, mortality and busulfan [10]. A detailed presentation of the results obtained on glutathione S-transferase Mu 1 (*GSTM1*), glutathione S-transferase P (*GSTP1*) and glutathione S-transferase A1 (*GSTA1*) genes using *in vitro* approach as well as gene association studies was given. The lecture closed by discussing the importance of replication and the need of prospective randomized trials. Professor Ansari presented the efforts taken in order to validate current results. He presented on behalf of European Group for Blood and Marrow Transplantation (EBMT) and ESPT an outline of a large ongoing prospective trial for children with an acute lymphoblastic leukemia receiving a HSCT that involves more than 20 countries with different ethnicity to validate the results.

The second speaker of the session was **Pierre-Yves Dietrich** (Geneva, Switzerland). In the beginning of his talk, he outlined the evidence for immune system involvement in the development of cancer, followed by a review of current understanding of T-cell biology and how these cells mediate the killing of cancer cells [11, 12]. Next, he outlined current understanding of cancer defense mechanism against immune system through inhibition of interactions, excretion of soluble mediators, modulation of cell response and significant changes of local microenvironment, resulting in suppression or modulation of the immune system activity. The lecture continued with detailed discussion of different options for immunotherapy against cancer: therapeutic vaccines, chimeric antigen receptors (CAR) T-cells, inductions of changes to tumor environment and immune-editing. During the lecture special weightage was given to CAR T-cells and different generations of their development with focus on advantages and problematic aspects that need further attention before implementation in clinical practice. The talk was concluded with discussion of other immune system modulators already used in clinical practice or under investigation in clinical trials.

Aurore Perrot (Nancy, France) presented a talk on multiple myeloma targets [13]. She began her talk by reviewing the clinical features of multiple myeloma. She continued with the discussion of oncogenesis and most frequent genetic changes occurring during the development of multiple myeloma. Next, she outlined and described in detail current major targets for drugs: proteasome, surface/receptor molecules, modulation of immune system, epigenetic modulation of oncogene or tumor-suppressor gene and immune checkpoint inhibitors. Most extensive discussion was dedicated to drugs currently used in clinics or being tested in clinical trials with special focus on daratumumab, elotuzumab, histone deacetylase (HDAC) inhibitors and nivolumab. She concluded by stressing the importance of genetic abnormalities associated with the choice of drug used for treatment and the challenges for research in the future.

Maja Krajinovic (Montreal, Canada), presented a lecture on pharmacogenomics of leukemia [14]. During the introduction, she presented general overview of leukemia, its treatment, problems associated with short- and long-term toxicities and differences in individual response to the drugs used. The talk continued with the presentation of a study where associations between methotrexate, thymidylate synthase, relapse and events-free survival were identified. The lecture continued with presentation

of next-generation sequencing technology and opportunities in the field of pharmacogenetics and presentation of her group's recent results. During the second part of the talk, genes associated with asparaginase treatment (MYBBP1A) and allergies, pancreatitis and thrombosis were discussed. Next, results on IL16 association with pancreatitis and BAHD1 association with vincristine, events-free survival and neurotoxicity were presented. The lecture concluded with a discussion of future direction where special attention to basic functional and *in vivo* studies should be given followed by retrospective and prospective clinical trial.

Genomics and proteomics of Alzheimer's disease

Chairs: Andreas Papassotiropoulos, Basel, Switzerland

The first presentation was given by **Lynn M. Bekris** (Cleveland, USA) about genetic variations within the apolipoprotein E (*APOE*) locus and their associations with late-onset AD risk [15, 16]. To explore whether *APOE* locus *cis*-regulatory elements might contribute to regional gene regulation, Bekris et al. produced regulatory region reporter constructs containing haplotypes of *APOE* locus promoters for *APOE*, *APOC1* and *TOMM40* as well as for other potential enhancers. Results demonstrate that multiple *APOE* locus *cis*-elements influence both *APOE* and *TOMM40* promoter activity, suggesting a complex regulatory structure. These results have important implications for AD therapeutic strategies that focus on targeting *APOE* expression.

Erich E. Wanker (Berlin, Germany) continued the session with a presentation on the protein misfolding diseases such as AD, Parkinson's disease and Huntington's disease, all of them characterized by the accumulation of insoluble protein aggregates in patient's brain. In order to better understand the exact nature of pathogenic aggregates and their mechanisms of toxicity in cells, Wanker et al. have developed a large interactome network, where they added multiple proteins involved in different neurodegenerative diseases [17, 18]. The use of this network in combination with various OMICs approaches, aid them to identify "neurodegenerative disease modules." These disease modules highlighted proteins that are abnormally aggregated in brains of AD patients, suggesting that interactome maps are valuable resources, which enable the elucidation of common disease mechanisms.

Continuing with the AD, **Ellen Umlauf** (Vienna, Austria) presented a case-control study, which included healthy individuals, patients with mild cognitive impairment (MCI) and late-onset AD patients. The goal of this study was to identify additional single nucleotide polymorphisms (SNP) candidates associated with MCI. The results obtained after an additive logistic regression showed novel genetic markers linked to the MCI [19]. They also emphasized the importance of carefully characterized controls in addition to well-diagnosed patients in case-control studies.

Omics studies of human phenotypes and environment

Chairs: Georges Dedoussis, Athens, Greece/Baishen Pan, Shanghai, China

Panos Deloukas (London, UK) focused on the genome-wide association studies (GWAS), which have identified several associations for coronary artery disease (CAD) explaining 15% of its heritability. Most of these signals are driven by common variants with low-effect associations. However, such variants seem to contribute minimally to the genetic architecture of CAD. The UK Biobank was established to improve understanding of the causes of common diseases including CAD and has completed the recruitment of 502,713 individuals [20]. Based on their NHS records and national registries, they identified 10,801 CAD cases and undertook an association analysis. Based on the initial results, there are several new CAD risk loci, which are, however, once again driven by common variants.

Robert Barouki (Paris, France), described the concept of exposome, which aims to integrate all environmental exposures over the life time [21]. This ambitious concept aims to be complemented with genome approaches. There are several different exposome projects, each one focused in different exposure fields, such as air pollution effects, water contaminants, early life exposure, etc. The different exposome projects in Europe and in the United states were briefly discussed.

Amalia Gastaldelli (Pisa, Italy) presented the importance of the interaction between the phenotype and environment in the non-alcoholic fatty liver disease (NAFLD) [22]. NAFLD is a metabolic disease, often associated with hepatic and systemic inflammation, insulin resistance and obesity. The -omics techniques including genomics, transcriptomics, epigenomics, metabolomics, exposomics and foodomics are currently used in clinical trials to identify mechanisms and risk factors.

Closing the session, **Laurent Becquemont** (Le Kremlin Bicêtre, France) made an introduction on the

human plasma-metabolome. This is an interesting project including 800 healthy volunteers, where the aim is to define the "normal" levels of 185 targeted plasma metabolites [23]. They identified total blood cholesterol, gender and age as the main components explaining the variability of the studied human metabolome. This study provides an essential baseline to define the normal metabolome profile and the main sources of variation.

Nutrition and metabolic health

Chairs: Roland P. Bühlmann, Basel, Switzerland/Mario Noyer-Weidner, Berlin, Germany

The introduction to this session was made by **Philippe Froguel** (Lille, France), who spoke about obesity, a genetic trait with 70% heritability [24]. GWAS have identified more than 100 loci increasing BMI and mutations in genes mainly part of the regulation of food intake have been found to cause monogenic obesity. There are evidences that different parts of the brain are highly expressing obesity genes, suggesting a role in food intake behavior. Genes involved in nutrient processing and/or on gut microbiote composition are also involved in obesity and metabolism. Finally, system biology approaches suggest that -omics data may help predict effects of nutrients on metabolism, thus opening new directions towards personalized nutrition.

In his lecture, **George Dedoussis** (Athens, Greece) presented a different approach for the management of NAFLD. Instead of using pharmacological means for managing the disease, a dietary regimen with bioactive phytochemicals from fruits, vegetables and plants or their products was proposed [25]. Fifty five patients with NAFLD were enrolled in a two isocaloric dietary treatments for 24 weeks. Anthropometric, NAFLD Fibrosis score and biochemical tests were conducted pre- and post-intervention. The results showed a significant improvement of the patients enrolled in the study. He also described the MAST4HEALTH program, which aims to explore the effect of Mastiha, a natural product of Greece, which was shown to possess antioxidant/anti-inflammatory and lipid lowering properties.

Pharmacogenomics and personalized/stratified therapy

Chairs: Pierre-Yves Dietrich, Geneva, Switzerland/Ron Van Schaik, Rotterdam, The Netherlands

Maurizio Simmaco (Rome, Italy) started the session by presenting a novel predictive tool for 5FU drug toxicity and efficacy (5-fluorouracil) [26]. This chemotherapeutic agent is widely used and life-threatening side-effects can arise in as high as 30% of the patients. One of the factors affecting the toxicity is the dihydropyrimidine dehydrogenase (*DPYD*) gene polymorphisms, associated with low activity of the DPD enzyme. Although these polymorphisms are accepted as a good predictive test to recognize patient at risk of severe toxicity, they can identify only a small fraction of those patients. Because of this, the ex-vivo assay, called 5FUDR, has been developed, which can identify an increased fraction of patients who developed severe toxicity compared to *DPYD* genotyping.

Markus Paulmichl (Salzburg, Austria) discussed the difficulties of the implementation of pharmacogenomics moving from "diagnose and treat" to "predict and pre-empt" [27]. The implementation conundrum was presented and steps for solutions of the difficulties were proposed including amelioration of knowledge and dealing with analytical challenges. Examples of analytical challenges were presented for CYP2D6 and for the development of anticoagulant prescription.

Michael Marschler (Prahealthsciences, Mannheim, Germany), in his lecture introduced us on the influence of pharmacogenomics on pharmacovigilance activities (not published results). Due to gene-environment interactions there exists large variability in responses to drug therapy. Thus, serious adverse drug reactions can happen in specific sub-populations who may have different sensitivity to medicinal products. Because of this, for the preparation of risk management plans it is essential to consider the potential risk of genomic variations and identify risk minimizations measures.

Robin Everts (San Diego, USA) did an introduction on the current pharmacogenomics research on the absorption, distribution, metabolism and excretion properties of drugs. Although the research in this field is increasing rapidly, the pharmacogenetics-related allele's standardization is lacking. The presentation was focused on the contribution that Agena Bioscience can provide through examples of participation in the Genetic Testing Reference Materials Coordination Program [28].

To finish the session, **Tiago Nava** (Montréal, Canada) gave a presentation about the study of *GSTA1* genetic variants and its importance in the evaluation of busulfan (Bu) first dose in children [29]. The busulfan is a key component of conditioning before HSCT in children. They aim to evaluate the role of *GSTA1* genotyping on performance of different models in predicting Bu first dose. For this, they enrolled 129 patients who underwent HSCT after

Bu containing conditioning was included. They demonstrated that *GSTA1* haplotypes clearly interfere in performance of dose prediction models in children, proposing that the development of new population PK study including *GSTA1* haplotypes are necessary to a more evenly distributed exposure among pediatric patients.

Genomic biomarkers and management of metabolic and inflammatory diseases

Chairs: Federico Innocenti, Chapel Hill, USA/Winfried Marz, Augsburg, Germany

Behrooz Z. Alizadeh (Groningen, The Netherlands) started the session with a presentation on C-reactive protein, as an example of biomarkers with causal associations and he argued on its importance in classification of individuals into homogenous groups for diseases/risk factors (not published results). These results are essential in population-based medicine, where careful assessment of the validity and causality of biomarkers becomes important, when critical decision beyond prediction for an individual or a patient, intervention and management will be made.

M. Pilar Francino (Valencia, Spain) continued the session with a presentation about the microbial groups present in the human gut and their contribution to several basic physiological functions, including nutrition, defense against pathogens and metabolic and immune homeostasis [30]. Consequently, disturbances in the microbiota can result in several metabolic health problems. During this presentation, M. Pilar Francino reviewed some of the complex relationships between gut microbiota alterations and metabolic health. She showed us the high importance that the microbiota has in the regulation of host metabolism, mainly in relation to energy homeostasis and adiposity. Thus, concluding that personalized medicine will need to take into account the composition and function of an individual's gut microbiota in order to better prevent, diagnose and treat metabolic disorders.

Steffen Gay (Zurich, Switzerland) presented the field of epigenetic modifications in inflammation in general and in particular in rheumatoid arthritis [31]. The interplay of epigenetics is best illustrated by the involvement of multiple regulatory biological processes, such as acetylation, methylation, phosphorylation, sumoylation and noncoding RNAs, including microRNAs (miR) and long noncoding RNAs (lncRNA). He presented results of his group investigating all these epigenetic processes and how they interact. One of their most recent finding has been the observation that synovial fibroblasts differ in their phenotype depending from the localization in the body through the differential expression of miRs and lncRNAs. These findings are of fundamental importance for the homing of immune cells in health and disease.

In the following presentation, **Blandine Comte** (St. Genes-Champannelle, France) presented their studies on the metabolic syndrome (MetS), which relates largely to increasing obesity and sedentary lifestyle but also to early metabolic life events (not published results). In order to identify predictive biomarkers of evolution toward MetS and to bring new knowledge about this pathological state, Comte et al. used an integrative multi -omics approach in a nested case-control study. They obtained numerous results, statistically identifying 93 discriminant metabolites and 47 proteins between MetS cases and controls. Moreover, the multi -omics approach improved performance and robustness of the prediction and correlation analyses with other data contributed to better understand the role of these biomarkers in the pathological processes and therefore to evaluate their potential clinical value.

The finishing lecture of the session was given by **Alexander V. Kryukov** (Moscow, Russian Federation), who spoke about the apixaban, an oral nonvitamin K anticoagulant, which is a substrate for P-glycoprotein, encoded by ATP-binding cassette sub-family B member 1 (*ABCB1*) gene and the polymorphism C3435T within this gene, which is correlated with the altered expression levels of P-glycoprotein (not published results). He postulated that the P-glycoprotein may influence the pharmacokinetic parameters of apixaban. The plasma concentration monitoring of nonvitamin K anticoagulants could improve safety. Thus, data about genetic factors altering pharmacokinetics of apixaban would help to develop algorithms for anticoagulant therapy personalization. The goal of this work was to determine if polymorphisms within *ABCB1* are associated with apixaban peak concentration in patients with acute cardioembolic stroke and atrial fibrillation. However, they did not find association between *ABCB1* C3435T polymorphism and apixaban peak concentration.

New biomarkers and companion diagnostics

Chairs: Lynn Bekris, Cleveland, USA/Panos Deloukas, London, UK

Starting the last of the sessions, **Tomris Ozben** (Antalya, Turkey) presented the potential use of the

cell-free DNA (cfDNA) in the prognostic assessment of different solid malignancies [32]. In this study, the quality and quantity of cfDNA were assessed, furthermore cancer-specific DNA mutations as prognostic biomarkers in prostate cancer patients were tested. The results showed that patients with high cfDNA concentration at baseline had worse disease-free time and overall survival. Thus, they concluded that cfDNA detection can be used as a prognostic and predictive tool for stratification, clinical management and follow-up of prostate cancer patients.

Klaus Lindpaintner (Waltham, USA) made an introduction to the Population Resources and the requirements for Advancing Health Care [33]. The maturation of powerful technology platforms allows rapid and comprehensive mapping of genetic variants and as well as of other biomarkers, the promise of precision healthcare is becoming increasingly tangible. However, to generate the requisite information resources, a new and challenging scale of data ecosystems will be required that puts major demands on operators, users and investors. Importantly, as genetic and genomic assays are becoming commoditized, the focus is once again shifting towards detailed and sophisticated phenotyping and the exploration and inclusion of real-world data.

In his lecture, **Georges Weryha** (Nancy, France) was focused on osteoporosis. After many extensive programs of fundamental and clinical research, nowadays there are efficient pharmacological treatments for bone frailty. Indeed, a huge amount of clinical data demonstrates the efficiency of osteoporosis treatments [34]. GWAS and whole-exome sequencing have identified genetic determinants of monogenic and complex conditions including osteoporosis and bone mass abnormalities. The insight provided by genetic studies is serving the identification of predictive biomarkers, redefining disease, response of treatment and discovery of new therapeutic targets for skeletal disorders. Osteoporosis therapies fully belong to the new field of genomics and predictive medicine.

Satellite meetings

ESPT WORKING GROUPS ON:

Pediatric individualized treatment in oncology hematology

This group has produced a first review/position/recommendation paper (International Journal of Molecular Science) on behalf of ESPT, published in order to help clinicians to understand the role of pharmacogenomics today in pediatric oncology. In this meeting sub groups and specific recommendations/review papers have been developed.

Curriculae and education for pharmacogenomics and personalized medicine

The working group discussed about education that is necessary for the implementation of pharmacogenomics and personalized medicine, challenges and plans that can be applied.

Endiobiotic and drug interactions

The working group discussed about the necessity of clinical guidelines, of the collaboration with Drug Regulatory Agencies and the issue of translating the German Guidelines. Studies on different aspects of pharmacogenomics implementation were proposed along with economical evaluation and training.

Transporter of drugs and metabolites

The working group discussed in detail the issues of drug transporters in cancer resistance and the clinical relevance of pharmacogenetics of drug transporters as well as general aspect of pharmacogenomics of transporters in drug treatment.

H2020 MSCA-RISE-2015 (MAST4HEALTH PROJECT)

The members of the MAST4HEALTH project discussed about practical issues in NAFLD/NASH diagnosis and management and presented the interventional study with a natural product to implement safety and efficacy, which is designed by this project. The progress of the project and the next steps were presented.

Round table

The future of personalized medicine, systems medicine and systems pharmacology: how to translate the big data for the clinicians and personalized therapy.

Discussion leaders: Sofia Siest, Nancy, France/Maurizio Simmaco, Rome, Italy

At the closing of the Conference, this round table was based on all previous sessions and thematics discussed and initiated an open discussion on the challenges of translation of big data in the clinical practice. Participants agreed that this a crucial issue for the implementation of personalized medicine and identified the domains that need to be reinforced so as to overcome the weak points of the

available strategies: education of clinicians and regulatory bodies agents, informatics tools and software that facilitate the translation of knowledge into clinical decision, translational research along with the progress of industrial products to support both research and clinical practice.

Conclusions

In conclusion, this conference raised major questions:

- Can genetic screening help identify individuals at greatest risk for cardio-metabolic diseases and cancer?
- What is the greatest clinical need with regard to diagnosis, prediction and patient stratification for these pathologies and how this is/can be addressed?
- Does a genetic risk score identify patients at highest risk and is its use justified in clinical practice?
- What are the challenges of the current clinical trials?
- What about comorbidities with aging?
- What is the impact of pharmacogenomics on:
- Deliverance of more predictable responses to drug therapy
- Minimization of the occurrence and severity of adverse drug reactions
- Conduction of more cost-effective clinical trials
- Drug discovery and the drug development process
- Do the existing diagnostic tools answer the needs of pharmacogenomics?

These questions will be addressed in the next Santorini Conference (santoriniconference.org), which will take place from 30 September to 3 October 2018.

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A transnational collaborative network dedicated to the study and applications of the Vascular Endothelial Growth Factor–A in medical practice: The VEGF Consortium

Short title: The VEGF Consortium

Authors

Maria G. Stathopoulou[1], Ting Xie[1], Daniela Ruggiero[2], Jerome Chatelin[1], Marc Rancier[1], George Weryha[1], Mary Jo Kurth[3], Alex-Ander Aldasoro Arguinano[1], Vesna Gorenjak[1], Alexandros M. Petrelis[1], Georges Dagher[4], George Dedoussis[5], Panagiotis Deloukas[6], John Lamont[3], Janja Marc[7], Maurizio Simmaco[8], Ron H.N. van Schaik[9], Federico Innocenti[10], Jean-Louis Merlin[11], Jochen Schneider[12], Behrooz Ziad Alizadeh[13], Marina Ciullo[2], Sudha Seshadri[14], Sophie Visvikis-Siest[1] - The VEGF Consortium

1. UMR INSERM U1122; IGE-PCV ‘‘Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire’’, Université de Lorraine, Nancy, France
2. Institute of Genetics and Biophysics, National Research Council of Italy, Naples, Italy.
3. Randox Laboratories Ltd, Crumlin, UK.
4. Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) / INSERM US 13, BIOBANQUES.
5. Department of Nutrition Dietetics, Harokopio University of Athens, Athens, Greece
6. Queen Mary University of London, London, UK
7. University of Ljubljana, Faculty of Pharmacy,
8. Sant’Andrea Hospital - Sapienza University of Rome, Italy

9. European Society of Pharmacogenomics and Personalised Therapy (ESPT)
10. University of North Carolina at Chapel Hill, NC, USA
11. Cancerology Institute of Lorraine, Vandoeuvre-les-Nancy, France
12. University of Luxembourg, Luxembourg
13. Department of Epidemiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
14. Department of Neurology, Boston University School of Medicine, Boston, Massachusetts, USA.

Corresponding author

Dr. Sophie VISVIKIS-SIEST

UMR INSERM U1122; IGE-PCV

Université de Lorraine

30 Rue Lionnois, 54000 Nancy

Tél : + 33 (0)6 07 60 25 69

Fax : + 33 (0)3 83 32 13 22

E-mail : sophie.visvikis-siest@inserm.fr

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Abstract

Background: The study of biomarkers with important roles in multiple pathways for the development of chronic diseases is an essential component for the implementation of personalised medicine in clinical practice. Vascular endothelial growth factor-A (VEGF-A), a key molecule of angiogenesis, represents a biomarker with such properties and potential. The Vascular endothelial growth factor European Genomic Federation – VEGF Consortium is a transnational collaborative network dedicated to large integrative and multidisciplinary genomic studies of the VEGF-A in order to generate applicable knowledge for medical practice.

Methods: The Consortium consists of 14 Partners from 13 institutions in Europe and the USA. It comprises 11 working groups. Prospective, longitudinal, family-based or population-based cohorts of healthy individuals and patients and case-controls studies are in the core of the Consortium. The methodological aim of the Consortium is to follow a strategy of "systems biology" by integrating a variety of experimental, theoretical and computational methodologies for the achievement of its goals.

Results: Genetic variants that explain more than half of the genetic variability of VEGF-A levels have been identified, while links between these variants have been established with a variety of chronic diseases intermediate phenotypes. Important findings have also been identified concerning the expression isoforms of VEGF-A gene.

Discussion: The VEGF Consortium is a novel Consortium with an innovating structure and original goals. It is expected to provide significant advances in the study and management of chronic diseases, and translation of the scientific information in clinical practice through personalised medicine.

Keywords: VEGF-A, collaborative network, multidisciplinary genomic studies, personalised medicine

Introduction

Chronic diseases represent a major health and development challenge with devastating social, financial and public health impact. They are characterized by complex pathophysiology and regulation processes and are expressed in diverse outcomes and prognosis; while a variety of environmental risk factors play a significant role in their development [1]. The biggest challenge in the fight against chronic diseases is to improve the disease risk prediction, prevention and therapy, fields which are the main targets of personalised medicine. Personalised medicine's objective is the individualisation of disease prediction, prevention, diagnosis, prognosis and therapy. This individualisation is becoming more and more feasible due to the recent technological progress in the "-omics" era, which allows the study of the interactions between complex biological pathways. This may lead to the identification of disease-specific biomarkers and to personalised medicine [2]. Furthermore, the study of biomarkers that are implicated in multiple molecular pathways with critical roles in the development of chronic diseases may be the key for the implementation of personalised medicine in clinical practice.

Vascular endothelial growth factor-A (VEGF-A) represents a biomarker with such properties and potential. It has a wide diversity of functions throughout life via its important role in angiogenesis, which is essential for the accumulation of new tissue and tissue remodeling processes [3, 4]. It is considered as a regulator of the most important chronic diseases, such as cardiovascular diseases (CVD), cancer, diabetes, chronic obstructive pulmonary disease (COPD), bone diseases and others [5-18].

Furthermore, VEGF-A is a highly heritable molecule. The Visvikis-Siest team has initially found that the heritable component of VEGF-A levels was detected in >60% of healthy individuals from the STANISLAS Family Study (SFS) [19]. The same team identified

multiple biological determinants of VEGF-A levels [20], as well as pre-analytical variations of VEGF-A gene expression and protein levels [21]. High heritability of VEGF-A serum levels was also reported in the first genome-wide linkage study on this quantitative trait conducted in the Cilento population study [22].

Moreover, VEGF-A gene expresses a large number of splice variants with both pro- and anti-angiogenic activities [23, 24]. These isoforms have been linked with the development and treatment of chronic diseases [25-29]. Additionally, treatments targeting the VEGF-A regulation system are commonly used in the treatment of several chronic diseases especially CVD, cancer, and diabetic complications [30-37]. Therefore, VEGF-A is a biomarker, that may offer applicable information with very promising role in implementation of personalised medicine in several common chronic diseases.

The Vascular endothelial growth factor European Genomic Federation – VEGF Consortium (www.vegfconsortium.org) was founded on June 2014 by an international group of researchers with an interest on VEGF-A and its implications in personalised medicine. The initiative for its founding was taken by Visvikis-Siest S, who is the leader of the Consortium.

Here we present the VEGF Consortium and to describe its objectives and ambitions, its structure and its components together with the methodologies employed in our projects and preliminary results.

Materials

Aim and objectives

The VEGF Consortium aims to develop a transnational collaborative network dedicated to large integrative and multidisciplinary genomic studies of the VEGF-A in order to generate applicable knowledge for medical practice. Given the wide range of the research field and the

need for application of different methodologies, the objectives can only be achieved through a consortium of scientists with different and complementary expertise and with a large range of resources such as large study populations, research materials, and harmonized data.

The specific objectives of the Consortium are:

- To combine data from multiple cohorts in order to identify VEGF-A '-omics' profiling in health and non-communicable diseases
- To elucidate the pivotal role of VEGF-A in the pathophysiology of non-communicable diseases
- To demonstrate the patients' stratification potential of VEGF-A '-omics' profiling
- To implement the research results into clinical practice and establish the role of VEGF-A as a predictive, preventive, diagnostic and prognostic biomarker
- To provide information on the effect of VEGF-A '-omics' profiling in side effects and response to therapy through pharmacogenomics studies
- To propose implementation strategies and European guidelines involving VEGF-A '-omics' profiling for the management of non-communicable diseases
- To share methodologies, data and knowledge in the field of '-omics' management and innovative statistics
- To develop standardized teaching and evaluation methods practiced and validated by the Consortium

Partners

The VEGF Consortium currently consists of 14 Partners (10 founding partners and 4 additional core partners) from Europe and the USA:

Founding partners:

- INSERM UMR U1122; IGE-PCV – Sophie Visvikis-Siest, Coordinator/ Consortium Leader
- UMR INSERM U1122; IGE-PCV –Maria Stathopoulou / Project Manager
- Boston University (BU) – Sudha Seshadri
- European Society of Pharmacogenomics and Personalised Therapy (ESPT) – Gérard Siest †, Ron H.N. van Schaik
- INSERM US13, BIOBANQUES / Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) – Georges Dagher
- National Research Council of Italy / Genetics of Complex Traits Laboratory at IGB-(CNR) – Marina Ciullo
- Queen Mary University of London (QMU) – Panagiotis Deloukas
- Randox Laboratories Limited (Randox) – John Lamont
- Sapienza University of Rome (UniRoma1) – Maurizio Simmaco
- University Medical Center of Groningen (UMCG) – Behrooz Z. Alizadeh
- University of Ljubljana (UL) – Janja Marc

Additional core partners:

- Harokopio University of Athens (HUA) – George Dedoussis
- Institut de Cancérologie de Lorraine (ICL) – Jean-Louis Merlin
- University of Luxembourg (ULux) – Jochen Schneider
- University of North Carolina at Chapel Hill, (UNC) – Federico Innocenti

Organisation

The Consortium is coordinated by an executive board that consists of the founding partners. The principles of the Consortium are described in the Consortium Agreement document, which is signed by all the partners of the Consortium.

Regular teleconferences are scheduled between partners of the Consortium and within specific working works to ensure the organization and execution of the projects. At least one face-to-face meeting is taking place each year.

It comprises 11 working groups, where the partners of the Consortium are participating based on their expertise:

1. Working group on VEGF-A '-omics' profiling in health (Sophie Visvikis-Siest, Sudha Seshadri, Marina Ciullo, Behrooz Z. Alizadeh)
2. Working group on VEGF-A '-omics' profiling in diseases (Sudha Seshadri, Behrooz Z. Alizadeh, Panagiotis Deloukas, George Dedoussis, Georges Dagher, Janja Marc, Maurizio Simmaco, Sophie Visvikis-Siest)
3. Working group on '-omics' technologies (Panagiotis Deloukas, Sudha Seshadri, Marina Ciullo, Behrooz Z. Alizadeh, Sophie Visvikis-Siest)
4. Working group on methodological aspects (Marina Ciullo, Behrooz Z. Alizadeh, Panagiotis Deloukas, Sudha Seshadri, Sophie Visvikis-Siest)
5. Working Group on VEGF-A clinical implementation (John Lamont, Maurizio Simmaco, Sophie Visvikis-Siest)
6. Working group on pharmagenomics (Sophie Visvikis-Siest, Federico Innocenti, Jean-Louis Merlin)
7. Working Group on Endothelins and Endothelial Factors (ESH) (Sophie Visvikis-Siest)

8. Working group on VEGF-A basic research (cancer cell lines, animal models) (Janja Marc, Sophie Visvikis-Siest)
9. Working group on VEGF-A and inflammation (Behrooz Z. Alizadeh, Sophie Visvikis-Siest)
10. Working group on communication and scientific/educational meetings (Ron Van Schaik, Sophie Visvikis-Siest, Janja Marc, Maurizio Simmaco)
11. Working group on raising awareness of populations (Maurizio Simmaco, Ron Van Schaik, Sophie Visvikis-Siest)

Cohorts

An important number of cohorts participate in the VEGF Consortium. Prospective, longitudinal, family-based or population-based cohorts of healthy individuals are in the core of the Consortium. These are:

1. The STANISLAS Family Study (SFS): This is longitudinal family structure cohort of community-based population of French origin recruited in 1993-1995, in the Lorraine region of France and followed up for 15 years. A number of 1006 nuclear families comprising two parents and at least two biological children over 6 years old are included in the study. Basic exclusion criteria are the absence of chronic or acute disease and the absence of personal history of CVD. Data were collected during medical visits every 5 years [38, 39].
2. The Framingham Heart Study (FHS): This is one of the most known cohorts worldwide and its results have been innovative in many fields and especially in the field of CVD. It is an ongoing, longitudinal, community-based, observational cohort study that was initiated in 1948 to prospectively investigate the risk factors for CVD.

It comprises three generations of participants: the Original cohort followed since 1948; their Offspring and spouses of the children, followed since 1971; and third generation composed of the children and their spouses from the largest Offspring families, enrolled in 2000 (Gen 3). The Original cohort enrolled 5,209 men and women who comprised two-thirds of the adult population then residing in Framingham, MA and survivors continue to receive biennial examinations. The Offspring cohort of 5,124 participants (including 3,514 biological offspring), have been examined approximately once every 4 years. Gen 3 included 4,095 individuals that have been examined on 2 occasions (between 2002-2005 and 2008-2011)[40-42].

3. Cilento study: This is a population-based study that aims at identifying genetic risk factors for common diseases and traits. The sample includes isolated populations from three villages (Campora, Gioi and Cardile). The overall sample size of individuals participating to the study is 2100. All inhabitants of the selected isolated populations are invited to participate to the study. The decision to participate is voluntary and all participants signed an informed consent [43, 44].
4. The LifeLines Cohort Study and Biobank (LLs): The general aim of the LLs Cohort Study is to unravel how life-time exposure to (universal) risk factors influences individual susceptibility to multifactorial diseases. LLs is a multi-disciplinary prospective population-based cohort study examining the health and health-related behaviours of 167,000 persons living in the North East region of The Netherlands in a three-generation design. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioural, physical and psychological factors which contribute to the health and disease of the general population, with a special focus on multi-morbidity and complex genetics. All survey participants were between 18 and 90 years old at the time of the enrollment [45-48].

5. The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS): This is a community-based population randomly selected from the general population in the town of Uppsala, Sweden between April 2001 and June 2004: An invitation letter was sent to participants selected in a randomised order (from the register of community living) within 2 months of their 70th birthday. Of the 2025 subjects invited, 1016 participated.

Furthermore cohorts of patients and case-control studies are also included in the Consortium:

6. The Hellenic Study of Interactions between SNPs and Eating in Atherosclerosis Susceptibility (THISEAS): This is case-control study of coronary artery disease (CAD). Participants were recruited from 3 hospitals found in the area of Athens, Greece. Cases were subjects with a first-ever myocardial infarction (MI) before age of 70 years presenting with either acute coronary syndrome (ASC) or stable CAD defined as >50% stenosis in at least one of the three main coronary vessels assessed by coronary angiography. ACS was defined as acute MI or unstable angina corresponding to class III of the Braunwald classification. ACS patients have also undergone coronary angiography examination that verified the presence of significant stenosis. Controls were age-matched subjects without MI/CAD history with negative coronary angiography findings (<30% stenosis), or negative stress test, or subjects without symptoms of disease that were admitted at the same hospitals as cases and were free of any cardiovascular disease, cancer, or inflammatory diseases. Subjects with renal or hepatic disease were excluded from both study groups.

7. Ljubljana patients: This is a group of different case-control studies recruited from Ljubljana, Slovenia that include healthy individuals and cases of osteoporosis, osteoarthritis, CVD, and diabetes [49-52].

In addition, partners of the Consortium have access to large biobanks such as the UK Biobank (Panagiotis Deloukas), Biobanking and Biomolecular Resources Research Infrastructure - European Research Infrastructure Consortium BBMRI-ERIC (including Sophie Visvikis-Siest with the Biological Resources Centre IGE-PCV (BB-0033-00051)) and the Alliance for Clinical Trials in Oncology (Federico Innocenti).

Expertise and methodologies

The Consortium's research is based on the idea that the exploitation of different molecular pathways, combined with the use of a variety of experimental, theoretical and computational techniques will lead to the identification of biomarkers, that can allow the analysis of "-omics" profiling and the identification of disease biomarkers and relevant functional genetic variants. Thus, the methodological aim of the Consortium is to follow a strategy of "systems biology".

The partners of the Consortium come from different and complementary disciplines and they provide a wide range of expertise to the Consortium in the fields of genomics, transcriptomics, epigenomics, "-omics" methodologies and analyses, research methodology, cardiology, oncology, chronic diseases prediction and prevention, drug development, clinical trials, computational modelling, bioinformatics, systems biology, personalised medicine, personalised therapy, pharmacogenomics, clinical implementation, infrastructures for diagnostic products development and manufacture, innovation and commercialisation

strategy, dissemination and education, raising awareness towards "-omics", and patients associations.

Based on this expertise, the Consortium is using the most up-to-date methodologies to achieve its objectives. The identification of genetic determinants of VEGF-A is performed by genome-wide association studies (GWAS) meta-analyses in large populations which is used in combination with the 1000 genomes imputation data so as to maximize the number of polymorphisms to be tested. Conditional analyses are being performed aiming to determine the most significant independent variables that determine VEGF-A levels variability. The strategy is complemented also by functional gene approach studies, as the partners of the Consortium acknowledge that GWAS are not the only useful methodology for genetic determinants identification. Relevant genes are considered those that are implicated in the regulation and signaling of VEGF-A, angiogenesis and related metabolic pathways linking VEGF-A to different diseases (e.g. inflammation). The VEGF-A genetic determinants are then being tested as predisposing genes for a variety of disease-related phenotypes, including intermediate phenotypes of chronic diseases such as blood lipids and blood pressure, disease phenotypes such as obesity, hypertension, diabetes and also disease clinical manifestations such as stroke. Gene × gene and gene × environment interactions are also being tested aiming to unravel a complete range of associations between VEGF-A genetic determinants and chronic diseases.

Transcriptomics studies are supporting the translation of the genetic determinants into functional products. The effects of polymorphisms on VEGF-A expression isoforms and the associations of these isoforms with the aforementioned disease-related phenotypes are used in order to build functional relationships from our findings. RNA sequencing will be used to achieve the highest transcriptome coverage, complemented by classical RT-PCR methodologies.

Functionality will also be assessed through in vitro methods and by bioinformatics approaches. The in vitro functional studies will provide additional elements on the cellular physiology, the regulation of pathways, the gene expression and validation of prognostic potential of the identified "-omics" determinants. Bioinformatics analyses will be used for the construction of VEGF-A networks aiming to identify network-based biomarker signatures across multiple "-omics". Several methodologies are being used including up-to-date pathway annotations, algorithm-based pathway analysis using graph-based statistic network analysis routines and classical machine learning techniques, gene mapping onto protein-protein interaction networks, gene prioritisation and identification of pathways, and literature mining analysis. Causality of identified variants will be assessed by Mendelian Randomisation methodology.

Genome-wide DNA methylation assays are available and will determine the effect of epigenetic modifications and assist in establishing the role of the environment in VEGF-A inter-individual variability and the VEGF-A disease-related traits through epigenome-wide association studies (EWAS) approaches.

Methodologies of computational biology (including modelling and simulations), and adapted algorithms models are being used for the development and the evaluation of stratification tools for disease risk prediction and targeted therapy.

Pharmacogenomic studies will be performed using clinical and genomic (GWAS) data from randomised, clinical trials of bevacizumab or other anti-VEGF-A treatments vs placebo in cancer patients at first and then in other patient groups. These studies will provide insights about the role of VEGF-A "-omics" determinants in response, side effects and toxicity of anti-VEGF treatments leading more rapidly towards the goals for personalised medicine.

Biochips measuring VEGF-A "-omics" determinants (protein and molecular arrays) have already been developed and will be updated and specialised based on the produced results.

These will allow the easy implementation of the results in clinical practice.

Methodological recommendations for each design and analytical step are strictly used to deliver robust and valid results. Standardised analytical procedures are followed and established analysis pipelines are used throughout the Consortium.

Furthermore, the inclusion of an industrial partner (Randox) and a European scientific society (ESPT) is a very original idea for a research consortium. These partners can offer their expertise and infrastructures including translation of results into a product with a well-established route to market through sales offices and distributors of Randox and the established dissemination platform of ESPT that assures the diffusion of the Consortium results in the general population, patients and health and care systems. This ensures that the results produced by the Consortium are used for evidence-based clinical decision-making and are integrated into the healthcare systems.

Results

Although the VEGF Consortium was officially founded on 2014, it was based on the long-term collaboration between some of its founding partners. Therefore, a number of significant results have been published already, while many projects are ongoing, with promising preliminary results.

Among the most basic steps was the identification through 2 GWAS of 10 genetic variants that explain >50% of the circulating VEGF-A levels variability [53, 54]. This is a unique finding among GWASs. In most GWASs, the identified variants do not explain >10% of the individual variability of the assessed traits. This finding has strengthened our belief that VEGF-A will indeed be used as strong biomarker for personalised medicine. Significant

associations between some of these polymorphisms and intermediate phenotypes of chronic diseases have been identified since then: high density and low density lipoprotein [55], L-selectin gene expression [56], and with free tri-iodothyronin (FT3) levels [57]. Significant epistatic interactions between these variants were observed for intercellular adhesion molecule 1 (ICAM-1), E-selectin, Interleukin 6 and tumor necrosis factor α (TNF-α) plasma levels [56]. Concerning specific disease risk, we have shown that these polymorphisms and/or their epistatic interactions can affect the risk for depression [58], and for auto-immune thyroid diseases [57], while no associations were found for diabetes type 2 [59].

Concerning the expression isoforms of VEGF-A gene, we have shown that these are significantly associated with ICAM-1, L-selectin and TNF-α expression [56] and with specific auto-immune thyroid diseases[60].

Furthermore, we have also identified associations between VEGF-A circulating levels and thyroid hormones levels [60] and with ICAM-1 and E-selectin levels[56].

Through a candidate gene approach of polymorphisms in genes involved in angiogenesis, we have identified direct and epistatic effects of variants on NOS3 (nitric oxide synthase 3), $CD14^{+}$ monocytes, MMPs (matrix metalloproteinases), and ILR4 (interleukin 4 receptor) genes with levels of VEGF-A and VEGF-A expression isoforms, but also gene × environment interactions[61].

An important result is also the production of two patents based on the results of studies performed by partners of the Consortium [62, 63].

Several projects are ongoing focused on CVD intermediate phenotypes, thyroid diseases, cancer, and stroke.

Four face-to-face meetings have been organised to date: Paris (2014), Budapest (2015), Santorini (2016) and Paris (2017).

Discussion

The VEGF Consortium is an ambitious international collaboration that aims to pave the way for the implementation of VEGF-A in personalised medicine and routine clinical practice. The designed projects take advantage of the wide expertise of its partners, the large infrastructures of cohorts and biobanks and a combination of the most up-to-date "-omics" approaches for generating multi-dimensional data, as well as systems medicine approaches, network analysis and computational modelling methodologies.

Apart from the scientific excellence, one major originality of the Consortium is that it integrates commercialization and communication platforms, targeting the valid and easy measurement of the identified biomarkers in large-scale settings but also the education of the general population, patients, scientists and health practitioners. One of its aims is to raise the awareness for strategies and applications of personalised medicine within health managers and health systems. The aim of these approaches is not only to produce valid scientific knowledge and applications but also to ensure that these applications will be successfully implemented.

The Consortium is also very active in submitting proposals for funding from national and international funding bodies in order to be able to finance its large-scale projects. The Consortium is open to new proposals and new partners, so as to augment furthermore its expertise and infrastructures.

The VEGF Consortium is a novel Consortium with an innovating structure and original goals. It is expected to provide significant advances in the study and management of chronic diseases, and translation of the scientific information in clinical practice through personalised medicine. The ultimate goal is to ameliorate life expectancy, quality of life for individuals, as well as financial benefits for health systems.

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Abstract
Cardiovascular diseases (CVD) are complex diseases where many environmental and genetic factors are involved. Thanks to the breakthroughs of the Hap Map and Human genome projects and the development of GWAS and PheWAS, the genetic etiology of the CVD has been extensively investigated the last two decades. However, there is still a big room of knowledge waiting to be discovered and alternative approaches are needed in order to keep advancing in the pathophysiology of CVD.
In this thesis, we propose an integrative approach to discover new genetic associations potentially involved in CVD. We chose previous GWAS hits and we focused on phenotypically homogeneous populations. We also centred our efforts in studying the pleiotropic and gene-gender interaction effects. Another main goal during this thesis was to defend the implementation of personalized genome-based therapy of the results obtained.
By using the above-explained approaches, new pleiotropic effects were discovered in the IL-6R and ABO genes. Within the IL-6R gene we found an antagonistic pleiotropy of this gene when it relates to CRP and lipid trait levels. We also found new pleiotropic effects within the ABO gene, by associating the SNP rs644234 with increased sE-selectin and HDL levels, and decreased ApoE levels. In addition, we studied the gene-gender interaction effects, finding some sex-specific associations in two of the genes studied (ABO and GNB3). Further, we centered our efforts in implementing the results obtained during the thesis at the clinical level. The SNP rs2234246 within the TREM-1 gene was associated with increased levels of its protein and could be used as a predictor or risk biomarker for different diseases (including CVD). Due to the high potential of this polymorphism, we applied a European patent and we are planning to start clinical trials in patients suffering from septic shock and acute myocardial infarction. Also the IL-6R haplotype rs4845628*T/rs4537545*C, which is increasing simultaneously CRP, LDL-C and ApoB levels, could be used in the treatment in the spirit of personalized medicine. Indeed the IL-6R gene is a target of the drug Tocilizumab and our results could help to prevent the side effects caused by this drug.
Following the goals and approaches synthetized above, we discovered new associations between genes of interest and intermediate phenotypes that are involved in CVD and other complex diseases. Our results help to better understand how the studied genes are exerting their effects at the molecular level, ultimately affecting the outcome of the individuals suffering from CVD. Our results will hopefully be taken into account in future personalized treatments.

Key words: Cardiovascular diseases, Pleiotropy, Gene-gender interactions, Genetic epidemiology, Personalized medicine, Intermediate phenotypes.

Résumé
Les maladies cardiovasculaires (MCV) sont d'une étiologie complexe et elles sont soumises à de nombreux facteurs environnementaux ainsi que génétiques. Les études d'association pangénomiques ou GWAS et les études d'association panphénotypiques ou PheWAS ont révolutionné l'épidémiologie génétique de la dernière décennie. Malgré les succès obtenus, pour réduire la morbidité et de la mortalité CV il est nécessaire l'identification de nouveaux biomarqueurs en utilisant des approches différentes.
Cette thèse propose une approche intégrative pour découvrir de nouvelles associations génétiques associés avec les MCV et autres maladies complexes. Nous avons d'abord réuni les résultats existants grâce à des GWAS précédents, puis nous les avons étudié au sein de populations phénotypiquement homogènes. Nous avons recherché la pléiotropie de ces gènes ainsi que leurs éventuels effets dus aux interactions gène-genre. De plus, nous avons dirigé nos efforts vers une possible traduction des résultats obtenus dans l'application clinique.
En utilisant des méthodes alternatives au-delà des GWAS classiques, nous avons détecté les effets pléiotropiques de différent gènes (gènes IL-6R et ABO). Concernant le gène IL-6R, nous avons trouvé une pléiotropie antagoniste pour les phénotypes lipidiques et les niveaux de CRP. Nous avons aussi trouvé un nouvel effet pléiotropique dans le gène ABO, où nous avons associé le SNP rs644234 avec une augmentation des niveaux soluble de E-sélectine et HDL, et avec une diminution des niveaux de ApoE. Par ailleurs, nous avons trouvé quelques associations gène-genre intéressantes pour certains gènes étudiés (ABO et GNB3). Concernant l'implémentation clinique des connaissances obtenues par cette thèse, le variant génétique rs2234246 dans le gène TREM-1, qui augmente les taux solubles de la protéine TREM-1, pourrait être utilisé comme un prédicteur ou un marqueur de risque pour différentes maladies (MCV inclues). Grâce au grand potentiel de ce polymorphisme, nous avons déposé un brevet Européen et nous envisageons de mener des essais cliniques chez des patients souffrant de choc septique et d'infarctus aigu du myocarde. D'autre part, nous avons associé l'haplotype rs4845628*T/rs4537545*C du gène IL6R à des taux élevés de CRP, de LDL-C et d'ApoB. Le produit pharmaceutique tocilizumab, dont la cible est le récepteur IL-6R, a comme effet indésirable une augmentation du taux de LDL-C. Nos résultats pourraient donc être utilisés afin d'éviter ces effets indésirables.
En suivant les objectifs et approches synthétisés ci-avant nous avons découvert de nouvelles associations entre gènes et phénotypes intermédiaires qui sont impliquées dans les MCV et d'autres maladies complexes. Nos résultats aident à mieux comprendre comment les gènes étudiés exercent leurs effets au niveau moléculaire, en influant finalement sur l'état des patients souffrant de MCV. Nous espérons que nos résultats vont être pris en compte pour faire progresser la médecine personnalisée.

Mots clés : Maladies cardiovasculaires, Pléiotropie, Interactions Gène-Genre, Epidémiologie génétique, Médecine personnalisée, Phénotypes intermédiaires.