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REGULATION GENETIQUE DE LA PRESSION ARTERIELLE - UNE APPROCHE DE GENOMIQUE MOLECULAIRE RELEVANT L'IMPLICATION DE L'INFLAMMATION DE FAIBLE NIVEAU

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A qui je dois tout le bien de ma vie…

A ma Maman!

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"… But remember throughout that no external cause is efficient without a predisposition of the body itself. Otherwise, external causes which affect one would affect all…"

Galen, 2000 years ago...

To be honest, I am not sure that Genetics was first introduced by Gregor Mendel..!

RESUME

L'hypertension artérielle est un trait polygénique ayant une héritabilité estimée entre 31% et 68%. Les variants génétiques découverts n'avaient qu'un faible effet et n'expliquaient qu'une infime partie (1%) de sa variabilité phénotypique. Ce résultat était à l'origine du concept d'héritabilité manquante. Nous pensons que l'héritabilité manquante pourrait s'expliquer par des interactions gène-gène (épistasiques), gène-environnement et la méthylation d'ADN. Une fois complétées par des études transcriptomiques, ces approches pourraient révéler les voies moléculaires impliquées. C'est le postulat principal que nous avons poursuivi au cours de cette thèse.

Ainsi, dans des populations européennes (en majorité françaises), nous avons identifié une nouvelle interaction épistasique fonctionnelle entre le rs5355C>T du gène *SELE* et le rs6046G>A du gène *F7* associée à la pression artérielle systolique. Dans cette même direction, nous avons rapporté deux autres interactions (rs1041163T>C dans *VCAM1* interagit avec rs1367117G>A dans *APOB* et rs3741240G>A dans *SCGB1A1* interagit avec rs1800590T>G dans *LPL*) pour être associées avec la pression artérielle. De plus, nous avons trouvé que le SNP intergénique rs7556897C>T dans le locus *SLC19A3-CCL20* interagissait avec l'obésité pour influencer la pression artérielle diastolique. En parallèle aux études précédentes, nous avons montré que le rs5030878T>C du gène *FPR1* et l'allèle KL-VS du gène *Klotho* pourraient être impliqués dans la régulation de la pression artérielle via leurs interactions avec l'âge et le traitement antihypertenseur respectivement. Contrairement aux autres variants génétiques étudiés, aucune association entre l'haplotype CAT1/2 du gène *CAT* et les niveaux de PA n'a été observée. Nous avons aussi participé à l'identification d'un nouveau SNP, le rs2000999G>A, expliquant quasiment la moitié de l'héritabilité de l'haptoglobine dont le rôle antihypertenseur est en cours d'investigation. Finalement, nous avons proposé une nouvelle catégorie de SNPs les eMethSNPs.

En conclusion, nous rapportons une panoplie de variants génétiques qui pourraient réguler la pression artérielle via des interactions gène-gène et gène-environnement. Les interactions identifiées dites 'fonctionnelles' montrent que l'inflammation de faible niveau est impliquée dans la régulation de la pression artérielle.

ABSTRACT

Essential hypertension is a polygenic trait, its heritability varies between 31% and 68%. Discovered genetic variants were shown to have small effects, consequently explaining a tiny fraction (1%) of its heritability and resulting to a missing heritability. The missing heritability could be explained by gene-gene (epistatic), gene-environment interactions and DNA methylation. Once conducted, these approaches should be further complemented by transcriptomic analyses, and thereby to reveal the involved molecular pathways. This is the postulate followed during our thesis.

Therefore, in European populations (mainly French), we have shown that rs6046G>A in *F7* interacted with rs5355C>T in *SELE* in order to influence systolic blood pressure levels. In the same direction, we have reported two other interactions (rs1041163T>C in *VCAM1* interacts with rs1367117G>A in *APOB* and rs3741240G>A in *SCGB1A1* interacts with rs1800590T>G in *LPL)* to be associated with blood pressure. We have also found the intergenic SNP rs7556897T>C, located between *SLC19A3* and *CCL20* locus, interacting with obesity in order to influence diastolic blood pressure. In parallel to those studies, we have shown that rs5030878T>C in *FPR1* and KL-VS allele in *Klotho* may be implicated in BP regulation through their interaction with age and antihypertensive drugs respectively. In contrast with the above-investigated genetic variants, CAT1/2 haplotype in *CAT* was not associated with blood pressure levels. We have also participated to the identification of a novel SNP, the rs2000999G>A explaining up to the half of haptoglobin's heritablilty, a protein currently under investigation for its antihypertensive role. Finally, we proposed a new category of functional genetic-epigenetic biomarkers, the eMethSNPs. In conclusion, we report a range of genetic variants that may regulate BP and influence its levels via gene-gene and gene-environment interactions. The identified 'functional' interactions show that low-level inflammation is involved in blood pressure regulation.

LISTE DES PUBLICATIONS

A- Articles originaux:

A-1 Articles originaux faisant partie de la thèse :

1. Said El Shamieh, Bernard Herbeth, Mohsen Azimi-Nezhad, Hamanou Benachour, Christine Masson, Sophie Visvikis-Siest.

Human Formyl Peptide Receptor 1 C32T SNP interacts with age and is associated with blood pressure levels.

Clin Chim Acta. 2012 Jan 18;413(1-2):34-8. Epub 2010 Dec 7.

2. Said El Shamieh[*], Rosine Nzietchueng[*], Hamanou Benachour, Carlos Labat, Bernard Herbeth, Ndeye Coumba Ndiaye, Christine Masson, Sophie Visvikis-Siest, Athanase Benetos.

Klotho KL-VS genotype is involved in blood pressure regulation.

* Contribution égale

Clin Chim Acta. 2011 Sep 18;412(19-20):1773-7. Epub 2011 Jun 1.

3. Said El Shamieh, Ndeye Coumba Ndiaye, Maria G Stathopoulou, Helena Murray, Christine Masson, John V Lamont, Peter Fitzgerald, Athanase Benetos, Sophie Visvikis-Siest.

Functional epistatic interaction between rs6046G>A in *F7* and rs5355C>T in *SELE* modifies systolic blood pressure levels.

PLoS ONE, 2012;7(7):e40777. Epub 2012 Jul 18.

4. Philippe Froguel*[¥], Ndeye Coumba Ndiaye*, Amélie Bonnefond, Nabila Bouatia-Naji, Aurélie Dechaume, Gérard Siest, Bernard Herbeth, Mario Falchi, Leonardo Bottolo, Rosa-Maria Guéant-Rodriguez, Cécile Lecoeur, Michel R Langlois, Yann Labrune, Aimo Ruokonen, **Said El Shamieh**, Maria G Stathopoulou, Anita Morandi, Claudio Maffeis, David Meyre, Joris R Delanghe, Peter Jacobson, Lars Sjöström, Lena MS Carlsson, Andrew Walley, Paul Elliott, Marjo-Riita Jarvelin, George V Dedoussis, Sophie Visvikis-Siest[¥].

A genome-wide association study identifies rs2000999 as a strong genetic determinant of circulating Haptoglobin levels.

* Contribution égale

¥ Auteur correspondant.

PLoS One. 2012;7(3):e32327. Epub 2012 Mar 5.

5. Said EL Shamieh*, Ndeye Coumba Ndiaye*, Amélie Bonnefond*, Maria G Stathopoulou, Cécile Lecoeur, David Meyre, Sébastien Dadé, Payman Shahabi, Gérard Siest, Athanase Benetos, George V Dedoussis, Philippe Froguel, Sophie Visvikis-Siest.

Obesity inverts the beneficial effect of newly identified rs7556897T>C on blood pressure regulation at childhood.

* Contribution égale

PLoS One, soumis.

6. Ndeye Coumba Ndiaye, **Said EL Shamieh**, Maria G. Stathopoulou, Gérard Siest, Michael Y Tsai, Sophie Visvikis-Siest.

Two epistatic interactions may be involved in blood pressure genetic regulation.

BMC Medical Genetics, Accepté avec modifications.

7. Valérie Nivet-Antoine, Carlos Labat, **Said El Shamieh**, Xavier Dulcire, Charles-Henry Cottart, Jean-Louis Beaudeux, Sophie Visvikis-Siest, Athanase Benetos.

Relationship between catalase rs769214 SNP and arterial aging.

Atherosclerosis, Accepté avec modifications.

A-2 Autres articles originaux :

8. Massimo Mangino, Shih-Jen Hwang, Timothy D Spector, Steven C Hunt, Masayuki Kimura, Annette L Fitzpatrick, Lene Christiansen, Inge Petersen, Clara C Elbers, Tamara Harris, Wei Chen, Sathanur R Srinivasan, Jeremy D Kark, Athanase Benetos, Sophie Visvikis-Siest, **Said El Shamieh**, Kaare Christensen, Gerald S Berenson, Ana M Valdes, Ana Vinuela, Melissa Garcia, Donna K Arnett, Ulrich Broeckel, Michael A Province, James S Pankow, Candace Kammerer, Yongmei Liu, Michael Nalls, Sarah Tishkoff, Fridtjof Thomas,

Elad Ziv, Bruce M Psaty, Joshua C Bis, Jerome I Rotter, Kent D Taylor, Erin Smith, Nicholas J Schork, Daniel Levy, Abraham Aviv.

Genome Wide Association points toward CTC1 and ZNF676 as telomere regulating genes

Human Molecular Genetics, 2012 Sep 25.

9. Maria G. Stathopoulou*, Amélie Bonnefond*, Ndeye Coumba Ndiaye*, Mohsen Azimi-Nezhad, **Said El Shamieh**, Abdelsalam Saleh, Marc Rancier, Gerard Siest, John Lamont, Peter Fitzgerald, Sophie Visvikis-Siest.

Contribution of vascular endothelial growth factor and related common genetic variants to the variation of blood lipids levels

Journal of Lipid Research, accepté avec modifications.

* Contribution égale

10. Maria Stathopoulou, Pedro Monteiro, Payman Shahabi, Eva Penas-Liedo, **Said El Shamieh**, Louis Silva Santos, Gerard Siest, Adrian Lerna, Sophie Visvikis-Siest.

Synergy between clopidogrel and calcium-channel blockers for blood pressure regulation possibly involving CYP2C19 rs4244285

Annals of Internal Medicine, Soumis.

B- Revues scientifiques faisant partie de la thèse :

11. Said El Shamieh*, Ndeye Coumba Ndiaye*, Mohsen Azimi Nehzad*, Maria G. Stathopoulou*, Sophie Visvikis-Siest*.

Cardiovascular diseases and genome-wide association studies.

* Contribution égale

Clinica Chimica Acta, 2011 Sep 18,412(19-20):1697-701. Epub 2011 Jun 7. Review.

12. Said El Shamieh and Sophie Visvikis-Siest.

Hypertension genetic biomarkers and future challenges integrating epigenomics

Clinica Chimica Acta, Sous presse.

C- Scènes de conférences:

13. Gerard Siest, Mohsen Azimi Nezhad, Denise Bagrel, **Said El Shamieh**, Daniel Lambert, Ndeye Coumba Ndiaye, Payman Shahabi, Sophie Visvikis-Siest.

Functional genomics towards personalized healthcare and systems medicine.

Personalized Medicine, May 2011, Vol. 8, No. 3, Pages 227-242.

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LISTE DES ABREVIATIONS

ACE: *angiotensin I converting enzyme*

ADN: acide désoxyribonucléique

ADNc: acide désoxyribonucléique complémentaire

ANOVA: analyse de variance

ARN: acide ribonucléique

CHARGE: cohorts for heart and aging research in genomic epidemiology

CCL20: *chemokine, cc motif, ligand 20*

cM: centiMorgan

CPAM: caisse primaire d'assurance maladie

CRB: centre de ressources biologiques

CRP: *c reactive protein*

DNase: désoxyribonucléase

DMR: differently *methylated region*

dNTP: désoxirubonucléotide triphosphate

ECA: enzyme de conversion de l'angiotensine

EDTA: *ethylene diamine tetracetique acid*

F7: *Factor 7*

fMLP*: formyl-methionine-leucine-phenylalanine*

FPR1: *formyl peptide receptor 1*

GWAS: genome-wide association study

GWS: genome-wide scan

HAS: haute autorité de santé

HDL-C: *high-density lipoprotein cholesterol*

HP: *haptoglobin*

HPR: *haptoglobin related protein*

HTA: hypertension artérielle

HWE: *Hardy Weinberg equilibrium*

ICAM-1: *inter-cellular adhesion molecule 1*

IGE-PCV: Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire

IL-1: *interleukin* 1

IL-6: *interleukin* 6

IMC: indice de masse corporelle

LISTE DES ABREVIATIONS

Kb: kilobase

Log: logarithme de base 10

LD: *linkage disequilibrium*

LDL-C: low-density lipoprotein cholesterol

LOD: *logarithm of odds*

MAF: fréquence de l'allèle mineur

MCP-1: *monocyte chemoattractant protein*

MCV: maladies cardiovasculaires

$MgCl_2$: chlorure de magnésium

mmHg: millimètre de mercure

MMLV-RT: *moloney murine leukemia virus reverse transcriptase*

NO: oxyde nitrique

O_2^-: anion superoxyde

Oligo dT: oligonucléotide de déoxythymine

NF-κB*: nuclear factor-kappaB*

PA: pression artérielle

PAD: pression artérielle diastolique

PAI-1: *plasminogen activator 1*

PAS: pression artérielle systolique

pb: paire de bases

PBMC: p*eripheral blood mononuclear cells*

PCR: p*olymerase chain reaction*

POLR2A: *polymerase (RNA) II polypeptide A*

RCV: risque cardiovasculaire

RNasin: inhibiteur de ribonucléase

ROS: espèces réactives d'oxygène

RT-PCR: *reverse transcription-polymerase chain reaction*

SAM: S-adenosyl methionine

SELE: *E-selectin*

SELP: *P-selectin*

SLC19A3: *Solute Carrier Family 19 Member 3*

SNP: *single nucleotide polymorphism*

LISTE DES ABREVIATIONS

SOD: superoxyde dismutase

STANISLAS: Suivi Temporaire Annuel Non Invasif de la Santé des Lorrains Assurés Sociaux

TaqPolymérase: polymerase isolée à partir de la bactérie *Thermus aquaticus*

TNFα: *tumor necrosis factor α*

VCAM-1: *vascular cell adhesion molecule-1*

VDR: *vitamin D receptor*

π: proportion d'héritabilité expliquée

%: pourcentage

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AVANT-PROPOS

L'hypertension artérielle (HTA) est une maladie qui touche la population du monde entier. Selon une publication sortie en 2005 dans le journal scientifique « *The Lancet* », 972 millions d'individus souffrent d'HTA (le quart de la population mondiale adulte), dont 333 millions dans les pays développés et 639 millions dans les pays en voie de développement. En 2025, le nombre d'adultes hypertendus devrait croître de 60% pour atteindre un total d'un 1,56 milliard. En France, l'HTA touche plus de 8 millions d'individus dont plus de la moitié sont âgés de plus de 60 ans et dont 16,5% ont plus de 20 ans. L'ensemble de ces observations fait en sorte que l'HTA est devenue un défi majeur de santé publique au niveau mondial.

Malgré que de nombreuses études cliniques étaient à l'origine d'une meilleure compréhension de l'étiologie d'HTA et par conséquent de la mise en place de mesures préventives ces taux de mortalité et morbidité ne cessent d'augmenter. Cette limite de l'effet des mesures préventives est bien évidemment due au fait que la communauté scientifique n'ait pas encore élucidé la totalité des phénomènes menant au développement d'HTA. Notamment la génétique sous-jacente.

Historiquement, les études de la génétique de la pression artérielle (PA) et de l'HTA étaient basées sur une approche 'gène candidat' définie selon une connaissance à priori de la physiopathologie de la maladie d'intérêt. Avec l'avènement des études pangénomiques ou *genome-wide scan*, une approche s'affranchissant de l'hypothèse préalable a vu le jour et a permis de mettre en évidence des cheminements physiopathologiques non soupçonnés et par la même occasion, des bio-marqueurs inédits. Néanmoins, la part d'ombre d'héritabilité génétique persiste, notamment l'effet cumulatif des « *single nucleotide polymorphims »* (SNPs) identifiés qui reste pour le moment très en deçà de la variation phénotypique estimée.

Nous pensons que ce fossé provient du nombre restreint des SNPs fonctionnels identifiés et de carences dans la modélisation génétique. Côté modélisation, la variabilité manquante pourrait s'expliquer par des interactions gène-gène (épistasiques) et gène-environnement. De même, les processus épigénétiques, notamment la méthylation d'ADN pourraient être impliqués. Une fois complétées par des études transcriptomiques dans des cellules primaires, ces trois approches

pourraient explorer les possibilités de passer de l'association statistique à la causalité et de révéler les voies moléculaires impliquées dans la régulation de la PA.

Ainsi, nous avons émis l'hypothèse que la compréhension de la génétique de la PA et de l'HTA est conditionnée par la nécessité de mettre en œuvre de tels designs intégrés. En parallèle, nous avons exploré l'association de trois variants génétiques (présents dans des gènes appartenant à des voies métaboliques liées au processus de l'inflammation) avec la PA et l'HTA. De même, nous avons participé à une étude pangénomique de la variabilité des taux plasmatiques d'haptoglobine, une protéine d'inflammation récemment considérée comme cible thérapeutique antihypertensive possible chez les patients ayant des taux élevés d'hémoglobine dans le sang.

L'équipe EA-4373 'Génétique Cardiovasculaire' possède un outil permettant de mener à bien ces investigations : la Cohorte STANISLAS regroupant à l'inclusion 1,006 familles nucléaires d'origine Lorraine depuis plus de deux générations. Ces familles, composées de deux parents ayant au moins deux enfants, ont fait l'objet de trois bilans de santé à cinq ans d'intervalle au Centre de Médecine Préventive de Vandœuvre-lès-Nancy. Au cours de ces bilans de santé, des données à la fois socio-économiques, biologiques et cliniques ont été recueillies. Au total, 600 variables différentes ont été obtenues pour chaque participant et des banques ADN / ARN_{totaux} / et lymphocytaires (PBMCs) ont été constituées. En plus de la cohorte STANISLAS, notre équipe dispose de nombreuses populations (individus hypertendus, individus âgés et des enfants). Toutes ces populations sont conservées dans un centre de ressources biologiques (CRB) mis en place à cette occasion: le CRB Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire (IGE-PCV).

Notez que lors de la rédaction de ce mémoire, nous avons choisi d'utiliser le 'nous' singulier. Notre contribution effective dans chaque étude est signalée dans le chapitre 'PRESENTATION DES TRAVAUX' à la fin de la page de résumé de chaque article. En règle générale, nous avons réalisé les expériences de génotypage et de transcriptomique et les analyses bio-informatiques (en ayant préalablement suivi une formation spécifique) et participé à l'interprétation des analyses statistiques, à la veille bibliographique, à la rédaction des articles et aux débriefings hebdomadaires fédérant l'ensemble des équipes collaboratrices. Nous avons également apporté

notre aide à la gestion des échantillons biologiques au sein du CRB : IGE-PCV en réalisant des aliquotages et en contribuant à la gestion de la traçabilité des échantillons biologiques.

Comme déjà annoncé, la PA et l'HTA sont conditionnées par des interrelations complexes existant entre les variants génétiques et l'environnement que les nombreuses études de populations n'ont permis d'élucider que en partie. En partie seulement, car l'approche classique était basée sur la présélection de variants génétiques impliqués dans des voies métaboliques connues.

Vu la révolution technologique traduite par la mise en place de GWAS depuis 2007, de nombreux variants inédits associés ont été mis en évidence [49]. **Néanmoins, le '*dark matter*' persiste, notamment l'effet cumulatif des SNPs identifiés qui reste pour le moment très en deçà de la variation phénotypique estimée**.

Nous pensons que ce fossé provient du nombre restreint des SNPs fonctionnels identifiés et de carences dans la modélisation génétique. Côté modélisation, la variabilité manquante pourrait s'expliquer par les **interactions épistasiques**. En effet, l'influence des SNPs n'est pas simplement additive, ou dominante/récessive, mais aussi interactive et seule une approche systémique par réseaux de gènes permettra de l'évaluer. De plus, les interactions **gène-environnement** ne sont pas encore intégrées lors de l'étude d'une dizaine de milliers de SNPs, alors qu'il est établi que dans certains cas, la présence de gènes parfaitement fonctionnels ne suffit pas à l'émergence d'un risque chez l'individu en l'absence de certaines stimulations environnementales. De même, l**es processus épigénétiques, notamment la mADN** peuvent être aussi importants. **L'ensemble de ces trois approches devrait être complété par une étude transcriptomique dans des modèles cellulaires primaires. Ces derniers imitent l'état 'in vivo' et génèrent par conséquent des voies moléculaires éventuellement impliquées dans la PA.**

Ainsi, nous avons émis l'hypothèse que **la compréhension de la génétique de la PA et de l'HTA est conditionnée par la nécessité de mettre en œuvre des designs intégrés, permettant d'appréhender les 'carences méthodologiques' qu'elle comporte,** notamment, étudier **les interactions épistasiques et gène-environnement**, **la mADN** et de plus, **explorer les possibilités de passer de l'association statistique à la causalité par les analyses moléculaires des effets des SNPs.**

Nous avons donc poursuivi **cinq objectifs principaux** :

A- Développer et expérimenter une stratégie globale exploratoire intégrant les interactions épistasiques et gène-environnement afin d'identifier des SNPs candidats de la PA et de l'HTA passés inaperçus dans les études génétiques précédentes en particulier pangénomiques.

En se basant sur le fait qu'une GWAS prenant en compte les effets épistasiques serait irréalisable à l'état actuel vu le manque des outils statistiques et logistiques adéquats, nous avons utilisé l'approche gène candidat et nous sommes donc focalisés sur des SNPs faisant partie de nos objectifs ultérieurs ou précédemment étudiés dans notre laboratoire. Dans un premier temps, nous avons étudié leurs associations avec la PA. Ensuite, nous avons analysé leurs interactions épistasiques permettant de réguler les niveaux de PA.

Dans le but d'élucider les interactions gène-environnement et plus spécifiquement les interactions gène-obésité et d'identifier de nouveaux SNPs candidats de PA via l'obésité, nous sommes focalisés sur l'étude des populations pédiatriques où l'âge et les facteurs d'environnement (tabagisme, consommation d'alcool, prise de la pilule, stress… etc.) sont moins importants.

B- Réaliser une étude fonctionnelle des SNPs identifiés par l'étape précédente dans des PBMCs.

Cette approche complémentaire peut révéler des nouvelles voies moléculaires impliquées dans la PA et l'HTA en expliquant une partie de leur variabilité phénotypique.

C- Déterminer l'éventuelle relation du rs5030878T>C du gène *FPR1,* de l'allèle KL-VS du gène *KLOTHO* et de l'haplotype CAT1 du gène *CAT* avec les niveaux de PA et l'HTA.

Vu l'importance du récepteur *FPR1* dans le processus immuno-inflammatoire, des variations génétiques au niveau de cette molécule pourraient affecter la production et les fonctions des molécules d'inflammation telles que la CRP [1]. Dans ce sens, il a été montré dans notre laboratoire que l'allèle C du rs5030878C>T dans FPR1 est associé avec une diminution du taux circulant de la SELE [2].

De nombreuses études ont proposé que l'allèle KL-VS du gène KLOTHO (qui a été démontre protecteur de la fonction endothéliale) est associé à une augmentation des niveaux de PA dans des populations non européennes mais jamais dans des populations françaises [3-5].

Le gène de la catalase (CAT) est connu pour jouer un rôle antioxydant en protégeant les cellules contre le stress oxydatif. Des études successives ont identifié au niveau de son promoteur proximal un haplotype CAT1 caractérisé par 3 SNPs ([rs769214 (-844 G/A), rs7943316 (-89A/T) et rs1049982 (-20T/C)]) et associé avec l'HTA dans des populations asiatiques (notamment chinoises) [6;7]. L'association avec l'HTA est possiblement médiée par le SNP rs769214G>A démontré capable d'altérer l'activité transcriptionelle du gène *CAT* via la modification du site de fixation de son facteur de transcription PAX-6 [8]. Aucune étude n'a été réalisée avec la PA et l'HTA dans des populations françaises.

Vu l'ensemble de ces observations, nous avons donc étudié l'association de ces trois variants génétiques avec la PA et l'HTA chez des individus de nationalité française.

D- Participer à la recherche de nouveaux variants génétiques susceptibles d'influencer la PA ou l'HTA-le cas d'haptoglobine: identidication de nouvaux SNPs contribuant à la variabilité de ces taux plasmatiques.

L'augmentation des taux plasmatiques d'haptoglobine (HP) semble capable de prévenir la survenue d'HTA [9]. Cette observation peut être expliquée par la liaison de l'HP à l'hémoglobine qui empêcherait les complications oxydatives qui pourraient induire l'HTA [9]. Identifier des variants génétiques responsables de la variation

interindividuelle des taux plasmatiques d'HP pourrait avancer les études génétiques de la PA et de l'HTA avec un intérêt diagnostique, préventif et thérapeutique.

E- Proposer une nouvelle stratégie intégrative de la PA et de l'HTA incluant l'épigénomique.

Comme discuté précédemment, plusieurs observations soutiennent la participation d'une composante épigénétique dans l'étiologie de PA et d'HTA. Cependant, aucune information concernant le rôle de la mADN dans la régulation de la PA et de l'HTA n'est dévoilée à l'échelle génomique.

Les méthodologies, les résultats détaillés, et les approches expérimentales utilisés pour la réalisation de nos objectifs sont présentés en détail dans la section « PRESENTATION DES TRAVAUX ».

I. ETAT DES LIEUX DE L'HYPERTENSION ARTERIELLE

I-1. Historique

L'extraordinaire force du sang décrite par Sénac , La première mesure de pression artérielle (PA) par Stephen Hales, en 1733 fut en effet rapidement commentée par le Français Jean Baptiste Sénac, médecin consultant du Roy et auteur du Traité de la structure du Cœur, de son action et de ses maladies publié en 1749 comptant parmi les livres fondateurs de la cardiologie [10]. Voici comment la langue scientifique du XVIIIème siècle décrit la «force du sang» avant même que l'on emploie le terme de «pression artérielle» [10].

«Le sang surmonte tous ces obstacles, mais en les surmontant il perd une partie de son mouvement , or on ne saurait évaluer cette perte, puisque qu'on ne saurait apprécier les obstacles (...). Quelques faits nous prouvent que le sang agit avec beaucoup de force (Figure 1) [10]. Quand il s'échappe des artères, il jaillit avec violence, il s'élance jusqu'à 10 ou 15 pieds, il va même jusqu'à 6 et 7 en forçant une veine : mais de telles observations prouvent seulement en général l'excès de la force qui pousse le sang en divers corps [10]. D'autres expériences nous montrent très sensiblement la force du sang , les vaisseaux les plus solides crèvent quelques fois dans des efforts et même sans ces efforts : or les parois des artères et des veines résistant à une pression violente [10]. Mr Hales ne put forcer les membranes d'une carotide de jument avec un instrument qui comprimait l'air , la veine jugulaire d'un autre cheval soutient une pression égale à la pression d'une colonne d'eau qui pèserait 97 livres (Figure 1), la veine jugulaire d'un chien ne céda pas au poids de 14 livres d'eau... On pousse le piston avec de grands efforts, cependant les vaisseaux y résistent ordinairement, même chez les enfants : ce qui prouvent que les artères ne cèdent pas alors à une action violente, c'est que le piston abandonné à lui-même revient à une grande rapidité [10]. Si par la résistance que trouvent les injections, on jugeait de la force du sang, elle paraitrait extraordinaire» [10].

Figure 1: Une première mesure de la pression artérielle réalisée par Stephen Hales en 1733.

Enregistrement de la pression artérielle à l'aide d'un capteur (tuyau en cuivre) introduit dans l'aorte.

I-2. Epidémiologie de l'hypertension artérielle en France et de part dans le monde

A- En France :

L'hypertension artérielle (HTA) touche plus de 8 millions d'individus dont plus de la moitié sont âgés de plus de 60 ans (Fondation de la Recherche sur l'Hypertension Artérielle, FRHTA) [10] et dont 16,5% ont plus de 20 ans. C'est le premier motif de consultation en médecine générale et un risque majeur de survenue de complications cardiovasculaires (CV). La prévalence de l'HTA augmente avec l'âge: après 70 ans, elle touche plus de 40% de l'ensemble des hommes et des femmes , or la proportion d'individus de plus de 65 ans, s'accroît rapidement depuis 1995 [1]. Malgré une importante prise en charge, les prévalences déclarées d'HTA sont largement sous estimées : ainsi, plus de 50% des hommes et 35% des femmes hypertendus (HT) sont méconnus [10].

En France, l'ensemble des maladies cardiovasculaires (MCV) représente une dépense majeure dans l'ensemble des dépenses de santé de l'ordre de 11 milliards d'euros (M€) [1]. L'HTA seule représente un coût de 2,6 M€ et représente la second poste de dépense après le cancer (4,5 M€) et devant le diabète (1,1 M€) [10]. Les dépenses de prévention des facteurs de risques liés à l'HTA coûtent sensiblement moins cher que celles du traitement de ses complications CV [10], ce qui rend l'investissement dans la recherche pour le développement de méthodes diagnostiques précoces de l'HTA et pour la mise en place de stratégies préventives efficaces primordiale [10].

B- Au niveau mondial : Selon le journal scientifique « *The Lancet* » [11], 972 millions d'individus souffrent d'HTA (le quart de la population mondiale adulte), dont 333 millions dans les pays développés et 639 millions dans les pays en voie de développement. En 2025, le nombre d'adultes HT devrait croître de 60% pour atteindre un total d'un 1,56 [11]. L'HTA est donc considérée comme un défi majeur de santé publique au niveau mondial. La prévention, la détection, le traitement et le contrôle de ce phénomène doivent être pris en considération avec une haute priorité [11].

I-3. Diagnostique de l'hypertension artérielle

De nos jours, Il n'existe pas de seuil de PA individualisé en deçà duquel le risque cardiovasculaire (RCV) que constitue ce trait peut être considéré comme nul. Le rôle délétère de la pression artérielle diastolique (PAD) a été le premier à avoir été mis en évidence mais c'est la pression artérielle systolique (PAS) qui a la signification pronostique la plus impactante. Quant à la pression pulsée (PAS-PAD) elle traduit une altération de la compliance (ou fonction d'amortissement) des gros vaisseaux.

L'HTA est également un composant permettant la détermination du RCV absolu [12] mais la PA est fréquemment mesurée en clinique, au cabinet du médecin, ce qui altère souvent sa signification pronostique [12]. Au niveau circulatoire, le processus d'artériosclérose, se traduisant par une atteinte pathologique de la média au niveau des gros et des petits vaisseaux, est un phénomène spécifiquement lié à l'HTA et au vieillissement à l'inverse du processus d'athérosclérose, dans lequel l'HTA n'intervient qu'en tant que facteur de risque [12]. Au niveau cardiaque, l'HTA favorise la formation de plaques athéromateuses au niveau des gros troncs coronariens, générant ainsi une authentique insuffisance coronarienne organique. De même, elle contribue en collaboration avec divers facteurs neuro-hormonaux, à l'apparition d'une hypertrophie ventriculaire gauche associant hypertrophie myocytaire et expansion du tissu de soutien [12]. Les traitements antihypertenseurs ont un effet direct sur la morbidité et la mortalité CV en réduisant nettement le taux de survenue des AVC (de 30 à 40% suivant les populations considérées) et des accidents coronariens (10 à 15%) [12]. Ainsi, l'haute autorité de santé [Prise en charge des patients adultes atteints d'HTA essentielle–Actualisation 2005] préconisait les objectifs tensionnels suivants :

- PAS/PAD< 140/90 mmHg chez l'adulte d'âge moyen ,

- PAS< 150 mmHg chez le sujet âgé avec HTA systolique ,

- PAS/PAD< 140/80 mmHg chez le diabétique de type 2 ,

- PAS/PAD< 130/85 mmHg chez l'insuffisant rénal sans protéinurie ,

- PAS/PAD< 125/75 mmHg chez l'insuffisant rénal avec protéinurie> 1g/j.

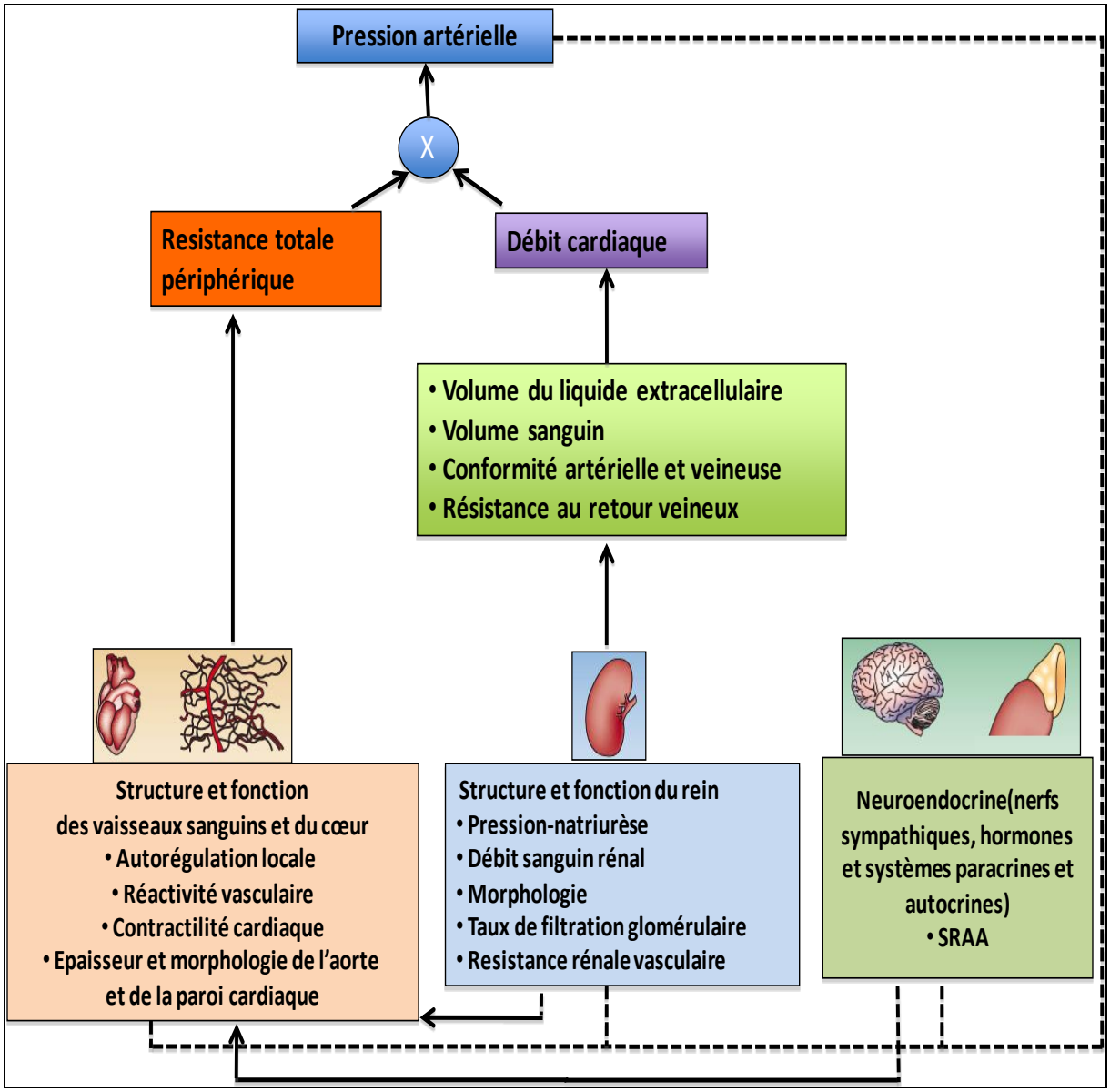
I-4. Bases physiopathologiques de l'hypertension artérielle

La PA est déterminée par une série de facteurs physiques tels que le volume sanguin, le débit cardiaque, la résistance et la conformité vasculaire [13] Ces éléments sont régulés par une série de facteurs neuronaux, endocriniens et paracriniens (Figure 2). Cependant, le rôle des reins dans la régulation de l'équilibre hydro-électrolytique est le déterminant le plus important de la PA [13]. De nombreux facteurs déterminent l'efficacité du rein dans l'accomplissement de cette tâche [13]. Parmi ces facteurs nous pourrons citer le fonctionnement des nerfs sympathiques rénaux, les niveaux d'hormones circulantes, les facteurs paracrines intra-rénaux et autocrines, et les nombreux mécanismes intrinsèques qui régulent le flux sanguin rénal et la filtration glomérulaire [13].

Les formes les plus courantes d'HTA sont diagnostiquées à l'âge moyen, une fois que de légères hausses répétées ou intermittentes de PA au-dessus des limites normales sont détectées [13]. Du fait que l'HTA progresse avec l'âge, des changements structuraux dans les vaisseaux sanguins se produisent et 'soutiennent' cette vasoconstriction [13]. Ces changements persistent même après le retrait du stimulus original, et déclenchent par la suite des événements qui peuvent conduire à un stress oxydatif et d'autres blessures [13]. Cette étape ultérieure d'HTA est généralement associée à la sensibilité aux sels [13]. Chez les Afro-Américains et Singapouriens-Asiatiques, le stade terminal de l'insuffisance rénale progresse rapidement, comparés aux Européens occidentaux et européens américains chez qui nous remarquons un stade plus lent [13]. Une telle variabilité observée lors de la progression de la maladie est également visible chez différents modèles de rat HT, ce qui suggère une prédisposition génétique à l'HTA [13]. Ces observations ont conduit à des efforts considérables pour essayer de révéler des gènes impliqués dans la régulation précoce d'HTA (lors des ses premiers stades) [13].

De même, des efforts sont en cours pour identifier les gènes prédisant l'atteinte des organes clés de régulation d'HTA, tels que le cœur, les vaisseaux sanguins, le cerveau et les reins.

Figure 2: Mécanismes de régulation de la pression artérielle (d'après Cowley AW Jr [4]).



La PA est très dynamique. A tout moment elle est définie comme le produit du débit cardiaque et de la résistance périphérique totale. Le débit cardiaque est déterminé par les relations complexes entre le volume du liquide extracellulaire, le volume sanguin, la conformité artérielle et veineuse et la résistance de la circulation sanguine autour de la circulation systémique. Au fil du temps, avec un apport normal de sel et d'eau tous les jours, le rein est le contrôleur principal de volume du liquide extracellulaire et de la PA par le mécanisme de pression-natriurèse. La résistance périphérique totale est déterminée par la structure et la fonction de la vascularisation et par des mécanismes locaux d'autorégulation. Les facteurs

neuroendocrines (nerfs sympathiques, hormones et systèmes paracrines et autocrines) ont une influence importante sur les reins et la fonction vasculaire. La rétroaction homéostatique négative (en pointillés) a également un rôle important dans la régulation de la PA, une variété de capteurs de PA produit des signalisations de réponse qui ont pour but de contrôler les fonctions neuroendocriniennes, vasculaires et rénales.

SRAA : système rénine angiotensine aldostérone, PA : pression artérielle.

I-5. Hypertension artérielle et facteurs de risque associés

Chez environ 10% des sujets HT, une cause unique, curable, peut être retrouvée [14]. À côté de facteurs non modifiables (âge, sexe, prédisposition génétique), l'HTA est aussi fortement corrélée à des facteurs environnementaux théoriquement modifiables tels que la consommation sodée, la sédentarité, l'obésité, l'inflammation, la consommation d'alcool et le stress, y compris professionnel. Parmi tous ces facteurs acquis, deux ont une importance toute particulière : l'obésité et l'inflammation.

I-5.1. Obésité

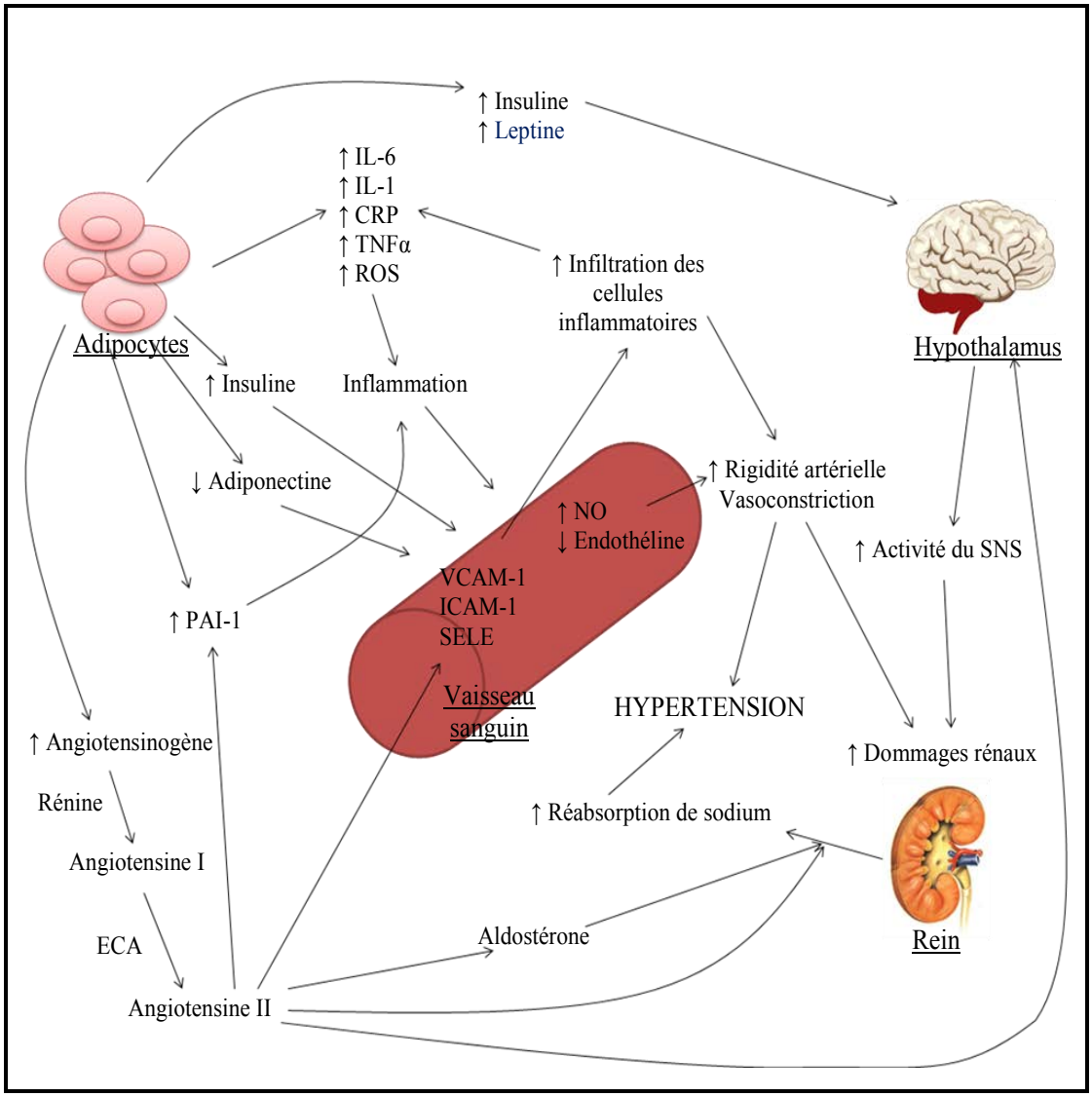
L'obésité est un facteur de RCV majeur étroitement lié à l'HTA , Des arguments épidémiologiques et moléculaires démontrent clairement que les individus obèses ont un risque significativement élevé de développer une HTA et même des complications CV [15;16]. Ainsi, dans la population française, la présence d'une surcharge pondérale multiplie le risque d'être HT par 1,5 chez les femmes et par 2 chez les hommes. La présence d'une obésité multiplie ce risque par 3 et par 5, respectivement [10]. Cela permet d'appréhender le problème majeur de santé publique qu'est l'augmentation progressive de la prévalence de l'obésité. Cette progression constante a été bien mise en évidence aux États-Unis à travers les différentes enquêtes de « *National Health and Nutrition Examination Survey »* qui montrent que la prévalence de l'obésité, définie par un indice de masse corporelle (IMC) supérieur ou égal à 30 kg/m^2, passe de 13% dans l'enquête 1960-1962 à 26% (1999-2000), puis 28% (2001-2002) et 32% (2003-2004) [17]. La fréquence de l'obésité en France métropolitaine a été précisée en 2009 par l'Enquête nationale Nutrition Santé [17]. La moyenne observée de l'IMC est de 25,6 kg/m² alors que 32% de la population ont une surcharge pondérale, et 17% sont obèses [10]. Aux Antilles, les anomalies pondérales sont un peu plus fréquentes, comme l'ont montré les enquêtes de population réalisées durant la même période en Martinique et en Guadeloupe [10]. La prévalence de l'obésité chez les sujets de plus de 18 ans est de

21% en Martinique, et de 23% en Guadeloupe [10]. Des différences de répartition en fonction du sexe sont également à souligner en France métropolitaine et aux Antilles. Ainsi l'enquête nutrition santé montre une fréquence de la surcharge pondérale deux fois plus élevée chez les femmes, comparées aux hommes (respectivement 41% et 24%) [10].

Les différentes voies métaboliques qui pourront être à l'origine de l'augmentation des niveaux de PA via l'obésité [18] sont résumées dans la Figure 3. Premièrement, l'activation du système nerveux sympathique est considérée comme cruciale dans la pathogénèse de l'HTA chez les individus obèses [18]. Deuxièmement, les anomalies rénales ont également été reconnues comme conduisant à l'augmentation des niveaux de PA, notamment la pression natriurétique, la voie du système rénine-angiotensine-aldostérone (SRAA) et la structure même du rein [18]. Troisièmement, des anomalies vasculaires pourront être induites par l'obésité [18]. Ces dérèglements métaboliques conduisant à une augmentation des niveaux de PA sont principalement reliés aux éléments lipidiques associés à l'obésité ainsi qu'aux mécanismes inflammatoires et hormonaux [18]. En effet, les cellules adipeuses produisent des adipokines telles que la *Leptin* (LEP), des espèces réactives d'oxygène (ROS) ainsi qu'un certain nombre de molécules pro et/ou inflammatoires (*interleukin-1*, IL-6 et TNF-α), des facteurs angiogéniques (*vascular endothelial growth factor*), des composants modulant l'homéostasie

(*plasminogen activator inhibitor-1*) et la CRP [18] qui sont à l'origine d'une dysfonction endothéliale (la dysfonction endothéliale sera abordée dans la suite du chapitre) [18]. De plus, la LEP a été identifiée comme molécule servant de lien entre le tissu adipeux et l'activation du système nerveux sympathique entrainant l'augmentation des niveaux de PA [19]. La leptine agit également sur l'endothélium vasculaire en diminuant l'expression de l'oxyde nitrique synthase [19]. A son tour, l'insuline, plus exprimée chez les sujets obèses que chez les sujets minces, est également associée à la dysfonction endothéliaux en entrainant la baisse de production d'oxyde nitrique ainsi qu'en favorisant l'effet vasoconstricteur de l'endothéline-1 (Figure 3) [18].

Figure 3: Voies métaboliques impliquées dans l'augmentation des niveaux de pression artérielle via l'obésité (d'après Ktosis V et al [9]).



L'obésité est associée à une augmentation des niveaux de pression artérielle conduisant à l'hypertension via l'inflammation. Ainsi le système rénine-angiotensine-aldostérone, la rigidité vasculaire, le système nerveux sympathique et le fonctionnement rénal sont perturbés par une série d'évènements inflammatoires.

CRP : *c reactive protein*, ECA : enzyme de conversion de l'angiotensine, ICAM-1 : *intercellular adhesion molecule-1*, IL-6 : *interleukine 6*, IL-1 : *interleukine 1*, NO : oxyde nitrique , PAI-1 : *plasminogen activator inhibitor-1*, ROS : espèces oxygénées réactives , SNS: système nerveux sympathique , TNFα : *tumor necrosis factor alpha*, VCAM-1 : *vascular cell adhesion molecule-1*, SELE : *E-selectin,* ↑ : augmentation, ↓ : diminution.

Le SRAA est également montré activé par l'obésité [18]. Particulièrement, la rénine, l'angiotensinogène, l'angiotensine II et le récepteur de type 1 de l'angiotensine II ont été trouvés en abondance dans le tissu adipeux [18]. Le SRAA serait donc activé de manière continue entrainant une augmentation chronique des niveaux de PA [18].

I-5.2. Inflammation

L'idée envisageant que l'HTA et l'inflammation sont en quelque sorte liées émane des études transversales et prospectives montrant que les taux des molécules inflammatoires circulantes sont augmentés chez les patients HT, et que leurs niveaux permettent de prévoir l'occurrence d'HTA [20]. Certaines études transversales ont montré que, par rapport aux normotendus (NT), les taux plasmatiques de marqueurs inflammatoires, notamment *C reactive protein* (CRP), les cytokines, telles que *tumor necrosis factor alpha* (TNF-α) et *interleukin 6* (IL-6), les chimiokines, telle que *monocyte chemoattractant protein* (MCP-1), et les molécules d'adhésion, telles que *P-selectin* (SELP) et *E-selectin* (SELE), sont augmentées chez les patients HT (16464884). Les mêmes associations ont été répliquées chez les sujets considérés pré-HT, donc ayant un seuil de PAS entre 120 mmHg et 139 mmHg et de PAD entre 80-89 mmHg [21].

Chez les rongeurs, il a été montré que l'HTA agit comme un déterminant majeur de la dysfonction endothéliale [22-24]. En accord avec cette théorie, il a été démontré que la réponse inflammatoire peut se développer dans les artères des rats modèles d'HTA. Ce phénomène est caractérisé par l'expression des molécules inflammatoires (notamment IL-1, IL-6, TNF-α) et des molécules d'adhésion *intercellular adhesion molecule-1* (ICAM-1) et *vascular cell adhesion molecule-1* (VCAM-1) via l'activation du facteur de transcription *nuclear factor-kappaB* (NF-κB) [22-24].

I-6. Dysfonction endothéliale

I-6.1. Définition

L'endothélium vasculaire est la couche la plus interne des vaisseaux sanguins en contact avec le sang et il forme avec la membrane basale l'intima [25]. En conditions normales, l'endothélium participe à la réaction immunitaire, contrôle la coagulation sanguine en participant à la fois à l'hémostase et à la fibrinolyse [25], exerce également un rôle prépondérant dans le contrôle du tonus vasculaire [25], de l'inflammation et du stress oxydatif en plus de moduler la migration et la prolifération cellulaire [25].

La dysfonction endothéliale est un état pathologique systémique de la paroi interne des vaisseaux sanguins qui est due à un déséquilibre entre la production des facteurs vasodilatateurs et vasoconstricteurs [25], la résultante détermine alors le tonus vasculaire (Figure 4) [25]. Il en résulte une augmentation de la perméabilité de l'endothélium vasculaire aux cellules du sang périphérique (telles que les monocytes, les lymphocytes T et les granulocytes) et aux lipoprotéines suite à la production de *E-selectin* (SELE) [25]. Cette dernière est une molécule d'adhésion cellulaire exprimée par les cellules endothéliales activées par les cytokines inflammatoires [25]. Par conséquent, elle est considérée comme biomarqueur de la dysfonction endothéliale [25].

I-6.2. Dysfonction endothéliale et liens avec l'hypertension artérielle

L'HTA est caractérisée par une dysfonction endothéliale et une réduction de la vasodilatation médiée par l'endothélium [26] chez les patients HT ainsi que chez les modèles animaux [26]. De nombreuses études ont montré qu'une dysfonction endothéliale est caractérisée par une diminution des relaxations endothéliales, dûe notamment à la réduction du monoxyde d'azote (NO), une étape clé qui se développe précocement lors d'HTA [26].

Lors de l'inflammation, la SELE joue un rôle important dans le recrutement des leucocytes (les monocytes, les lymphocytes T et les granulocytes) au site de lésion (Figure 5) [27]. La libération locale de cytokines inflammatoires par les cellules

endommagées induit la surexpression de la SELE à la surface des cellules endothéliales vasculaires se trouvant à proximité (Figure 5) [27]. Les leucocytes sanguins vont lier la SELE par leurs ligands spécifiques, permettant aux leucocytes de «rouler» le long de la surface interne du vaisseau sanguin (Figure 5) [27]. Suite à la progression de la réponse inflammatoire, les chimiokines libérées par le site de lésion activent les leucocytes « roulants », qui sont maintenant capables de se lier fortement à la surface endothéliale et de commencer à faire leur chemin dans les tissus enflammés (Figure 5) [27].

I-7. Espèces réactives de l'oxygène

I-7.1. Biosynthèse

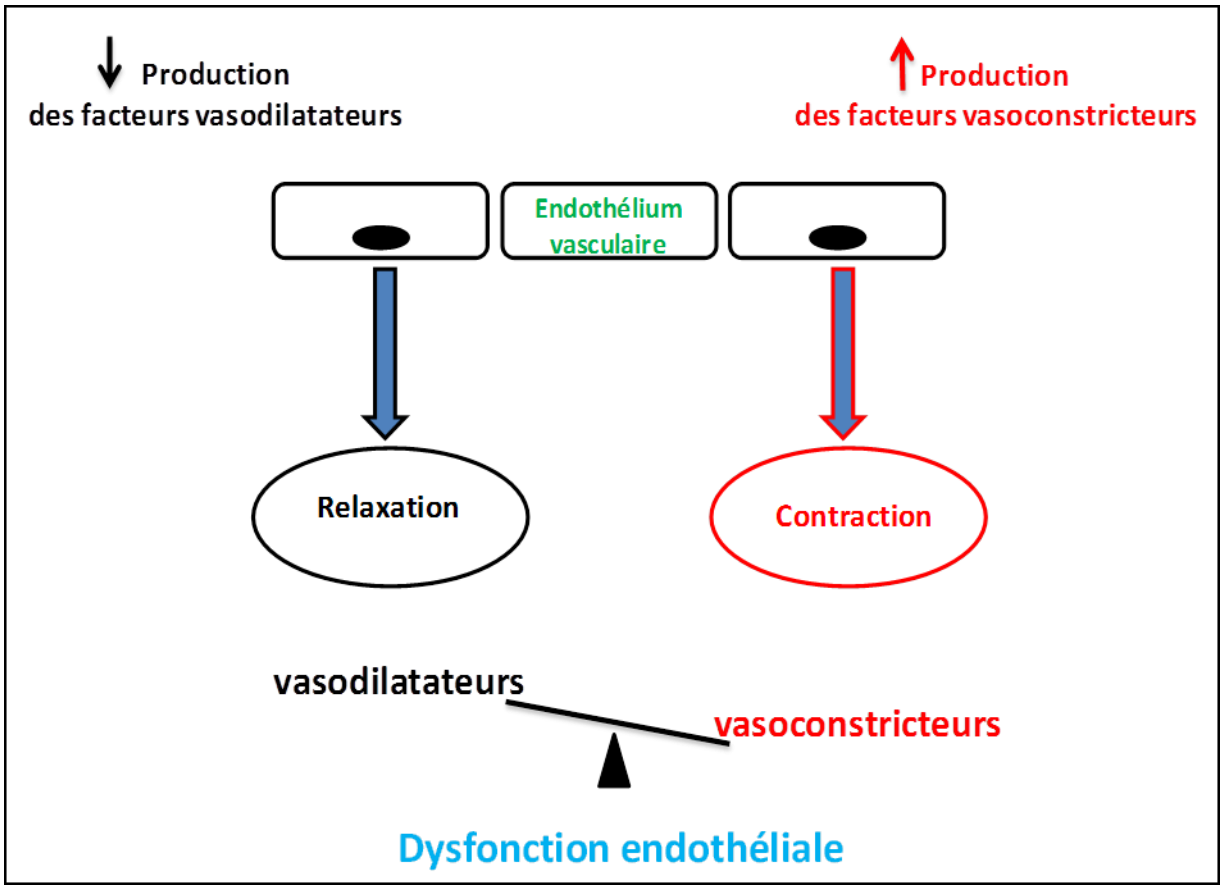
L'oxygène, si essentiel à la respiration et à la production d'énergie, est aussi la source du stress oxydatif. Les ROS sont les radicaux libres [anion superoxyde (O_2^-) et radical hydroxyl OH^-] et les substances chimiques oxydantes non radicalaires (peroxyde d'hydrogène H_2O_2 et peroxynitrites ONO_2^-) dérivés de l'oxygène [28]. Les ROS sont capables de modifier entre autres l'ADN et les protéines pour conduire à des anomalies de multiplication cellulaire [28].

Les O_2^- est produit par la réduction d'un électron sur la molécule d'oxygène survenant en majeure partie au niveau des cytochromes mitochondriaux [28]. Leur contrôle nécessite des mécanismes d'éliminations afin de se débarrasser de leurs effets délétères [29]. La Figure 6 illustre un de ces mécanismes qui consiste à la réduction complète des radicaux libres de l'oxygène en eau (H_2O) [29]. Les superoxydes dismutases (SOD) catalysent la réaction entre l'O_2^-, un électron et deux protons pour former le H_2O_2 alors que la catalase et les peroxydases transforment ce dernier en H_2O [29].

I-7.2. Espèces réactives de l'oxygène et liens avec l'hypertension artérielle

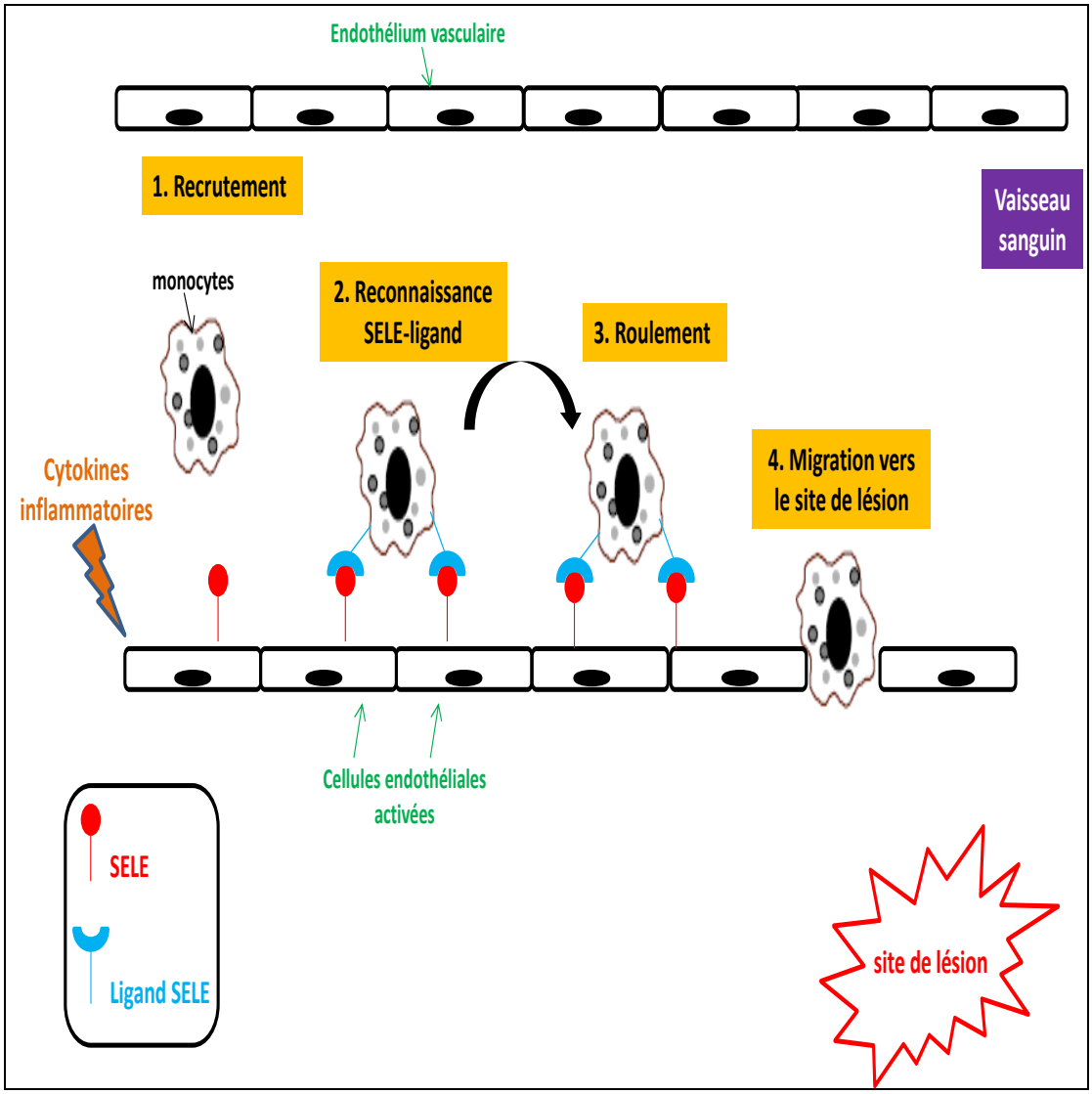
Au niveau vasculaire, les ROS contribuent à la dysfonction endothéliale [30], à la genèse et au maintien de l'HTA par plusieurs mécanismes [30]: via l'inactivation du

Figure 4: La dysfonction endothéliale.



La dysfonction endothéliale résulte de l'augmentation de l'expression des vasoconstricteurs produits par l'endothélium vasculaire.

Figure 5: Recrutement des monocytes au site de lésion lors d'une dysfonction endothéliale.



Suite à la libération des cytokines inflammatoires, la SELE joue un rôle important dans le recrutement des monocytes au site de lésion. Les cellules circulantes qui subissent l'inflammation induisent la surexpression de la SELE à la surface des cellules endothéliales vasculaires à proximité. Les monocytes sanguins vont lier la SELE par leurs ligands spécifiques, causant leur roulement tout au long de la surface interne du vaisseau sanguin. Ensuite les monocytes commencent à faire leur chemin pour joindre le site de lésion.

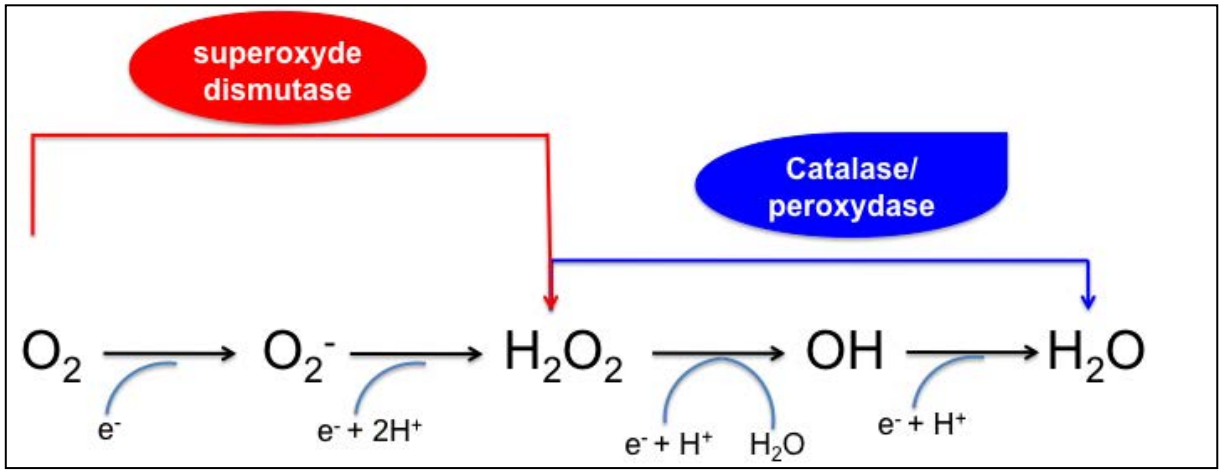
SELE : *E-selectin.*

NO [30-32], via la peroxydation de l'acide arachidonique [31] et via la déplétion du cofacteur BH4 [22]. La biodisponibilité des ions O_2^- est liée aux mécanismes endogènes enzymatiques [catalase, SOD et glutathione peroxydase] et non-enzymatiques (hème, transferrine, vitamines anti-oxydantes) qui les piègent et les désactivent [33]. En ce sens, il a été démontré que l'Angiotensine II stimulait la synthèse de O_2^- en augmentant l'activité de l'enzyme NADP(H) oxydase tant dans les cellules vasculaires musculaires lisses en culture que dans les aortes de rats rendus HT par infusion d'Angiotensine II [34]. L'HTA et la réactivité vasculaire induite ont pu être renversées par l'administration exogène de SOD [34].

I-8. Héritabilité génétique de l'hypertension artérielle

Au contraire des formes rares d'HTA familiales, l'HTA essentielle est un trait polygénique ayant une héritabilité (variance phénotypique imputable à la variation génétique) estimée entre ≈31 à ≈68 % [35]. D'autre part, les formes rares sont des traits monogéniques causées par des mutations exerçants un large effet phénotypique [35;36]. La contribution génétique dans l'HTA essentielle se présente sous la forme de plusieurs allèles géniques (de nombres et nature inconnus) qui pourront altérer la fonction et/ou l'expression des protéines codées, et par conséquence créer des phénotypes intermédiaires qui se compliqueraient en HTA [37]. Les études familiales d'HTA essentielle ont montré que son héritabilité est de ≈31 % (mesure unique de la PAS et PAD), ≈57 % (moyenne à long terme du phénotype PAS et PAD) et ≈68 % (profil de la PAS et de la PAD sur 24 heures) [35].

Figure 6: Réduction de l'O_2 en H_2O



Cascade de réduction de l'oxygène en eau. (e^-) électron, (H^+) proton, (SOD) superoxyde dismutase, (O_2^-) anion superoxyde, (H_2O_2) peroxyde d'hydrogène

II. ETIOLOGIES GENETIQUES DES FORMES MONO ET POLYGENIQUES DE L'HYPERTENSION ARTERIELLE ET APPROCHES EMPLOYEES POUR LES REVELER

II-1. Polymorphisme génétique ponctuel

Plusieurs formes de marqueurs génétiques existent tout au long du génome, les plus fréquents étant les polymorphismes génétiques ponctuels (SNPs) ou *Single nucleotide polymorphisms*. Le SNP est le changement d'un seul nucléotide au niveau d'une paire de bases (pb) composée habituellement de deux allèles, et concernant des individus d'une même espèce [38]. Ces variations sont très fréquentes [38]. Les SNPs représentent 90% de l'ensemble des variations génétiques humaines, et présentent une fréquence allélique supérieure ou égale à 0,5 %. Ils sont présents chaque 1,000 pb en moyenne dans le génome humain [38].

Les SNPs peuvent se retrouver au sein des exons, des introns, ou même dans des régions intergéniques (entre les gènes) [38]. Dans le cas où les SNPs se retrouvent au sein des régions codantes, celles-ci ne vont pas obligatoirement modifier la séquence d'acides aminés de la protéine produite, et ce, grâce à la redondance du code génétique [38].

Les SNPs peuvent également se retrouver dans des régions génomiques non codantes qui pourraient avoir des conséquences sur l'épissage génique, les sites de liaisons des facteurs de transcription, ou sur le temps de demi-vie des acides ribonucléiques (ARN). On parlera de formes allélique *synonymes* dans le cas où un autre allèle d'un SNP mène à la même séquence polypeptidique, et de formes *non synonymes* dans le cas où les séquences polypeptidiques produites sont différentes [38].

La fréquence allélique et le nombre de variants génétiques agissant sur un même trait déterminent leurs impacts sur ce même trait (Tableau 1).

Tableau 1: Effets phénotypiques et fréquences allèliques correspondantes dans le cas des maladies génétiques complexes : le cas de l'hypertension artérielle (d'après Ehret GB [26]).

Variant génétique	Type	Fonction	Effet phénotypique	Fréquence allélique
Nonsense	Codant	Terminaison prématurée du mécanisme de traduction	Très large	Très rare
Non-synonyme et non conservé	Codant	Changement d'acide aminé avec altération de la structure protéique	Moyen-très large	Rare
Non-synonyme et conservé	Codant	Changement d'acide aminé sans altération de la structure protéique	Faible-très large	Rare
Insértion/délétion avec changement du cadre de lecture	Codant	Changement du cadre de lecture de la séquence protéique	Très large	Rare
Insértion/délétion sans changement du cadre de lecture	Codant ou non codant	Changement de la séquence protéique	Faible-très large	Rare
Synonyme	Codant	Altération possible du mécanisme d'épissage génique	Faible-large	Moyenne
Promoteur ou séquences non traduites	Promoteur, 5'UTR/3'UTR	Modification éventuelle de l'expression ou de la stabilité de l'ARNm concerné	Faible-large	Rare-Moyenne
Site d'épissage/ inerface intron-exon	Exonique	Altération du mécanisme d'épissage génique	Faible-large	Rare
Intronique	Intronique	Modification éventuelle de l'expression ou de la stabilité de l'ARNm concerné	Très faible	Moyenne
Intergénique	Non codant	Modification éventuelle de l'expression des gènes à proximité	Très faible	Haute

Selon son effet phénotypique exercé sur l'organisme, la pression de la sélection détermine la fréquence d'un allèle dans une population générale. Ainsi les maladies génétiques les plus graves sont causées par des allèles rares comme dans les cas d'HTA Mendélienne. Par contre, les maladies polygéniques à faible effet phénotypique sont les plus répandues comme dans les cas d'HTA essentielle.

II-2. Recombinaison génétique

L'ensemble des gènes se distribue le long des chromosomes. *A priori*, lors de la transmission du matériel génétique à la descendance, les allèles d'un même gène (ou locus) devraient être transmis en un seul bloc grâce au lien physique existant entre eux sur le même chromosome [39]. Cependant en prophase de méiose 1, un échange des séquences génomiques aura lieu entre les chromosomes homologues une fois appariés [39].

La recombinaison ou *crossing over* indique le phénomène aléatoire par lequel apparaissent des combinaisons génétiques nouvelles dans une cellule d'un individu donné [39]. Ces combinaisons d'allèles seront différentes de celles observées chez les cellules des individus parentaux [39]. La Figure 7 illustre la recombinaison entre 3 différents allèles d'une paire de chromosomes.

La distance physique séparant la séquence d'ADN est directement proportionnelle au nombre de recombinaisons entre différents allèles d'un même chromosome [39]. Il est alors possible de calculer une distance génétique estimée à partir du taux de recombinaisons [39]. Son unité de mesure est le centiMorgan (cM).

II-3. Déséquilibre de liaison

Le déséquilibre de liaison ou *linkage disequilibrium* (LD), est l'association non aléatoire entre les allèles d'un ou plusieurs loci présents sur un même chromosome au sein d'une population donnée [39]. Le LD est calculé à partir des allèles pour définir des haplotypes [39]. Le LD est observé habituellement sur des distances courtes de moins de 10 kilobase (Kb), mais il pourrait être également présent sur de longues distances couvrant jusqu'à 100Kb [39].

A l'origine, le LD provient du lien physique existant entre les loci présents sur un même chromosome et dont leurs allèles sont transmis d'une génération à une autre [39]. Deux facteurs, physiques et statistiques, pourront modifier le LD. Au fur et à mesure des générations, des évènements de *crossing over* rompent ce lien entre les

allèles et entrainent par la suite une disparition du LD [39]. Etant de nature aléatoire, le nombre de *crossing over* entre les allèles dépend de la distance qui les sépare [39]. Plus les allèles sont proches, plus le taux de recombinaison est faible, et plus le LD serait fort [39].

Un deuxième facteur important, qui pourrait fortement altérer le LD, est le phénomène de stratification des populations observé lors des études d'association génétiques [39]. Ce facteur crée des faux positifs entre différents allèles, à cause de la présence de plusieurs sous-populations présentant des fréquences alléliques différentes, au sein de la population étudiée [39]. De même, nous pourrons également distinguer d'autres facteurs qui pourront moins significativement modifier le LD, tels que la sélection, la consanguinité, les mutations et la taille de l'échantillon étudié [39].

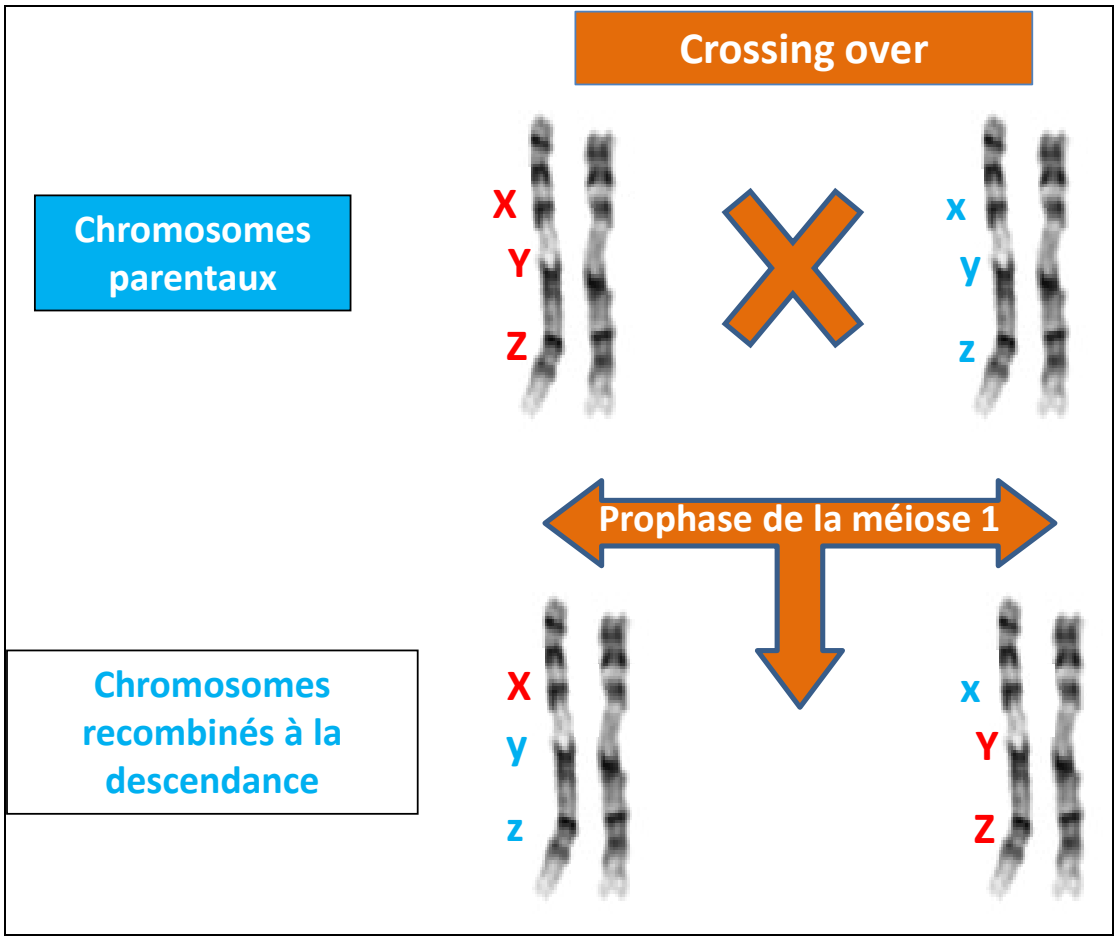
Les mesures de LD sont des mesures d'association qui quantifient l'écart entre les fréquences haplotypiques observées et celles attendues sous hypothèse d'indépendance entre les allèles [39]. Les mesures de LD les plus couramment utilisées sont D' et r^2 [39]. Ce sont des mesures réalisées sur des paires d'allèles [39]. La Figure 8 illustre les mesures de LD entre les différents allèles des SNPs d'une région génomique de 12Kb.

II-4. Approche gène candidat

Les gènes candidats sont des gènes codant pour des protéines pouvant être impliquées dans la régulation de la PA [40]. L'approche gène candidat implique l'utilisation des techniques épidémiologiques classiques, les études transversales et longitudinales de population pour les traits continus (PA) et les études cas-témoins pour les traits dichotomiques (HTA).

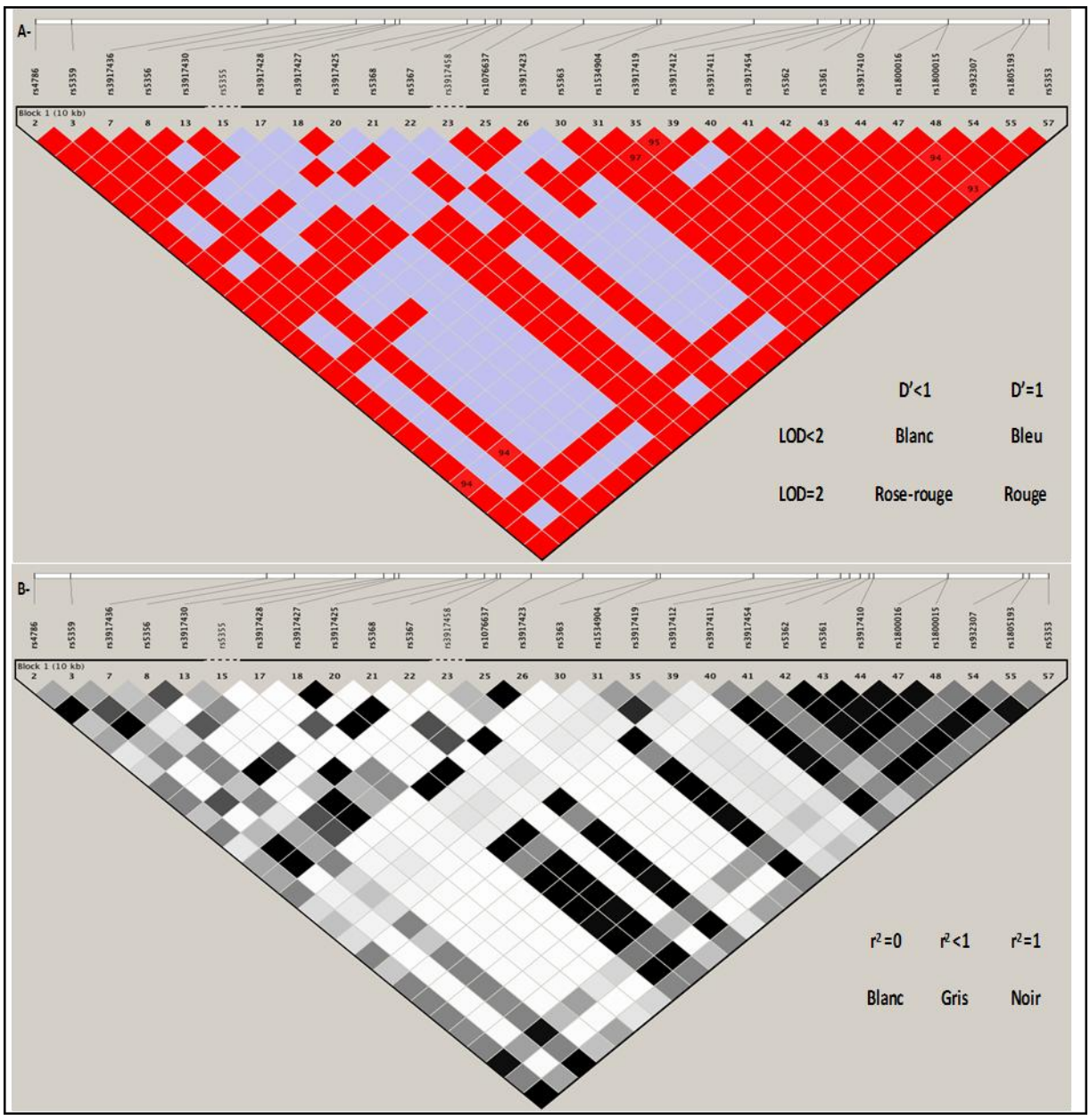
Cette approche peut se concentrer sur un ou plusieurs gènes et a pour but de déterminer si l'HTA est associée à certains marqueurs génétiques, constitués le plus fréquemment de SNPs [40].

Figure 7*:* Schéma d'un *crossing over* entre deux chromosomes homologues en prophase de méiose 1.



Trois allèles sont étudiés. Chez les parents, le premier chromosome, possède une combinaison d'allèles de séquence XYZ tandis que le second chromosome, possède la séquence xyz. Après recombinaison entre les différents allèles, la séquence du chromosome 1 est Xyz et celle du chromosome 2 est xYZ.

Figure 8: Les mesures D' et r^2 entre les allèles de paires de *single nuceotide polymorphism* sur une séquence génomique de 12Kb**.**



La matrice triangulaire des dépendances est représentée pour les mesures de D' (A) et r^2 (B). Le LD est directement proportionnel aux valeurs de D' et r^2**.**

A : Plus la couleur des triangles devient rouge, plus la valeur de D' augmente et plus le LD entre les allèles d'une paire de SNP est fort.

B : Plus la couleur des triangles devient noir, plus la valeur de r^2 augmente et plus le LD entre les allèles d'une paire de SNP est fort.

LD : linkage disequilibrium, SNP : *single nuceotide polymorphism.*

II-4.1. Formes monogéniques d'hypertension artérielle

Une dizaine de formes héréditaires rares d'HTA (formes mendéliennes) ont été identifiées depuis les années soixante. Les formes les plus connues sont montrées dans le Tableau 2 avec leurs phénotypes et leurs mutations [26]. Il s'agit souvent de mutations de gènes impliqués dans les mécanismes de réabsorption de sel au niveau des reins [26], et leur découverte a aidé à mieux comprendre les mécanismes impliqués dans la régulation de la PA [25]. Les formes monogéniques sont caractérisées par des mutations à haute pénétrance (à savoir une forte probabilité de développer la maladie chez la personne concernée), résultant en une perte ou un gain de fonction important.

II-4.2. Formes polygéniques d'hypertension artérielle

Elles représentent 95% des cas d'HTA [41]. Il existe à l'heure actuelle plus de 110 gènes candidats d'HTA essentielle, identifiés grâce aux études de gène candidat chez l'Homme. L'un des plus étudiés est probablement le gène *angiotensin I converting enzyme* (*ACE*). Les personnes portant le génotype Délétion/Délétion ont une concentration plasmatique d'enzyme de conversion de l'angiotensine deux fois plus élevée que les personnes portant un génotype insertion/insertion ou insertion/délétion [42]. Le Tableau 3 montre une liste de quelques gènes candidats trouvés associés avec la PA.

II-5. Etudes de liaison génétique au sein des familles

En raison du processus de recombinaison ayant lieu pendant la méiose entre les brins des paternels et maternels, des allèles dont les loci sont topographiquement proches sur un même chromosome, seront moins souvent séparés que des allèles dont les loci sont éloignés [29]. Ce qui fait que les SNPs adjacents localisés sur le même brin chromosomique forment un haplotype [29].

Tableau 2: Formes monogéniques d'hypertension artérielle.

HTA	Mode de transmission	Gène	Mutations et conséquences fonctionnelles	phénotype
Hyperaldostéronisme de type 1	AD	Fusion de *CYP11b1* et *CYP11b2*	Gène chimérique sous contrôle d'ACTH	HTA, hypoK, hyperA
Hyperaldostéronisme de type 2	AD	Locus chromosome 7p22	Surproduction d'aldostérone dans glandes surrénaliennes	HTA, hypoK, hyperA
Syndrome de Liddle	AD	*SCNN1B*, *SCNN1G*	Activation constitutive du canal sodique épithéliale dans le tube distal	HTA, hypoK, hypoA
Hyperplasie congénitale surrénalienne	AR	*CYP11B1*	Déficit de l'enzyme11b hydroxylase	HTA, hypoK, hypoA
Déficit d'11 β-OH stéroïd déhydrogénase type 2	AR	*HSD11B1*	Déficit de désactivation de cortisol	HTA, hypoK, hypoA
Syndrome de Gordon	AD	*WNK1*, *WNK4*	Activation constitutive du cotransporteur Na/Cl dans le tube distale	HTA, hyperK, hypoA
Mutations de récepteur PPAR-γ	AD	*PPARG*	Perte de fonction du récepteur	HTA, résistance à l'insuline, diabète
Syndrome d'HTA, hypercholestérolémie, hypomagnésémie	Mitochondriale	Non identifié	Substitution de cytidine dans les ARNt mitochondriaux	HTA, hypercholestérolémie, hypomagnésémie

HTA : Hypertension artérielle, AD : autosomal dominant, AR : autosomal récessif, K :Kaliémie, A : Aldostéronisme.

Tableau 3: Liste des gènes candidats (probablement) impliqués dans la régulation de la pression artérielle.

Voies métaboliques	Symbole	Localisation	Nomenclature
Système rénine-angiotensine-aldostérone	*ACE*	17q23	*Angiotensin converting enzyme*
	AGT	1q42-q43	*Angiotensinogen*
	AGTR1	3q21-q25	*Angiotensin II receptor, type 1*
	AGTR2	Xq22-q23	*Angiotensin II receptor, type 2*
	CYP11B1	8q22	*Cytochrome P450, family 11, subfamily B, polypeptide 1*
	CYP11B2	8q24.3	*Cytochrome P450, family 11, subfamily B, polypeptide 2 , aldosterone synthase*
	REN	1q32	*Renin*
Système nerveux sympathique	*ADRB1*	10q24-q26	*Adrenergic, beta-1-, receptor*
	ADRB2	5q32-q34	*Adrenergic, beta-2-, receptor*
	ADRB3	8p12-p11.2	*Adrenergic, beta-3-, receptor*
	DRD1	5q35.1	*Dopamine receptor D1*
	DRD2	11q23	*Dopamine receptor D2*
	DRD3	3q13.3	*Dopamine receptor D3*
	DBH	9q34	*Dopamine beta-hydroxylase (dopamine beta-mono-oxygenase)*
	NPY	7p15.1	*Neuropeptide Y*
	NPY1R	4q31.3-q32	*Neuropeptide Y receptor 1*
	PNMT	17q21-q22	*Phenylethanolamine N-methyltransferase*
Transporteurs du sodium	*SCNN1A*	12p13	*Sodium channel, nonvoltage-gated 1 alpha (alpha ENaC)*
	SCNN1B	16p13-p12	*Sodium channel, nonvoltage-gated 1 beta (beta ENaC)*
	SCNN1G	16p13-p12	*Sodium channel, nonvoltage-gated 1 gamma (gamma ENaC)*
	SLC12A3	16q13	*Solute carrier family 12 (sodium/chloride transporters), member 3*
	SLC12A1	15q15-q21.1	*Solute carrier family 12 (sodium/potassium/chloride transporters), member 1*
Stéroïdes	*HSD11B2*	16q22	*Hydroxysteroid (11-beta) dehydrogenase 2*
	NR3C2/ MLR	4q31.1	*Nuclear receptor subfamily 3, group C, member 2 , mineralocorticoid receptor*
Peptides natriurétiques	*NPPB*	1p36.2	*Natriuretic peptide precursor B*
	NPPA	1p36.21	*Natriuretic peptide precursor A*
	NPPC	2q24-qter	*Natriuretic peptide precursor C*
	NPR3	5p14-p13	*Natriuretic peptide receptor C/guanylate cyclase C*
Autres voies	*ABCB1*	7q21.1	*ATP-binding cassette, sub-family B (MDR/TAP), member 1*
	ADD1	4p16.3	*Adducin 1 (alpha)*

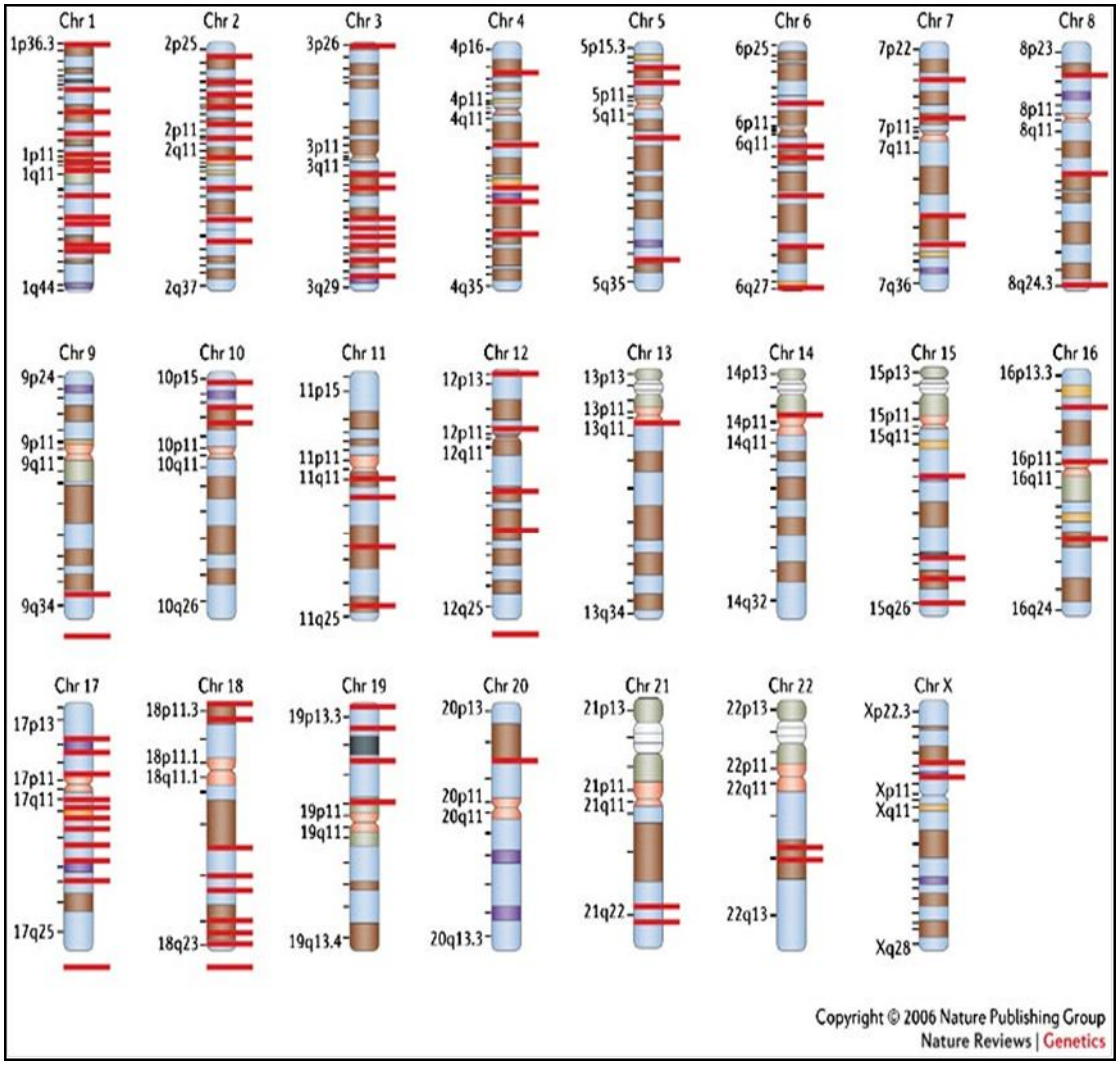
ADD2	2p14-p13	*Adducin 2 (beta)*
ADD3	10q24.2-q24.3	*Adducin 3 (gamma)*
CYP3A5	7q22.1	*Cytochrome P450 family 3, subfamily A, polypeptide 5*
GNB3	12p13	*Guanine nucleotide binding protein (G protein), beta polypeptide 3*
EDN-1	6p24.1	*Endothelin-1*
LPL	8p22	*Lipoprotein lipase*
NOS3	7q36	*Nitric oxide synthase 3*
PPARG	3p25	*Peroxisome proliferator-activated receptor gamma*

L'analyse de liaison ou *Linkage analysis* étudie la fréquence avec laquelle les allèles des SNPs sont transmis avec le locus du trait d'intérêt (PA ou HTA) à la prochaine génération (parents-enfants), sans qu'il n'y ait eu de recombinaison pendant la méiose [29].

Dès les années 1990, les généticiens se sont concentrés sur des analyses de liaison génétique, afin d'identifier des gènes impliqués dans la régulation de la PA et l'HTA. L'analyse de liaison se fait habituellement sur l'ensemble du génome, ainsi nommé *genome-wide linkage analysis* (*GWLAs*). Du fait que les GWALs ne sont pas basées sur des connaissances *a priori*, elles peuvent éventuellement identifier de nouveaux gènes candidats. Ceci génère de nouvelles hypothèses sur la pathogenèse de l'HTA. A l'heure actuelle, il a été montré que tous les chromosomes humains contiennent des régions significativement liées à la PA et à l'HTA (Figure 9) [13]. Cependant, seul le bras long du chromosome 2 (chr2.q) est lié à la PA et/ou l'HTA dans 9 populations européennes différentes [43-51], et 5 non-européennes notamment , africaines [52], afro-américaines [53], chinoises [54;55] et samoanes [56].

Il est possible d'établir l'occurrence génétique de la PA et de l'HTA au sein d'une famille montrant une transmission génétique d'une génération à une autre [29]. Ceci est fait, en calculant une probabilité de co-ségrégation entre le phénotype et les blocs de recombinaison de tous les membres de la famille à l'échelle génomique [29]. Pour calculer cette probabilité, les généticiens utilisent un *LOD-score* (pour *logarithm of odds* ou logarithme des probabilités) [29]. Le *LOD-score* compare la liaison observée avec la probabilité que celle-ci soit un événement aléatoire et permet donc d'obtenir une mesure de la certitude de l'observation sur une échelle logarithmique [29]. Si le *LOD-score* est supérieur à 3, il est alors possible de combiner les limites des blocs de recombinaison de chaque individu afin de déterminer l'étendue génétique du locus ainsi identifié (Figure 10). Les régions génomiques identifiées par *genome-wide linkage analysis* pourront contenir une multitude de gènes et donc des études plus approfondies seront nécessaires pour identifier le(s) gène(s) candidat(s).

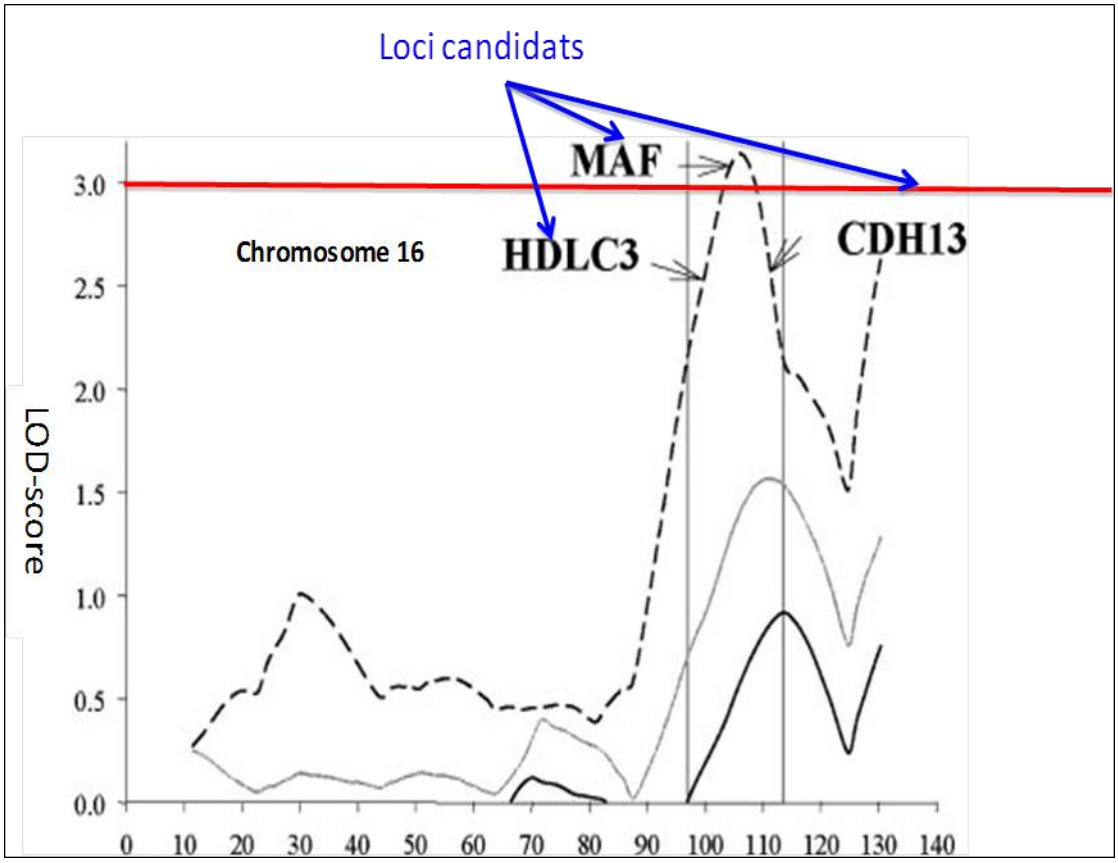
Figure 9: Régions génomiques liées à l'hypertension artérielle dans le génome humain (d'après Cowley AW Jr [4]).



Les régions chromosomiques liées à l'HTA à travers le génome sont représentées par des barres rouges. Les Barres rouges situées en dessous des chromosomes caractérisent les régions pour lesquelles aucun emplacement n'a été identifié.

HTA : hypertension artérielle.

Figure 10: Exemple d'un graphique représentant le LOD-score d'une liaison génétique.



Le LOD-score compare la liaison observée avec la probabilité que celle-ci soit un événement aléatoire. Supérieur à 3, il permet de combiner les limites des blocs de recombinaison de chaque individu afin de déterminer l'étendue génétique du locus identifié.

II-6. Approche pangénomique

Les études d'association pangénomique ou *genome-wide association studies* (GWAS) utilisent des cartes denses de SNPs localisés sur l'ensemble du génome humain (sauf le chromosome Y) afin de rechercher des associations entre ces différents variants génétiques et le phénotype étudié [35]. Contrairement à l'approche gène candidat qui cible un ensemble de gènes potentiels, les GWAS investiguent une grande partie du génome sans aucun *a priori* sur l'identité des gènes impliqués [35]. Cette approche présente l'avantage de permettre la découverte de nouvelles connaissances sur les SNPs causaux non identifiés auparavant [35]. Le concept des GWAS est basé sur l'idée suivante : 'une maladie commune, des variants génétiques communs', ce qui implique que les maladies complexes communes sont gouvernées par plusieurs SNPs présents chez plus de 1-5% de la population étudiée [35]. Le nombre de SNPs que nous pouvons étudier simultanément grâce à des puces d'ADN a rapidement augmenté ces dernières années et, actuellement, un million de SNPs sont génotypés simultanément dans les GWAS [35]. Ainsi, les GWAS ont permis un grand pas en avant par rapport aux études de liaison génétiques, car elles ont été conduites sur des milliers d'individus ethniquement homogènes, en identifiant des centaines de milliers de SNPs à la fois [35]. A ce jour, le catalogue des GWAS a répertorié 1,596 études effectuées sur 249 traits (Figure 11) [57]. Quant aux GWAS, une valeur de P inférieure à 0,05 (seuil habituellement utilisé en statistique) entre un SNP et un trait testé, ne suffit pas pour être considérée comme significative [25]. Celle-ci doit avoir une valeur inférieure au seuil Bonferoni, et donc $Pvalue \leq 5x10^{-8}$ (Figure 12) [25].

En 2009, une GWAS effectuée par le *Global BPgen consortium*, incluant 34,433 individus Caucasiens, a pu identifier une association entre la PA et 8 loci [58]. Ces loci étaient *CYP17A1*, *CYP1A2*, *MTHFR*, *SH2B3*, *PLCD3*, *ZNF652*, *FGF5* et *c10orf107* [58]. Seul le gène *CYP17A1* codant pour une protéine qui régule l'activité de l'enzyme stéroïde 17α-hydroxylase (essentielle pour la synthèse de minéralo et glucocorticoïdes), était auparavant associé à une forme rare d'HTA monogénique.

Figure 11: Répertoire des signaux d'association révélés par les études pangénomiques publiées jusqu'au jour.

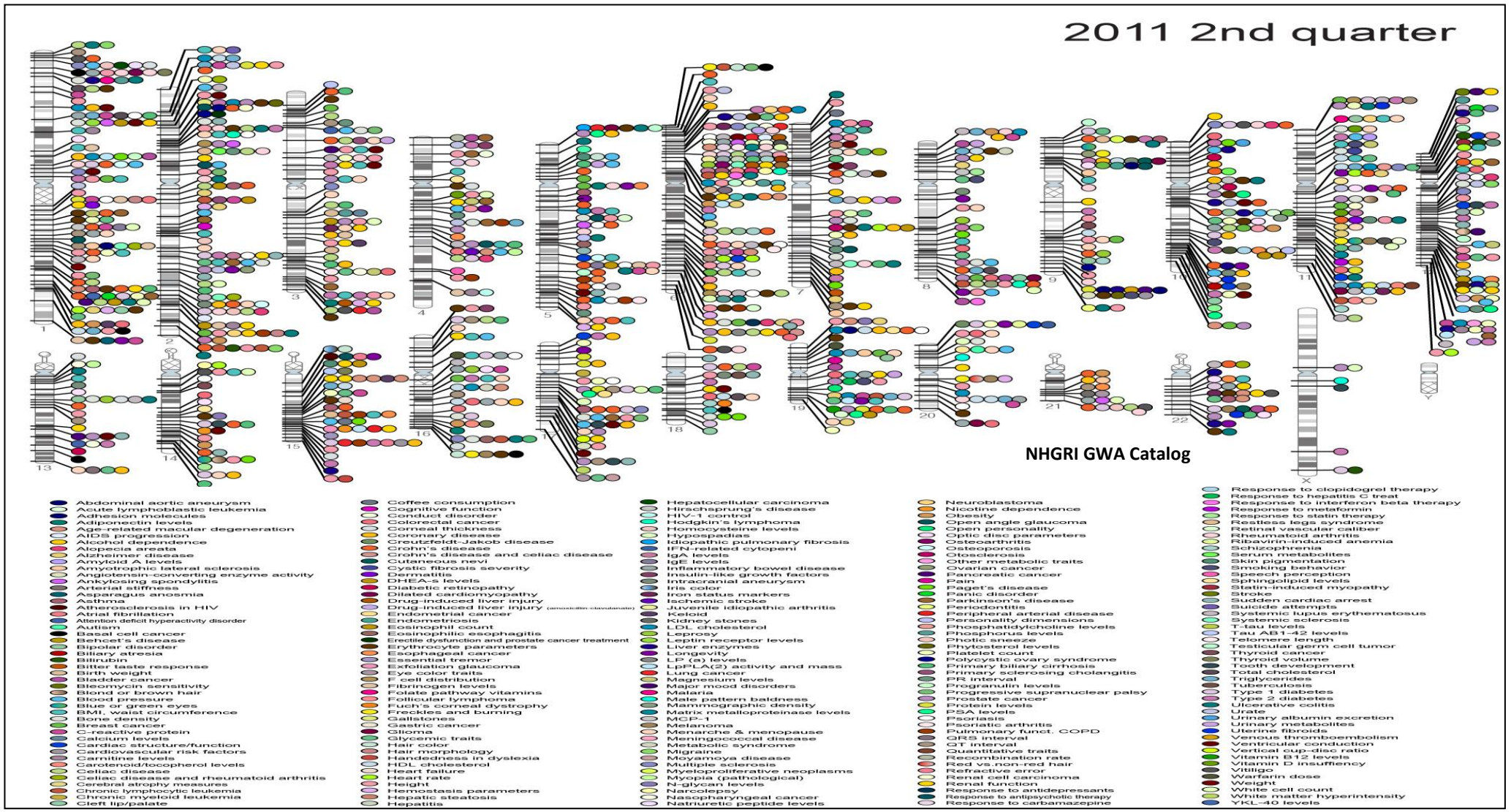
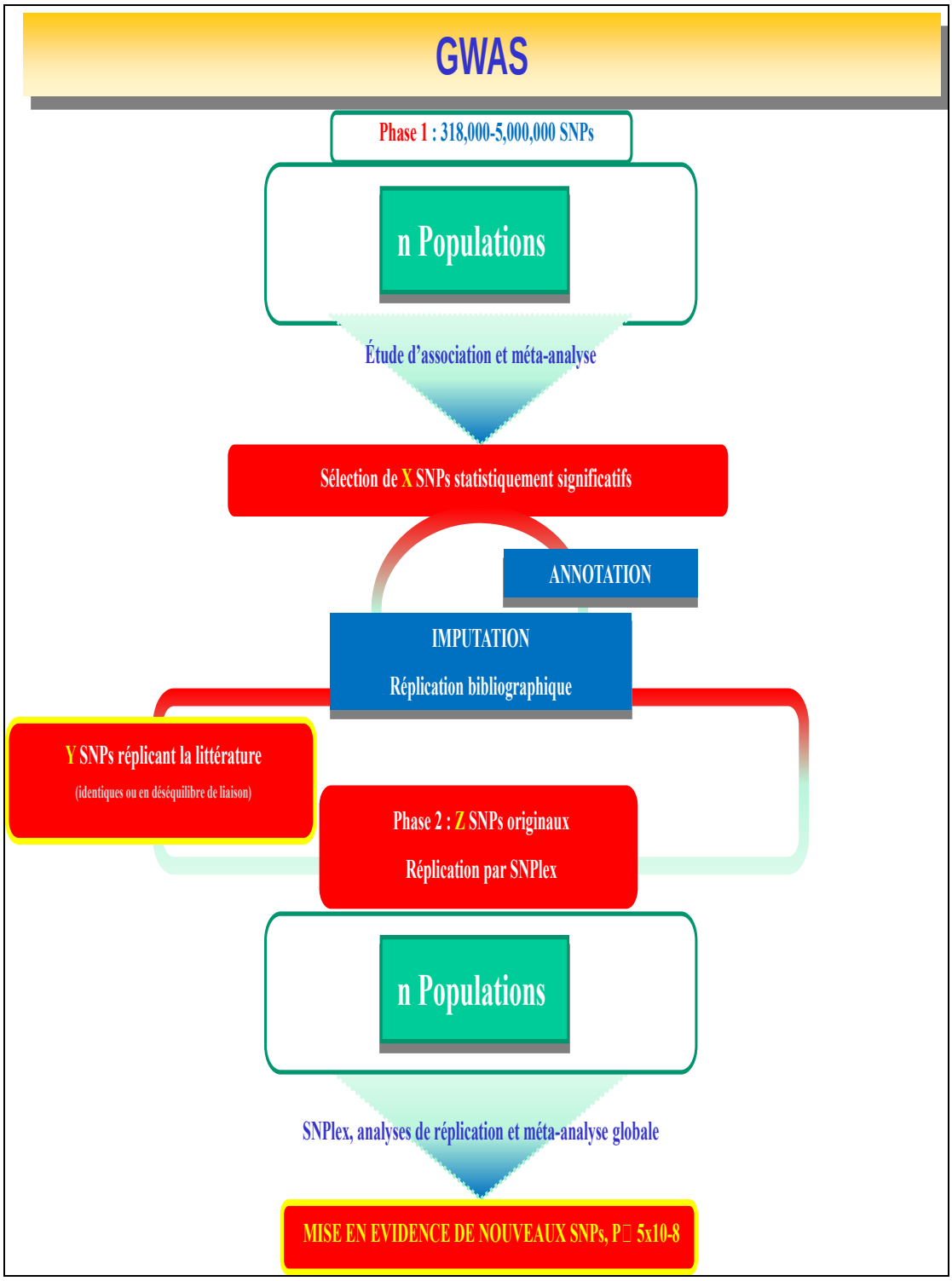


Figure 12: Représention schématique de l'approche pangénomique.



Les différentes étapes des GWAS sont résumées dans la figure ci-dessus. Le seuil $P \leq 5x10^{-8}$ correspond au seuil de Bonferroni.

GWAS : *Genome-wide association studies*.

Le gène *MTHFR* a été déjà associé à l'HTA essentielle chez l'homme [58]. Par contre, *CYP1A2*, *SH2B3, ZNF652*, *FGF5* et *c10orf107* étaient de nouveaux gènes, qui auparavant n'avaient jamais été impliqués dans l'HTA essentielle [58].

De même, *CHARGE consortium* [41], incluant 29,136 individus, a identifié plusieurs régions génomiques pour contenir des gènes candidats , plus précisément treize SNPs ont été associés avec la PAS, 20 avec PAD et 10 avec l'HTA ($P< 4\times10^{-7}$) [41]. Ce même consortium a fourni une méta-analyse des 10 loci montrant les plus fortes associations avec la PA et l'HTA dans *CHARGE et Global BPgen consortiums* [41]. Il a pu identifier, de façon reproductible (*Pvalue*$< 5\times10^{-8}$), 4 loci associés avec la PAS (*ATP2B*1, *CYP17A1*, *PLEKHA7* et *SH2B3*), 6 loci associés avec la PAD (*ATP2B1, CACNB2, CSK-ULK3, SH2B3, TBX3-TBX5* et *ULK4*) et un seul locus (*ATP2B1*) associé avec l'HTA (Tableau 4).

Récemment en 2011, une GWAS conduite sur 200,000 individus caucasiens a révélé que 29 SNPs dans 28 loci étaient associés à la PA (erhet gb, 2011) (Tableau 4). Parmi les 29 SNPs, 13 étaient précédemment décrits dans les GWAS de Levy et al [41] et Newton Cheh et al [48]. L'ensemble des 28 loci et leurs effets estimés sont présentés dans la Figure 13. Voici la liste par ordre alphabétique des gènes identifiés :

ADM : Codant pour le peptide hypotensif nommé *Adrenomedullin*, exprimé au niveau des phéochromocytomes humains.

ATP2B1 : Codant pour l'enzyme *Plasma membrane calcium-transporting ATPase 1*, exprimée dans les cellules endothéliales et impliquée dans la régulation de l'homéostasie intracellulaire du calcium.

***BAT2–BAT5* :** Codant pour les protéines *Large proline-rich protein BAT*. Celles ci pourraient être impliquées dans le processus inflammatoire menant à la destruction des cellules bêta pancréatiques lors du développement d'un diabète insulino-dépendant.

C10orf107 : Codant pour une protéine hypothétique nommée *chromosome 10 open reading frame 107*. A ce jour, le mécanisme d'action de ce gène reste inconnu.

***CACNB2* :** Codant pour la protéine *calcium channel, voltage-dependent, beta 2 subunit,* connue pour contribuer au bon fonctionnement des canaux de calcium.

Tableau 4: Méta-analyse des dix meilleurs *single nucleotide polymorphisms* associés avec la pression et l'hypertension artérielles dans 2 consortiums *CHARGE* et *Global Bpgen* (d'après Levy D et al [32]).

SNP ID	Chr	Position	Gene à proximité	Allèles (F/M)	FAF	Beta	e s	P	Beta	e s	P	Beta	e s	P
PAS						Méta-analyse *CHARGE*			Méta-analyse *Global Bpgen*			Méta-analyse *CHARGE* et *Global Bpgen*		
rs12046278	1	10,722,164	*CASZ1*	T/C	0,64	−0,84	0,18	$1,84x10^{-06}$	−0,29	0,15	$5,71x10^{-02}$	−0,53	0,12	$4,77x10^{-06}$
rs7571613	2	190,513,907	*PMS1*	A/G	0,82	−0,96	0,19	$7,24x10^{-07}$	−0,23	0,16	$1,59x10^{-01}$	−0,54	0,13	$1,90x10^{-05}$
rs448378	3	170,583,593	*MDS1*	A/G	0,52	−0,71	0,15	$1,28x10^{-06}$	−0,36	0,13	$4,76x10^{-03}$	−0,51	0,1	$1,18x10^{-07}$
rs2736376	8	11,155,175	*MTMR9*	C/G	0,13	−1,08	0,23	$1,90x10^{-06}$	−0,06	0,19	$7,36x10^{-01}$	−0,48	0,15	$9,15x10^{-04}$
rs1910252	8	49,569,915	*EFCAB1*	T/C	0,18	−0,93	0,19	$1,70x10^{-06}$	−0,07	0,17	$6,80x10^{-01}$	−0,43	0,13	$6,13x10^{-04}$
rs11014166	10	18,748,804	*CACNB2*	A/T	0,66	0,74	0,16	$2,11x10^{-06}$	0,33	0,13	$1,31x10^{-02}$	0,5	0,1	$7,03x10^{-07}$
rs1004467	10	104,584,497	***CYP17A1***	A/G	0,9	1,2	0,25	$1,99x10^{-06}$	0,94	0,21	$1,08x10^{-05}$	1,05	0,16	$\mathbf{1,28x10^{-10}}$
rs381815	11	16,858,844	***PLEKHA7***	T/C	0,26	0,84	0,17	$5,76x10^{-07}$	0,52	0,14	$2,72x10^{-04}$	0,65	0,11	$\mathbf{1,89x10^{-09}}$
rs2681492	12	88,537,220	***ATP2B1***	T/C	0,8	1,26	0,19	$3,01x10^{-11}$	0,5	0,17	$4,07x10^{-03}$	0,85	0,13	$\mathbf{3,76x10^{-11}}$
rs3184504	12	110,368,991	***SH2B3***	T/C	0,48	0,75	0,15	$5,73x10^{-07}$	0,45	0,13	$6,36x10^{-04}$	0,58	0,1	$\mathbf{4,52x10^{-09}}$
PAD														
rs13423988	2	68,764,770	*GPR73-ARHGAP25*	T/C	0,17	0,59	0,12	$1,09x10^{-06}$	0,11	0,11	$3,22x10^{-01}$	0,33	0,08	$5,00x10^{-05}$
rs13401889	2	190,618,804	*MSTN*	T/C	0,79	−0,54	0,11	$9,70x10^{-07}$	−0,10	0,11	$3,55x10^{-01}$	−0,31	0,08	$4,82x10^{-05}$
rs9815354	3	41,887,655	***ULK4***	A/G	0,17	0,6	0,12	$7,80x10^{-07}$	0,4	0,11	$3,79x10^{-04}$	0,49	0,08	$\mathbf{2,54x10^{-09}}$
rs7016759	8	49,574,969	*EFCAB1*	T/C	0,83	0,57	0,12	$1,87x10^{-06}$	0,06	0,11	$5,79x10^{-01}$	0,3	0,08	$2,29x10^{-04}$
rs11014166	10	18,748,804	***CACNB2***	A/T	0,66	0,46	0,09	$8,69x10^{-07}$	0,28	0,09	$1,46x10^{-03}$	0,37	0,06	$\mathbf{1,24x10^{-08}}$
rs11024074	11	16,873,795	*PLEKHA7*	T/C	0,72	−0,50	0,1	$2,83x10^{-07}$	−0,17	0,09	$6,82x10^{-02}$	−0,33	0,07	$1,20x10^{-06}$
rs2681472	12	88,533,090	***ATP2B1***	A/G	0,83	0,64	0,12	$3,74x10^{-08}$	0,36	0,12	$2,43x10^{-03}$	0,5	0,08	$\mathbf{1,47x10^{-09}}$
rs3184504	12	110,368,991	***SH2B3***	T/C	0,48	0,5	0,09	$1,68x10^{-08}$	0,45	0,09	$2,83x10^{-07}$	0,48	0,06	$\mathbf{2,58x10^{-14}}$
rs2384550	12	113,837,114	***TBX3-TBX5***	A/G	0,35	−0,48	0,09	$1,32x10^{-07}$	−0,23	0,09	$1,06x10^{-02}$	−0,35	0,06	$\mathbf{3,75x10^{-08}}$
rs6495122	15	72,912,698	***CSK-ULK3***	A/C	0,42	0,45	0,09	$8,05x10^{-07}$	0,35	0,09	$3,98x10^{-05}$	0,4	0,06	$\mathbf{1,84x10^{-10}}$

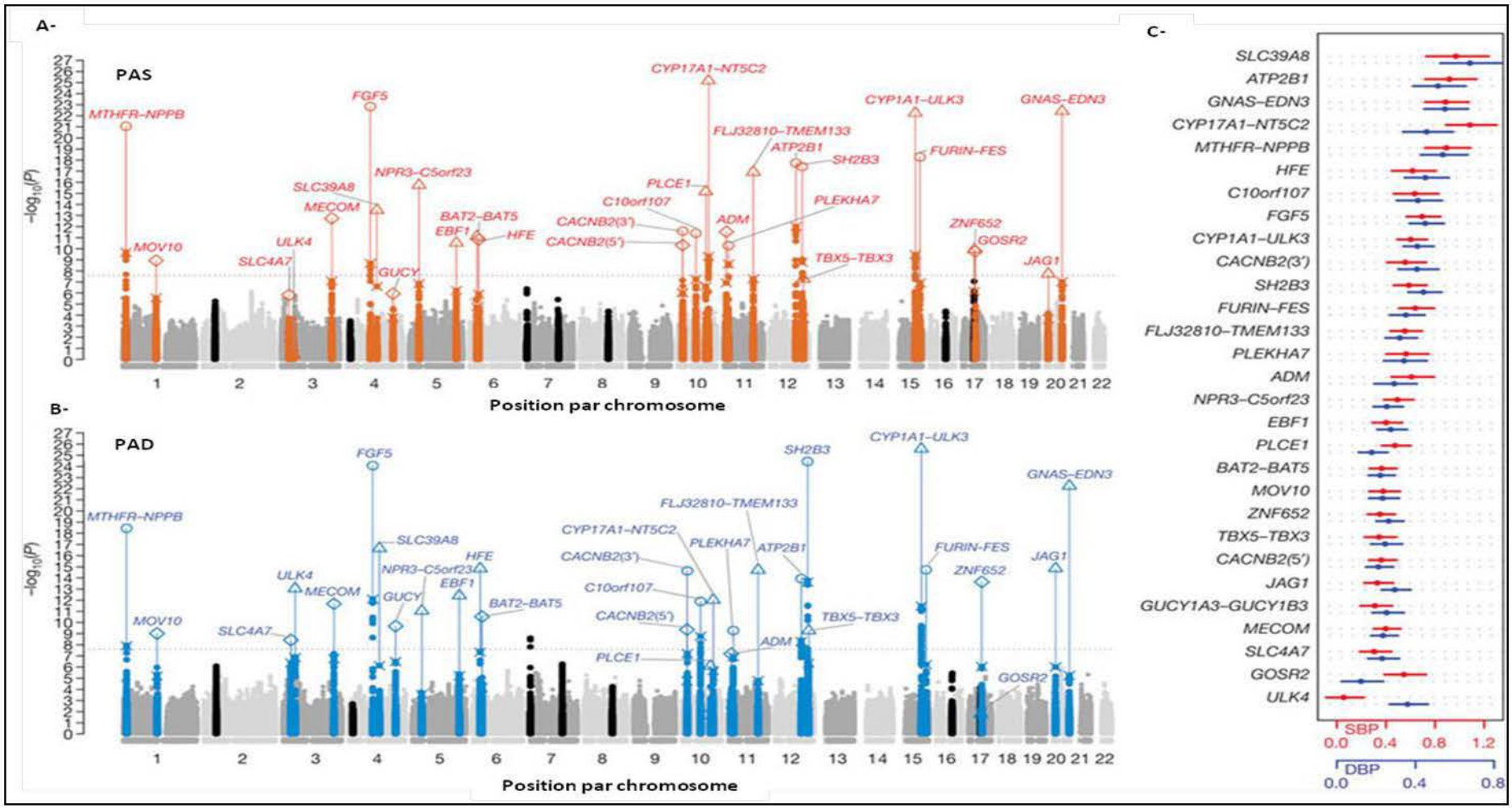
HTA

SNP ID	Chr	Position	Gene à proximité	Allèles (F/M)	FAF	Beta	e s	P	Beta	e s	P	Beta	e s	P
rs17806132	2	190,416,532	*PMS1*	A/G	0,16	0,14	0,03	$1,14x10^{-05}$	0,04	0,04	$2,87x10^{-01}$	0,1	0,02	$4,70x10^{-05}$
rs305489	3	11,986,163	*SYN2*	A/G	0,55	0,1	0,02	$4,20x10^{-06}$	0,01	0,03	$7,75x10^{-01}$	0,06	0,02	$1,70x10^{-04}$
rs7640747	3	37,571,809	*ITGA9*	C/G	0,62	−0,12	0,02	$4,81x10^{-07}$	−0,02	0,03	$4,12x10^{-01}$	−0,08	0,02	$1,24x10^{-05}$
rs11775334	8	10,109,030	*MSRA*	A/G	0,32	0,1	0,02	$1,03x10^{-05}$	0,05	0,03	$5,86x10^{-02}$	0,08	0,02	$4,05x10^{-06}$
rs899364	8	11,366,954	*FAM167A-BLK*	T/G	0,32	−0,10	0,02	$6,95x10^{-06}$	−0,04	0,03	$1,32x10^{-01}$	−0,08	0,02	$1,01x10^{-05}$
rs11014166	10	18,748,804	*CACNB2*	A/T	0,66	0,11	0,02	$7,79x10^{-07}$	0,07	0,03	$1,06x10^{-02}$	0,09	0,02	$5,72x10^{-08}$
rs2681472	12	88,533,090	***ATP2B1***	A/G	0,83	0,16	0,03	$1,65x10^{-08}$	0,13	0,04	$2,15x10^{-04}$	0,15	0,02	$\mathbf{1,75x10^{-11}}$
rs278126	12	118,620,100	*CIT*	T/G	0,28	0,11	0,02	$8,34x10^{-06}$	−0,01	0,03	$6,72x10^{-01}$	0,06	0,02	$1,74x10^{-03}$
rs11612893	12	129,290,572	*FZD10-PIWIL1*	T/G	0,1	−0,19	0,04	$7,62x10^{-06}$	−0,04	0,06	$4,19x10^{-01}$	−0,14	0,03	$5,50x10^{-05}$
rs16982520	20	57,192,115	*ZNF831-EDN3*	A/G	0,88	−0,14	0,03	$4,95x10^{-06}$	−0,11	0,04	$7,44x10^{-03}$	−0,13	0,02	$1,59x10^{-07}$

La GWAS d'HTA a été réalisée uniquement dans CHARGE consortium.

PAS : pression artérielle systolique, PAD : pression artérielle diastolique, HTA : hypertension artérielle, chr : chromosome, M/F : mineur/fréquent, FAF : fréquence de l'allèle fréquent, e.s : erreur standard, Beta : coefficient de régression linéaire.

Figure 13: Vingt neuf *single nucleotide polymorphisms* dans 28 loci associés *($P \leq 5x10^{-8}$)* avec la pression artérielle (d'après Ehret GB et al [50]).



Les valeurs *Genome-wide-log$_{10}$* sont montrées pour la PAS (A) et la PAD (B). Les SNP des loci atteignants le seuil de significativité sont étiquetés en rouge pour la PAS et en bleu pour PAD. Les SNPs proches des loci non confirmés sont de couleur noire. La ligne horizontale pointillée correspond à un $P< 2.5x10^{-8}$. Les effets estimés de chaque allèle mineur des 29 SNP associés avec la PAS (rouge) et la PAD (bleu) sont présentés en mmHg par allèle.

PAS : pression artérielle systolique, PAD : pression artérielle diastolique, SNP : *single nucleotide polymorphism.*

EBF1 **:** Codant pour la protéine *early B-cell factor 1*, connue pour être un facteur activateur de transcription.

FGF5 : Codant pour la protéine *Fibroblast growth factor 5*, faisant partie de la famille des facteurs de croissance des fibroblastes. Cette protéine est sécrétée par les fibroblastes et connue pour activer la multiplication cellulaire et le développement embryonnaire et l'angiogenèse.

GUCY1A3–GUCY1B3 **:** Codant pour l'enzyme *guanylate cyclase 1, soluble*. Elle joue un rôle important dans la régulation de l'hyperplasie cellulaire, l'hypertrophie et la différentiation cellulaire.

GOSR2 **:** Codant pour la protéine *Golgi SNAP receptor complex member 2*, connue pour être impliquée dans le trafic intracellulaire, notamment dans le rôle des protéines à travers les corps de Golgi.

HFE : Codant pour la protéine membranaire *Human hemochromatosis* qui pourrait réguler l'absorption ionique via la régulation de la liaison de la transferrine avec son récepteur.

JAG1 **:** Codant pour la protéine *Jagged 1*, qui joue le rôle d'un ligand pour de nombreux récepteurs Notch. Elle est également impliquée dans la régulation de la voie de signalisation Notch.

MECOM **:** Codant la protéine *MDS1 and EVI1 complex locus protein EVI1,* connue pour être un facteur de transcription jouant un rôle dans l'hématopoïèse, l'apoptose ainsi que la prolifération et la différentiation cellulaire.

MOV10 **:** Codant pour l'enzyme *Moloney leukemia virus 10,* jouant un rôle d'hélicase d'ARN.

PLCE1 **:** Codant pour l'enzyme *phospholipase C, epsilon 1*. Via son activité catalytique, cette enzyme est impliquée dans des mécanismes moléculaires activant la prolifération et la différentiation cellulaire.

PLEKHA7 : Codant pour la protéine *pleckstrin homology domain containing, family A member 7* requise pour la biogenèse et la maintenance des cadhérines et des caténines.

SH2B3 : Codant pour la protéine *SH2B adaptor protein 3*, impliquée dans l'inhibition de la cascade des cytokines et jouant un rôle critique dans l'hématopoïèse.

SLC39A8 : Codant pour la protéine *solute carrier family 39 (zinc transporter), member 8,* connue pour être impliquée dans l'importation cellulaire de l'ion de zinc et dans l'initiation de l'inflammation.

SLC4A7 **:** Codant la protéine régulatrice *solute carrier family 4, sodium bicarbonate cotransporter, member 7*, qui maintient le pH intracellulaire.

TBX5–TBX3 **:** Codant pour les facteurs de transcription *T-box transcription factor 3 and 5*. Ces 2 protéines font partie d'une famille de facteurs de transcription régulant le développement embryonnaire. De plus, *TBX5* a été associé à des malformations cardiaques.

ULK4 : Codant la protéine *unc-51-like kinase 4.* Très peu d'informations sont connues actuellement sur le rôle de cette protéine.

ZNF652 **:** Codant pour le facteur de transcription *zinc finger protein 652, connu pour être un facteur répresseur de transcription.*

En plus des 18 gènes décrits ci-dessus, 10 SNPs inter-géniques appartenant à 10 loci différents ont été identifiés par Ehret et al [59]. Ces loci étaient , *CYP1A1–ULK3*, *CYP17A1-NT5C2*, *GNAS-EDN3*, *FLJ32810-TMEM133*, *FURIN-FES*, *MTHFR-NPPB*, *NPR3-C5orf23*. A noter que chaque locus ici rapporté sous-entend l'association d'un seul SNP avec la PA. D'une façon générale, les généticiens rapportent le gène le plus proche du SNP (présentation des travaux , chapitre 3, publication N^{o}9). Cependant, les études d'expression eQTL (quantitative trait loci) basées sur l'analyse de l'association du génome avec le transcriptome (dans des tissus spécifiques), ont révélé certains SNPs capables de réguler l'expression des gènes topographiquement loin (régulation en trans) (présentation des travaux , chapitre 3, publication N^{o}9).

III. LA PART D'OMBRE D'HERITABILITE GENETIQUE

Les *GWAS* ont certes mis en évidence un nombre important de nouveaux loci candidats de régulation de la PA et de l'HTA (jamais une technique n'en avait révélé autant, en si peu de temps). Cependant, tous les SNPs identifiés avaient un faible effet sur le risque de survenue d'HTA [59]. Vingt neufs SNPs répertoriés jusqu'à aujourd'hui n'expliquent que 0,9% de la variabilité de ce phénotype [59]. Ce qui laisse 99,1% de part d'ombre ou '*dark matter*' dans l'étiologie de la PA.

III-1. Variants génétiques rares

Une grande partie de «l'héritabilité manquante» pourrait être dévoilée par l'étude des SNPs rares et de faibles fréquences alléliques. Ce type d'études n'a pas été illustré jusqu'à présent (présentation des travaux , chapitre 3, publication N°1). Les GWAS classiques ont jusqu'à présent examiné les SNPs fréquents (fréquence allélique> 5% dans la population générale) (présentation des travaux , chapitre 3, publication N°1). Ceux ayant de faibles fréquences alléliques (entre 0,5%-5% dans la population générale) et ceux ayant des fréquences alléliques rares (< 0,5% dans la population générale), censés avoir un plus grand effet, ont été ignorés (présentation des travaux , chapitre 3, publication N°1). Contrairement aux SNPs fréquents, qui sont apparus précocement dans l'histoire de l'humanité, les variants rares sont plus récents et ne sont donc pas répandus géographiquement (présentation des travaux , chapitre 3, publication N°1). Récemment, le séquençage à haut débit (nouvelle génération) a révélé une myriade de SNPs rares délétères (présentation des travaux , chapitre 3, publication N°1). La prochaine étape sera d'évaluer leurs associations à un risque accru de morbidité et mortalité CV de façon à mieux comprendre leurs implications. L'étude des SNPs rares par GWAS nécessite de très grandes populations et l'utilisation de méthodes statistiques adaptées (présentation des travaux , chapitre 3, publication N°1). Ces dernières devraient analyser l'ensemble des variants génétiques dans un locus entier au lieu de tester individuellement chaque SNP (présentation des travaux , chapitre 3, publication N°1). La prochaine génération de puces à haute résolution rendra cet objectif possible grâce à une couverture de l'ensemble de l'échelle génomique (présentation des travaux , chapitre 3, publication N°1).

III-2. Variabilité du nombre de copies d'un gène

La variabilité du nombre de copies d'un gène ou *copy number variation* (*CNV*) , désigne en génétique une forme particulière de polymorphisme dans laquelle le nombre de copies d'un ou plusieurs gène(s) est variable entre les individus d'une même espèce. La présence de différentes copies de gènes est due aux événements de duplication et de délétion locaux.

Les CNVs sont des régions génomiques rares de grande taille (>500kb) qui modifient l'expression génique et par conséquent celle des voies moléculaires (Publication). Elles pourront être soumises à la pression de la sélection naturelle. En effet, le CNV modifie la quantité de protéines produites dans une cellule. L'augmentation ou la diminution du nombre de copies peut donc être sélectionnée au sein d'une espèce ou d'une population en fonction des contraintes environnementales [60]. Ainsi, nous observons une augmentation du nombre de CNVs du gène *Amy1* (gène codant pour une enzyme salivaire responsable de la dégradation de l'amidon) dans les populations humaines ayant un régime plus riche en amidon. Il semblerait que cette augmentation ait été le fruit de la pression de sélection [60].

Les CNVs surviennent à une fréquence allélique de < 0,05% et sont présentes dans environ 8% de la population générale (présentation des travaux , chapitre 3, publication N°1), ce qui les rend rares mais collectivement communes. Les résultats publiés suggèrent que les CNVs modifient fortement l'expression génique, un évènement moléculaire affectant l'épissage et la transcription génique (présentation des travaux , chapitre 3, publication N°1).

III-3. Interactions gène-environnement

Les interactions gène-environnement (GxE) sont également considérées comme des éléments clés contrôlant la PA et l'HTA (présentation des travaux , chapitre 3, publication N°1). L'intégration des GxE dans les GWAS pourrait aider à mettre en évidence de nouveaux loci modifiant le risque d'HTA. Cette stratégie nécessitera la mise en place de grands consortiums (présentation des travaux , chapitre 3, publication N°1). Au-delà des difficultés habituelles soulevées par les GWAS, une étude GWAS-GxE doit essentiellement prendre en compte la taille de l'échantillon, l'évaluation et l'hétérogénéité de l'exposition (présentation des travaux , chapitre 3, publication N°1). Ainsi, les GWAS-GxE nécessitent de très larges

populations (Publication). Smith et Day [61] expliquent que les GWAS-GxE doivent avoir un échantillon au moins 4 fois supérieur à celui nécessaire pour les GWAS classiques, ceci étant critique pour détecter une interaction GxE d'une ampleur comparable à celle d'un SNP unique [61]. Bien que difficile, l'étude des interactions GxE nous aidera à mieux comprendre les résultats d'associations génétiques contradictoires de PA et d'HTA dus à l'exposition hétérogène.

III-4. Interactions gène-gène (épistasie)

L'épistasie est définie comme une interaction entre deux ou plusieurs loci. Elle est importante, du fait que son existence peut modifier ou même masquer l'effet d'un locus. L'épistasie peut également identifier des gènes non trouvés par l'approche consensuelle à locus unique (présentation des travaux , chapitre 3, publication N^{o}9). Ce concept a été largement revu par les méthodes paramétriques et non paramétriques qui ont été développées au cours de ces dernières années pour détecter ce type d'interactions (présentation des travaux , chapitre 3, publication N^{o}1). Selon la littérature, les interactions épistasiques jouent un rôle dans la prédisposition au cancer et aux maladies auto-immunes. Cependant, leur investigation à l'échelle génomique (*genome-wide coverag*e), y compris l'étude de tous les SNPs imaginables, reste un défi crucial en absence de logiciels adaptés et d'une large population (présentation des travaux , chapitre 3, publication N^{o}1).

IV. EPIGENETIQUE

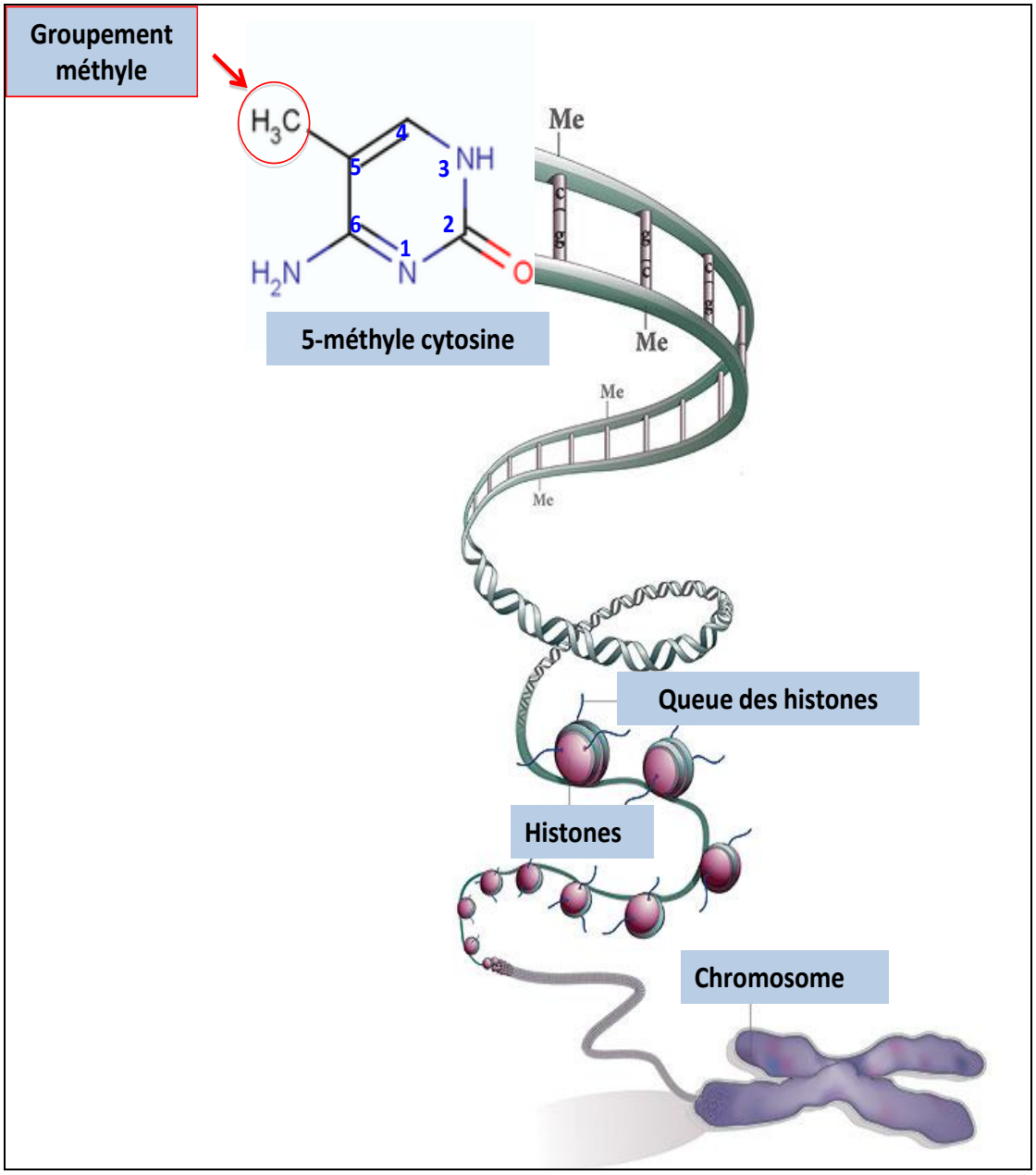
Au cours de la dernière décennie, il est devenu de plus en plus évident que la compréhension du génome ne s'arrête pas avec l'élucidation du code génétique [62]. Des informations régulatrices sont nécessaires pour déterminer les parties génomiques actives, et pour former un système de mémoire transmissible au cours des divisions cellulaires [63]. Cette information qui sous-tend la régulation héréditaire des gènes est connue sous le terme épigénétique [54].

L'épigénétique est l'étude des altérations transmissibles des phénotypes et de l'expression des gènes qui ne s'accompagnent d'aucune modification des séquences nucléotidiques [64]. Les mécanismes sous-tendant les phénomènes épigénétiques font intervenir des facteurs génomiques flexibles qui peuvent non seulement modifier la fonction du génome en réponse à l'environnement, mais aussi fournir un substrat moléculaire permettant la transmission stable de l'expression des gènes d'une génération cellulaire à l'autre [64].

Bien que l'épigénétique soit considérée comme un nouveau domaine de recherche, la première mention de ce terme remonte à plus de soixante ans. En 1942, Conrad Hal Waddington (1905-1975) a utilisé le terme épigénétique pour décrire ce qu'on appelle aujourd'hui la biologie du développement [64]. Il décrit «qu'aussi bien le phénotype que les propriétés morphologiques et fonctionnelles d'un organisme se posent de manière séquentielle dans un programme défini par le génome et soumis à l'influence de l'environnement» [64].

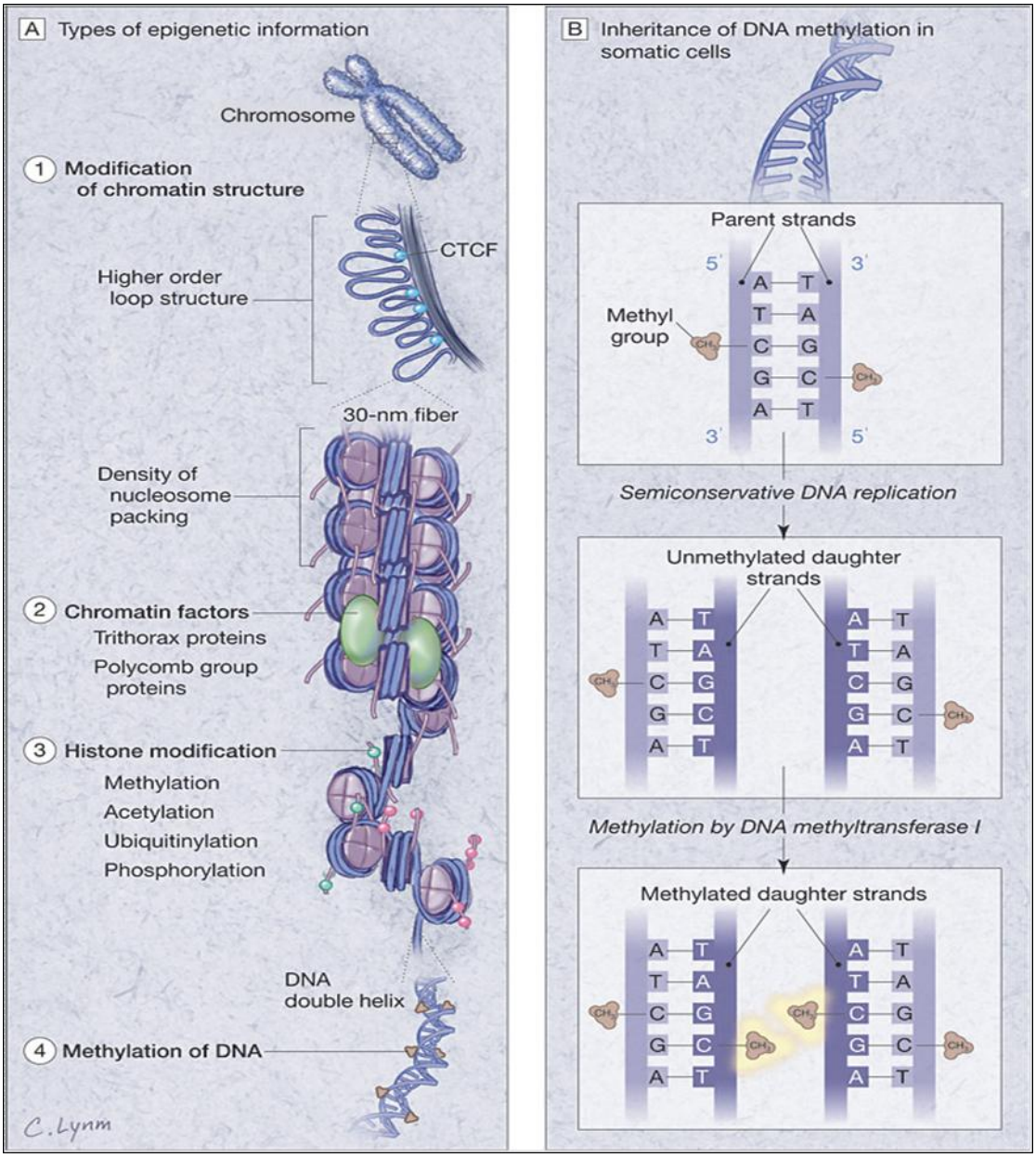
Le processus épigénétique le mieux connu est la méthylation de l'ADN (mADN), une addition covalente d'un groupement méthyle au niveau du carbone 5 d'une cytosine (Figure 14). La mADN est maintenue pendant la division cellulaire, chez les mammifères seulement, aux dinucléotides CpG, en vertu de l'enzyme ADN méthyltransférase 1 (DNMT1) [53]. Cela se produit du fait que pendant la réplication d'ADN, un dinucléotide CpG méthylé sur le brin d'ADN parental est apparié à un CpG non méthylé nouvellement synthétisé sur le brin fils [53]. Par conséquent, la DNMT1 cherche l'ADN hémi-méthylé et ajoute un groupement méthyle sur le nouveau dinucléotide CpG (Figure 15) [53].

Figure 14: Méthylation de l'ADN (d'après Feinberg AP, [54]).



Addition covalente de groupement méthyle au niveau du carbone 5 d'une cytosine.

Figure 15: Différents types d'informations et d'hérédité épigénétiques (d'après Feinberg AP, [54]).



A- Les informations épigénétiques. Le terme épigénétique correspond à l'étude des modifications de l'ADN ou de ses protéines associées qui transportent l'ensemble des informations pendant la division cellulaire, sans prendre en compte l'altération de la séquence ADN. B- Hérédité de la méthylation épigénétique. Dans les cellules somatiques, l'information épigénétique est répliquée lors de la mitose avec la séquence d'ADN. Le mécanisme de réplication de méthylation de l'ADN est bien maitrisé au contrairement au mécanisme de modifications des histones.

La mADN est présente au niveau des sites CpG, connue sous le nom *methylation variable position* (MVP) et considérée comme l'équivalent épigénétique d'un SNP [65]. Une fois la mADN altérée dans de multiples sites CpG adjacents, elle est considérée comme région différemment méthylée ou *differentially methylated region* (DMR). Les DMRs varient considérablement en longueur : elles sont généralement de longueur inférieure à 1kb, mais peuvent dépasser parfois les 1 Mb. Jusqu'à ce jour, les MVPs et les DMRs ont été principalement étudiés au niveau des promoteurs principaux et des ilôts CpG, mais il devient de plus en plus clair que la mADN est très dynamique, même en dehors de ces régions [65].

Un second exemple bien étudié du processus épigénétique est la modification de la chromatine, ou plus précisément des modifications covalentes au niveau des histones qui font partie des nucléosomes, autour desquels la double hélice d'ADN est enroulée [63]. Les histones sont de petites protéines pouvant subir une modification post-traductionnelle au niveau des résidus d'acides aminés spécifiques. Ainsi, les histones 3 et 4 peuvent être modifiées sur un mode covalent par méthylation, ubiquitination, phosphorylation ou sumoylation. Ces processus modifient la manière dont l'histone considérée interagit avec l'ADN et les autres protéines nucléaires [55]. En conséquence, selon leur type et la position du résidu d'acide aminé intéressé, les modifications subies par les histones ont pour effet de réprimer la transcription ou, au contraire, de l'activer. Contrairement à la mADN, le mécanisme de maintien des modifications de la chromatine pendant la division cellulaire reste très peu compris [53]. Aucune enzyme n'a été identifiée pour pouvoir reconnaître les modifications de la chromatine de la cellule mère et les reproduire dans la cellule fille [53].

Un lien important de l'épigénétique avec l'environnement, est que la source des groupements méthyle dans cette réaction est la méthionine. Un acide aminé essentiel est converti en donneur de groupements méthyle par un mécanisme impliquant l'acide folique [53].

D'une façon générale, la mADN (surtout au niveau du promoteur proximal) a été capable de réprimer la transcription des gènes [53]. De même, la relaxation des nucléosomes condensés est une étape clé pour l'expression génique [53].

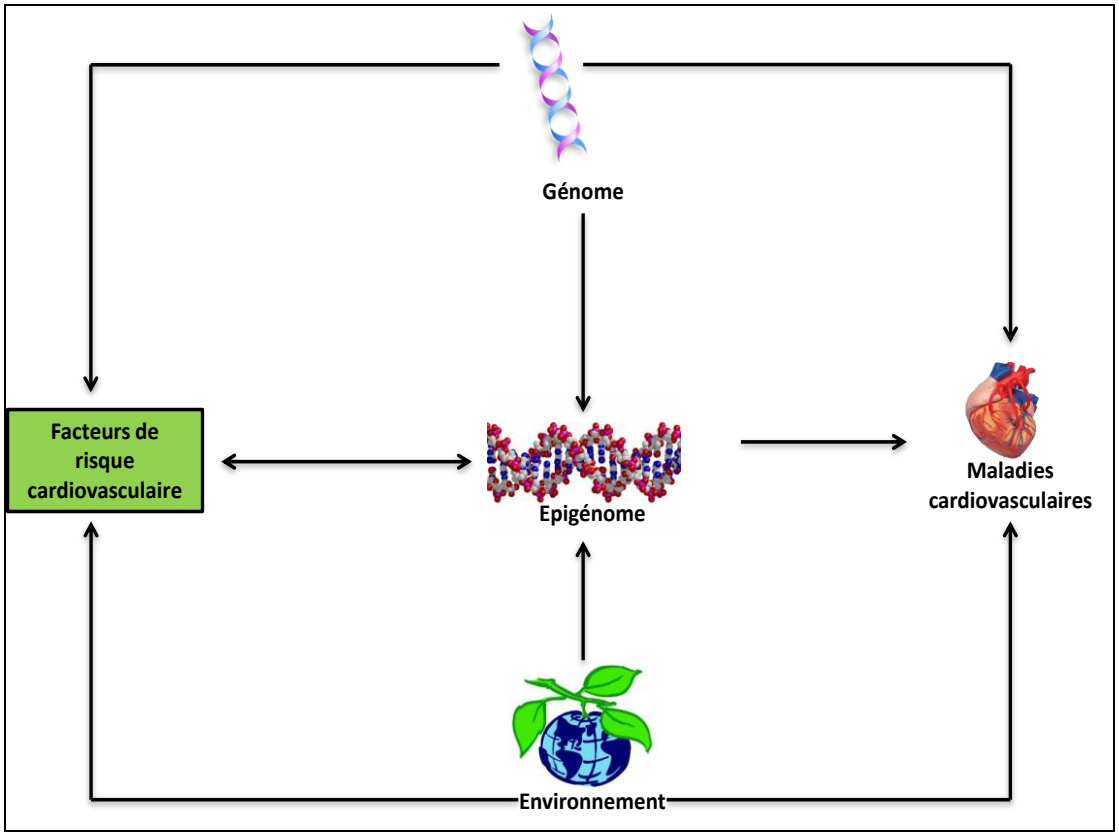
IV-1. Méthylation d'ADN et maladies cardiovasculaires

Les études animales ont démontré que la mADN joue un rôle majeur dans le développement de l'athérosclérose et des MCV [55]. Chez les souris privées de gènes qui codent normalement pour les enzymes méthylantes telles que les DNMT ou la méthylène-tétrahydrofolate réductase (MTHFR), nous constatons une hypo-mADN [55]. Les leucocytes des souris ne possédant pas de DNMT expriment plus intensément les médiateurs d'inflammation [66]. Il a également été établi que, chez la souris dépourvue du gène *MTHFR*, l'hypo-mADN précède la formation des stries graisseuses aortiques [67].

IV-1.1. Modèle théorique de l'épigénome cardiovasculaire

L'épigénomique constitue un lien majeur entre le codage génomique et l'expression phénotypique qui subit l'influence aussi bien des caractéristiques génétiques sous-jacentes que des facteurs environnementaux (Figure 16) [55]. A la différence du génome, l'épigénome est soumis, tout au long de la vie, à des modifications dynamiques pouvant promouvoir et entretenir des états adaptatifs et déviants dans l'expression des gènes [55]. Certaines données suggèrent que les facteurs de RCV pourraient affecter et remodeler les profils épigénomiques [55]. Ces profils sont susceptibles de contribuer à la survenue de troubles CV [55]. L'épigénome est, par son essence-même, en liaison avec la génétique dans la mesure où les modifications épigénétiques peuvent altérer l'expression des variations génétiques, lesquelles représentent l'un des processus clé conditionnant la mADN et les remaniements des histones [55].

Figure 16: Modèle théorique de rattachement de l'épigénomique aux maladies cardiovasculaires et aux facteurs de risque cardiovasculaire (d'après Bacarelli et al, [55]).



Les facteurs de risque cardiovasculaire pourraient affecter et remodeler les profils épigénomiques. Ils sont susceptibles de contribuer à la survenue de troubles cardiovasculaires. L'épigénome est en liaison avec la génétique dans la mesure où les modifications épigénétiques peuvent altérer l'expression des variations génétiques.

IV-1.2. Méthylation d'ADN et hypertension artérielle

Plusieurs observations directes et indirectes soutiennent la participation d'une composante épigénétique dans l'étiologie d'HTA. En effet, dans les leucocytes du sang périphérique des patients HT, des études ont objectivé une diminution globale des processus de méthylation au sein du génome [68] ainsi qu'une hyper-méthylation du gène *HSD11B2* [69]. De plus, Il a été établi que l'âge et le sexe influent sur le profil de mADN [63;70], par conséquent, la méthylation différentielle liée à l'âge et au sexe pourrait modifier la réponse génique face à l'HTA.

Cependant, aucune information concernant le rôle de la mADN dans la régulation de la PA et de l'HTA n'est dévoilée à l'échelle génomique

IV-2. Techniques d'analyses de la méthylation de l'ADN à l'échelle génomique

L'introduction des technologies génétiques de haut débit, en particulier les puces de génotypage, était le principal avantage qui a permis la réalisation des GWASs à grande échelle. Ce n'est que récemment, que les technologies de profilage épigénomiques ont atteint le même stade, où l'investigation de la mADN au niveau génomique est de plus en possible. Ceci constitue une étape motivante pour rechercher des DMRs impliquées dans la régulation de l'HTA.

La gamme complète des marqueurs épigénétiques est actuellement inconnue, mais les épigénéticiens estiment qu'elle est potentiellement énorme, en se basant sur le fait que l'épigénome humain diploïde contient plus 100 millions de cytosines (dont plus de 10 millions sont des ilots CpG) et plus de 100 millions de queues d'histones [65]. Le marqueur le plus approprié pour effectuer une analyse épigénomique est la mADN [71].

Une brève description de chacune des techniques utilisées pour analyser la mADN à l'échelle génomique actuellement disponibles est présentée dans cette section. Les techniques sont sous-divisées en différentes catégories en fonction du type d'analyse (digestion enzymatique, conversion chimique et enrichissement d'affinité) et du type des systèmes de détection (électrophorèse sur gel, bio-puces et séquençage nouvelle génération) [71]. Le choix d'une catégorie bien précise est dépendant de nombreuses variables, y compris le

nombre d'échantillons interrogé, la quantité d'ADN disponible, le coût et la disponibilité des équipements, la recherche de méthylation absolue ou différentielle, le besoin de résultats quantitatifs régionaux ou d'autres ayant une résolution plus profonde (pb unique) [71].

IV-2.1. Techniques basées sur la conversion chimique au bisulfite

La désamination chimique des cytosines, mais pas des 5meC, par le bisulfite de sodium transforme efficacement les cytosines non méthylées en uraciles [71]. Après PCR, les uraciles seront amplifiés comme étant des thymines, tandis que les résidus 5meC seront considérés comme étant des cytosines [71]. La découverte et l'optimisation de cette conversion (entre 1992-1994) ont depuis révolutionné le domaine de l'analyse des mADN, car cette méthode définit l'état de méthylation de chaque résidu cytosine dans la séquence cible, avec une résolution très profonde (pb unique) [71]. Du fait que la majorité des résidus de cytosine dans le génome sont non méthylés, il en résulte une réduction de la spécificité d'hybridation, ce qui rend cette approche moins appropriée pour conduire des analyses d'hybridation, comme les bio-puces [71].

Deux approches complémentaires (les bio-puces et le séquençage nouvelle génération) sont exclusivement couplées aux techniques de conversion chimique utilisées lors de l'analyse des mADN [71].

Approche de séquençage nouvelle génération : Jusqu'à ce jour, 3 approches combinant la conversion au bisulfite de sodium avec le séquençage nouvelle génération ont été publiées [71]. *Whole genome shotgun bisulfite sequencing* est actuellement le moyen le plus complet mais très coûteux, permettant d'élucider les niveaux de mADN [71]. Dans ce cas, la mADN est identifiée par séquençage *shotgun* suite au traitement par bisulfite [71].

IV-2.2. Techniques basées sur l'enrichissement d'affinité

Les techniques sur l'enrichissement d'affinité sont considérées comme des outils puissants pour étudier la mADN à l'échelle génomique [71]. Celles-ci utilisent soit des anticorps

spécifiques des résidus 5meC soit des domaines de liaisons aux groupements méthyle (MBD) [71].

Les techniques d'enrichissement d'affinité ne fournissent pas forcément des informations précises concernant la méthylation différentielle (au niveau d'un seul nucléotide), mais plutôt absolues [71]. Du fait que les régions génomiques riches en CpG abritent le plus grand nombre de sites de liaison potentiels, les résultats d'analyses basés sur les techniques d'enrichissement d'affinité, seront affectés par la densité locale en CpG [71]. En effet, dans le but de valider les résultats d'enrichissement d'affinité, une étape ultérieure de conversion chimique au bisulfite suivie d'un séquençage est nécessaire [71].

Une différence majeure existe entre les deux approches d'enrichissement d'affinité [53]. Concrètement, les MBD reconnaissent les dinucléotides CpG méthylés au niveau de l'ADN double brin, tandis que l'anticorps monoclonal anti 5meC ne reconnaît que les résidus 5meC présents au niveau de l'ADN simple brin [71]. Contrairement aux techniques de restriction et de conversion chimique au bisulfite, les approches basées sur l'enrichissement d'affinité sont sélectives pour les résidus 5meC [71]. En effet, les approches MBD sont exclusivement sélectives pour les résidus 5meC mais pas pour les 5hydroxy-meC [71]. En revanche, les anticorps sont spécifiques au type de résidu, selon le type d'anticorps monoclonal utilisé (anti 5meC ou anti 5hydrocymeC) [71]. A titre d'exemple, L'approche d'immunoprécipitation de l'ADN ou *methylated DNA immunoprecipitation assay* (MeDIP) utilise un anticorps spécifique des résidus 5meC présents dans l'ADN dénaturé, simple brin [71]. Les fragments ainsi enrichis peuvent être traités ultérieurement par bio-puces ou par séquençage nouvelle génération.

V. HYPERTENSION ARTERIELLE ET TRANSCRIPTOME

Le génome humain contient un ensemble complet des gènes nécessaires à la construction d'un être humain fonctionnel [39]. Toutefois, le génome n'est qu'une source d'information [39]. Pour fonctionner, il doit être exprimé [39]. La transcription d'un gène pour produire l'ARN constitue la première étape de l'expression génique. Le transcriptome est donc l'ensemble des transcrits d'ARN produits par le génome à un moment donné [39]. Contrairement au génome, le transcriptome est extrêmement dynamique [39]. La plupart de nos cellules contient un génome identique quels que soient le type cellulaire, le stade de développement ou les conditions environnementales [39]. Inversement, le transcriptome varie considérablement dans des circonstances différentes en raison de différents modèles d'expression génique [39]. L'étude du transcriptome est donc une manière globale de regarder les profils d'expression génique dans les maladies complexes [39].

Un SNP peut modifier l'expression d'un ou plusieurs transcrits conduisant au changement d'un phénotype donné [39]. Malgré leurs avantages, les études d'associations SNP-ARNm pourront avoir des résultats discordants, ou même contradictoires [39]. Toutefois, ces associations non répliquées doivent être considérées avec un œil critique [39]. L'approche transcriptomique apporterait une information intermédiaire entre des gènes et leurs protéines, une étape essentielle pour identifier des cibles physiopathologiques d'intérêt thérapeutique dans l'HTA [39].

V-1. Modèles cellulaires humains

La majorité des études ayant pour objectif l'identification de marqueurs spécifiques d'une maladie, se focalise sur les protéines circulantes (plasmatiques ou sériques) [72]. Pourtant, les concentrations plasmatiques et sériques correspondent à la résultante de molécules provenant de différents types de tissus et reflètent par conséquent, d'une façon globale, l'état physiopathologique d'un individu [72]. En effet, les taux plasmatiques et sériques ne reflètent pas leur production spécifique par leurs tissus-sources [72]. Dans le cas des cytokines, l'état sous-clinique installé, n'est éventuellement pas mis en évidence par la mesure du taux des protéines circulantes. Ainsi, les différents modèles et types cellulaires

pourraient être un modèle alternatif pour décrypter les voies moléculaires impliquées [72]. De nombreux organes étant impliqués dans la régulation de la PA [73], plusieurs modèles et types cellulaires sont utilisés :

A- Les cellules endothéliales subissent directement la PA et sont impliquées dans la régulation de la vasomotricité par la production d'oxyde nitrique ou d'endothéline [26]. Ces cellules sont également impliquées dans l'inflammation en permettant l'adhésion des cellules immunitaires et leur passage dans les organes en réponse au stress comme l'HTA [8].

B- Les cellules cardiaques répondent aux différents systèmes nerveux par leurs récepteurs adrénergiques, et leur prolifération, causée par l'angiotensine, entrainant l'hypertrophie ventriculaire gauche [63].

C- Les cellules rénales sont impliquées dans la filtration et la réabsorption de l'eau et des sels. Ces mécanismes influent sur le volume sanguin et donc la PA [63].

D- Les cellules hépatiques produisent l'angiotensinogène, considéré comme le premier élément du SRAA et ayant un important rôle de régulation de la PA [8].

E- Les cellules mononuclées du sang périphérique (*Peripheral Blood Mononuclear Cells* – PBMCs) sont intéressantes vue grâce à leur accessibilité [74]. De plus, elles interagissent avec l'intégralité des organes via la circulation sanguine. Une interaction qui pourrait fournir un message des organes non accessibles [74].

Les PBMCs sont composées de deux types cellulaires, majoritairement des lymphocytes (97%), et des monocytes (2,5%). Ces cellules étant donné qu'elles produisent de très nombreuses cytokines pro-inflammatoires. Un nombre important d'études transcriptomiques a utilisé les PBMCs comme modèle d'étude de la PA et de l'HTA [74-76]. Ces études ont suggéré les PBMCs comme modèle reflétant l'exposition aux facteurs

environnementaux et l'HTA. L'ensemble de ces observations, font de ce type cellulaire un modèle intéressant pour l'étude du rôle de l'inflammation dans la PA et l'HTA [74].

F- Les adipocytes matures, principales cellules du tissu adipeux, sont le site de l'activité de lipogenèse qui permet le stockage de l'énergie par l'organisme [77]. Lors de leur multiplication et de leur différenciation, ces cellules entrainent l'augmentation de l'IMC connu pour son lien fort et proportionnel avec les niveaux de PA. De même, le tissu adipeux viscéral est de plus en plus considéré comme une glande endocrine produisant un certain nombre de cytokines pro-inflammatoires (TNFα, l'IL-1 ou l'IL-6) qui pourront être impliqués dans la régulation de la PA [8].

I. Quels défis attendent les études pangénomiques ?

Cardiovascular diseases and genome-wide association studies.

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Malgré le grand nombre de SNPs identifiés au cours de ces dernières années par les GWAS des MCVs et de leurs facteurs de risque associés à cause de l'hétérogénéité des phénotypes cardiovasculaires cliniques et des populations investiguées, les résultats ne sont que partiellement répliquées. De nouveaux mécanismes physiopathologiques ont certes été mis en évidence mais l'identification du variant causal reste problématique. Dans les prochaines années, les approches pangénomiques devraient donc intégrer les interactions gène-gène et gène-environnement au sein de stratégies globales impliquant des approches complémentaires telles que la transcriptomique et la protéomique. L'ère des GWASs est pleine de promesses et nous n'en avons vu que les préludes.

Mots clés: GWAS, maladies cardiovasculaires, interactions gène-gène, interactions gène-environnement, facteurs de risque cardiovasculaires.

Contribution: *Il s'agit d'une revue de la littérature pour laquelle nous avons effectué des recherches bibliographiques portant notamment sur l'ensemble des GWASs réalisées dans le domaine de l'HTA et émis les hypothèses pour une stratégie d'étude intégrée dans ce domaine complexe. Nous avons également fait partie de l'équipe rédactionnelle et avons révisé et complété la version finale de l'article.*

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Invited critical review

Cardiovascular diseases and genome-wide association studies

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ABSTRACT

Genome-Wide Association Studies (GWAS) on cardiovascular diseases and related quantitative traits revealed numerous genetic variants, which however have been partially replicated, probably due to the heterogeneity of the clinical phenotypes and the populations studied. Even if novel biological pathways have been identified through these studies, there is still a long way until the validation of causal variants and their use in clinical practice as factors for prevention, risk assessment and as targets for the development of new medications. GWAS methodologies should, in the following years, integrate gene–gene and gene–environment interaction analyses in a global research strategy and also involve subsequent transcriptomic and proteomic investigations. The GWAS era is very promising but it is just at the beginning.

Contents

1. Introduction: cardiovascular diseases: common disorders with important genetic component

Cardiovascular diseases (CVDs) are the leading cause of death in the world. Based on World Health Organization, 17.1 million people died from CVDs in 2004 and it is estimated that by 2030, approximately 23.6 million deaths will be recorded due to CVDs, mainly from heart disease and stroke [1]. The aetiology of CVDs is multifactorial, where a complex combination of environmental, genetic and clinical risk factors seems to play determinant role [2]. In fact, the pathogenesis of coronary heart disease (CHD) is known to be influenced by smoking, diabetes, hypertension, obesity, physical inactivity, alcohol intake and psychosocial conditions [3–5]. Genetic elements contribute to the development of CAD [6,7]. This complex architecture and its genetic background [8,9] are still poorly understood and substantial discrepancies remain in estimating the heritability of numerous CVDs-related quantitative traits (QTs) [10]. Risk algorithms such as the Framingham Risk Score [11] have traditionally incorporated classical clinical and environmental risk factors such as age, gender, blood lipid concentrations, blood pressure (BP), body mass index, family history and

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smoking habit for primary prevention strategies purposes. The incorporation of genetic risk factors in the risk assessment algorithms can be beneficial for the improvement of preventive public health strategies and could open new landscapes for more effective treatment (personalised medicine) and reduced complications [12]. The identification and validation of genetic variants that predispose to CVDs and related QTs may lead to better understanding of the fundamental pathogenetic pathways and to the establishment of new biological pathways and novel risk factors.

Over the last 2 decades, genetics of chronic diseases and related QTs were assessed by candidate gene approaches [13]. Twins and families studies were used to estimate heritability, while population-based studies were used to identify variants that are associated with a phenotype and to explore interactions between genetic and environmental factors. Candidate genes are those considered to influence complex phenotypes due to their participation in biological pathways or due to their location close to a region of interest [13]. This "hypothesis-driven" research has provided the foundation for the development of genetic CVDs risk profiles [12]. However, since 2007, genome-wide association studies (GWAS) have brought a revolution in the field of genetic background of chronic diseases like CVDs and their associated QTs [14–16]. The recent technological advances are enabling the rapid and accurate assessment of millions of single-nucleotide polymorphisms (SNPs) in large populations, leading to an increased number of GWAS for different CVDs phenotypes and related traits. These studies resulted in the identification of hundreds of genetic factors, some robustly replicated, however with small individual effects [17,18]. Moreover, GWAS revealed previously unsuspected pathological pathways [8,19]. Nevertheless, the identification of causal variants explaining the high heritability of the different CVDs phenotypes, which could be used for prevention, diagnosis, treatment and prognosis of CVD patients, has not yet been achieved with satisfactory results through the GWAS [20].

In this work we review GWAS findings concerning CVDs and their related QTs, the reasons for missing heritability and future challenges.

2. Methods

2.1. Literature search

The GWAS investigator of HuGENavigator engine [21] and the NHGRI Catalog of published GWAS (http://www.genome.gov/gwasstudies, assessed May 2011) [22] were used in order to assess GWAS published between 2007 and 2011, using the keywords of "cardiovascular diseases", "coronary heart disease", "coronary artery disease", "diabetes", "obesity", "hypertension", "blood pressure", "anthropometric measurements", "response to medication" and "lipids". In order to confirm the retrieved findings, we have checked the related articles and their reference lists in the MEDLINE engine using the same previous keywords along with 'GWAS' or 'single nucleotide polymorphism (SNPs)'. In addition, we restricted the search results to articles published in English language and conducted on humans.

2.2. Data extraction

Two authors independently extracted the following information from each study: CVDs traits, variants and their near gene(s), genomic region, corresponding trait(s), reference(s), P-values, number of subjects in each study (discovery and replication), the variant risk allele and the effect size. Genome-wide significance was considered when P-value $< 5 \times 10^{-8}$.

3. Brief overview of GWAS on cardiovascular diseases and their related quantitative traits

To date, a vast number of GWAS focusing on about 150 distinct diseases and QTs have reported several hundreds significant SNP-trait associations. GWAS have been conducted on cardiovascular events (CHD) and specifically to myocardial infraction (MI) and they have reported the association of at least 16 genetic variants found in 15 genes (Supplementary Table 1). They have identified SNPs on chromosomal regions 1p13, 1q41, 2q36, 3q22, 6p24, 6q25, 7q22, 9p21, 10q11, 10q23.2, 10p11.23, 11q22.3, 12q24, 15q25 and 15q22 that are associated with risk of CHD or its main complication, MI [23–28]. The associations between SNPs on 9p21 and CHD or MI were the most strongly replicated findings in the majority of the assessed ethnic groups [29–31].

However, caution is needed when interpreting data found in GWAS for CVDs, given the weak sample size of the majority of these studies. The genetic variants are rarely robustly linked to the cardiovascular events. Similarly, the lack of suggestive associations stressed out the value of using accurate clinical subtype classification for the phenotypes and standardised methodology for multi-centre studies.

GWAS of CVDs-related QTs could provide an alternative approach for better comprehending the phenotypic diversity of a given CVD as stated by the Wellcome Trust Case Control Consortium [32].

A significant proportion of GWAS has been focused on obesity. The identified genetic variants, including some paediatric studies, have been replicated in less than 2/3 of them. Interestingly, the *FTO* gene was identified as the first locus harbouring common variants with an unequivocal impact on obesity predisposition, diabetes and fat mass in population studies. The most important reports are summarised in Supplementary Table 1.

More than 1/4 of all GWAS for CVDs related QTs have studied fasting plasma glucose and diabetes (Supplementary Table 1). Up to 90% of them have been replicated in European, American and Asian populations. In two distinct studies, Zeggini et al. [33,34] reported the association of type 2 diabetes with 22 SNPs close to the following genes: *CDKN2A, CDKN2B, FTO, HHEX, IGF2BP2, KCNJ11, CDKAL1, TCF7L2, THADA, TSPAN8, LGR5, VEGF, NOTCH2, ADAM30, PPARG, SLC30A8, JAZF1, SYN2, CDC, ADAMTS9, CDC123, CAMK1D*. Of these, 2 SNPS in the vicinity of *TCF7L2* had the greatest association with type 2 diabetes (Supplementary Table 1).

Also, lipid profile and BP studies represent a considerable part of CVD GWAS. About 2/3 of SNPs associated with lipid levels have been successfully replicated, notably 3 in the vicinity of *CETP* (Supplementary Table 1). It is important to mention that, although rs3764261 in *CETP* was strongly associated with total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) (P-value $= 7 \times 10^{-380}$) [35], it was not associated with CHD. Interestingly, a number of GWAS have achieved to find some loci which were associated with both lipid levels and CHD risk. For example, there are some replicated SNPs in *CETP, APOC1, APOB, APOA1* genes that are associated with more than one related trait in CVD risk (Supplementary Table 1).

It should be noted also that the meta-analyses on hyperlipidaemic and diabetic individuals [25,35,36], have included some common subsamples. Therefore, similar association results will not be surprising [37].

In addition to lipid profiles, replication studies have been relatively successful in confirming BP and hypertension (HTN) key loci [38–40]. In the largest joint meta-analysis published in May 2009, by CHARGE [41] and GlobakBPgen [8] consortia, researchers discovered 14 loci associated with systolic BP, diastolic BP and/or HTN. Recently, Takeuchi et al. [38] succeeded in replicating 7 loci in Japanese subjects, whereas Liu et al. [39] and Hong et al. [40] have confirmed some of them in Chinese Hans and Koreans respectively. These main

findings constitute a milestone in bringing genome knowledge into BP regulation in diverse populations, as it will be discussed below [42].

In 2009, a GWAS assessed the possible associations between several hundred thousand SNPs and warfarin dose in about 1000 Swedish patients. The results showed 3 significant SNPs around *CYP2C9* and *VKORC1*. After removing the effects of those SNPs through multiple regression adjustment, an additional signal was observed, implicating another SNP around the cytochrome P450 gene (*CYP4F2*) [43]. Similar results were presented in Japanese population [44]. These findings raise the probability that testing patients for variations in the two mentioned genes might provide information that could improve clinical algorithms currently used to guide the administration of warfarin.

Also, the role of genetic factors in differences of lipid-lowering response to statin treatment seems to be influenced by several loci with small individual contributions that are compounded when simultaneously inherited. Although several loci have been investigated in relationship with efficacy of statin using the candidate gene approach, the combined contribution of these genotypes explains a relatively small proportion of the variation in statin lipid-lowering efficacy [45– 47]. GWA analysis of lipid-lowering response to statin treatment has previously failed to identify novel loci. In order to find eventual genetic variants, Barber MJ et al. [48] have done a combined analysis on 3 statin GWA studies. They have eventually found that the most provocation is the association of rs8014194 in *CLMN* gene with changes in total cholesterol in response to statin treatment. They have observed strongest association of this SNP in one of three populations. However, this genetic variation was less strongly associated with LDL-C reduction.

4. GWAS strengths and limitations

4.1. Current GWAS designs

GWAS involve various tiered designs including case–control and cohort studies for the identification of trait/disease-SNP associations. These strategies allow affordable procedures to be carried out by conducting a genome-wide scan in a discovery set in order to select a core of significantly associated SNPs that will be subsequently genotyped in larger replication sets for confirmation [49]. These intricate designs involve more and more tiers according to the complexity and originality of the trait of interest [50] and allow the elimination of previous spurious associations (false positives) [18]. Traditionally, genetic associations having causal functionality and appearing in multiple populations and studies are considered reliable [18,49].

Testing the association of millions of SNPs with a specific trait or disease implies a large number of statistical tests (at least one per SNP) making nominal significance (0.05) inappropriate for originally selecting associated variants. Such designs also require large populations [51] and this need led to the recruitment of large international consortia, in which substantial pooled GWAS have been conducted. Indeed, significant progress in the management of the methodological weaknesses of GWAS has been made under the auspices of large consortia providing numerous independent populations for pooling analyses and replications in specific fields, such as the Glucose and Insulin Related Traits Consortium (MAGIC) [52]. These pooling strategies required controls for differences in allele frequencies [13] to minimise false positive findings and reinforce the validity of genetic variants found to be associated with specific traits or pathologies.

Only 12% of SNPs discovered by all the GWAS performed to date and associated with traits are located in the vicinity of protein-coding regions of genes, although SNPs in protein-coding regions are heavily over-represented on genotyping arrays [22]. Forty percent falls in intergenic regions, and another 40% is located in introns. The repeated replication of signals falling consecutively in the so-called "gene deserts", although it initially raised concern in the scientific community, has sharpened the focus on the potential roles of intronic and particularly intergenic regions in regulating gene expression [14].

4.2. Future challenges

Over the past few years, GWAS have reported a large number of novel genetic variants associated with CVDs [53,54]; nevertheless, many challenges remain.

There is a need to perform GWAS on populations with diverse geographic ancestries, which have undergone more mutations and greater recombination events. This type of studies could give greater degrees of genetic variation and shorter stretches of linkage disequilibrium allowing better localisation of genome-wide association signals [20,32]. Several different ethnicities such as Japanese [38], African Americans [55] and mainly Europeans [8,41] have been examined. European populations are closely related [42], and their genome has undergone less recombination events than for example those of Africans [56], an advantage that has increased the number of studies concerning this specific descendent. Will the initial findings be replicated in non-European populations? Recent GWAS do not support this probability. The researchers believe that different populations have each one a unique population structure (different modifier genes and different gene-gene and gene-environmental interactions), different lifestyles and different environmental factors, which may expose or mask the risk of a SNP, for example rs6903956, in CAD [28]. Moreover, based on GWAS report in 2009 [57] there is a significant association between rs1252453 in *PHACTR1* on chromosome 6p24 and early onset MI in some European and American populations. However, according to HapMap data, not only the minor allele frequency of rs1252453 in Chinese population is 0%, but also none of the 113 SNPs in or near *PHACTR1* was associated with CAD in Chinese population [28]. Therefore, the public health policies and strategies, if they will include genetic factors in preventive medicine, should depend on ethnic group and may not be applicable worldwide [58].

Another interesting question is why numerous worldwide replication studies confirmed loci but not SNPs. Genetic variants having high occurrence in Europeans may have different frequencies across different populations. Also, many SNPs are rare in non-Europeans [59]. Moreover, allelic frequencies may be different even if the same SNP is found in diverse populations [60].

In this context, GWAS involving African populations are potentially of interest. Low linkage disequilibrium between genetic variants is one of their main characteristics, due in part to a long history of recombination and mutations. When SNPs extend over short genomic regions, further sequencing may be very effective in defining the true risk variant [42].

What will be the application of these findings in clinical practice and for predicting CVDs risk?

Based on the success of GWAS, some commercial companies are already offering to the public *in vitro* diagnostic genotyping assay to assess for example the CHD risk, although the risk attributable to any individual variant has been modest to date [61]. We believe that commercial assays do not give complete genomic coverage, as certain regions are not covered. Therefore, the influence of variation at these missing regions in determining CHD risks is very likely to have been underestimated, and some novel loci may have been missed completely. Nevertheless, even if valid genetic variants with modest effects explain a substantial population-attributable risk fraction they do not necessarily provide clinically useful prediction for individuals or specific population, groups [62]. However, combining multiple SNPs having modest effect into a global genetic risk score could improve the identification of high-risk populations and improve individual's risk assessment. Anderson et al. [61], have demonstrated the feasibility and the potential utility of simultaneously considering the joint effects of 5 different SNPs (rs599839 in *CELSR2*, rs2383206 in

9p21.3, rs289715 in *CETP*, rs78739461 in *ApoF* and rs1799963 in *F2*) retrieved from candidate gene studies and GWAS in not only improving the net risk classification of intermediate-risk individuals but in also predicting the risk of premature CHD [61].

Similarly to Anderson et al. [61], Kathiresan et al. [63] integrated several SNPs highly associated in GWAS with LDL-cholesterol (rs7575840 in ApoB, rs4420638 in APOE cluster, rs12654264 in HMGCR, and rs1529729 in LDLR) and HDL-cholesterol levels (rs3890182 in *ABCA1*) into a cardiovascular risk score [63,64]. However, in the study of Kathiresan et al. [63] the clinical risk prediction was not improved by genotype scores, nevertheless, there was a significant improvement in risk classification.

From a pharmacogenomics point of view, more studies taking into account the GWAS results are needed to prouve the clinical usefulness of genotyping to medication.

4.3. Missing heritability in cardiovascular diseases, an Achilles' heel

Although recent GWAS have an enormous sample size reaching up to 200,000 participants and revealing hundreds of associations with extremely impressive P-values, much of the genetic risk remains unexplained, and this represents the so-called 'dark matter' of genetic risk.

A large "hidden heritability" of unknown nature that may be explained by both low minor frequency alleles (MAF) and rare variants exists. However, the role of rare variants in population screening for CVDs has not been illustrated until now. GWAS have predominantly focused on common variants (allelic frequency >5% in the general population) [56] and ignored variants with low MAF (0.5%<MAF<5% in the general population) and rare frequency (MAF<0.5%), which are believed to have a greater risk effect [56]. In contrast to common variants, which are thought to be old in the history of humanity, rare variants are more recent and therefore not geographically extended [42]. Recently, deep sequencing revealed a myriad of rare, deleterious variants [65] and assessing their associations with increased risk will be the first step to better understand their role in CVDs. When focusing on rare genetic variants, GWAS require very large populations and different statistical methods that jointly analyse variants in a locus instead of testing each variant individually. The next generation of high resolution arrays with more genome-wide coverage is making this goal feasible.

Structural large variants (>500 Kb) such as deletions and duplications (Copy number variants, CNV) represent rare variants that may affect gene expression and molecular pathways in humans [9]. These genomic imbalances occur at an allele frequency of <0.05% and are present in about 8% of the total population [9], making them rare but collectively common. Results available suggest that CNVs strongly affect gene expression, thereby affecting mRNA splicing and transcriptional activity [66].

Gene–environment interactions (GxE) are also issues that might blind many GWAS. Integrating GxE could help in highlighting different loci effects according to modifiable risk factors, particularly in CVDs. This strategy needs large consortia, methodological efforts and stratified designs in order to improve the existing tools of GWAS that do not yet incorporate complex statistics. Although, environmental pathways acting through epigenetic mechanisms to modify gene expression [67] and DNA methylation [66] have given some fruitful results in other chronic diseases [68] and exposures [69], they are yet to be studied in CVDs. In addition to the usual challenges reported for genetic association studies, a successful GxE study should take into account sample size, exposure assessment and heterogeneity, described in full elsewhere [67]. GxE in GWAS requires enormous populations [67]. Smith and Day [70] explained that detecting an interaction needed a sample size at least 4 times greater than that required for detecting a main effect of comparable magnitude. Although difficult, studying GxE interactions could help in understanding discrepancies due to heterogeneous exposure.

Gene–gene (epistatic, GxG) interactions may also play an important role in discovering genes that have not yet been found by the consensual single-locus approach. This statement has been extensively reviewed [15,71,72] and both parametric and non-parametric multi-locus methods have been developed to detect such interactions [73] in the last years. Epistatic interactions have been documented for susceptibility to cancer [74], morphology [75] and autoimmune conditions [76]. However, current epistatic designs do not have genome-wide coverage, so that their application to high-dimensional genome-wide data including all imaginable SNPs remains a crucial challenge [77].

Furthermore, we advise that all loci associated with CVDs and related QTs should be replicated in paediatric populations, as a common variant could have a continuous impact throughout life. Epidemiological and pathophysiological evidence suggests that the precursors of CVDs originate in childhood [78,79]. The atherosclerotic process starts in childhood [80] and numerous studies have shown that CVDs risk factors during childhood can affect the risk in adulthood. For example, increased BP levels during childhood strongly predict HTN in adults [81]. Isolating genetic variations that may influence a given risk factor for CVDs at childhood, where many environmental factors such as alcohol intake, stress and smoking are absent, might have major implications for public health and could be a challenge in designing primary preventive strategies.

5. Concluding remarks and perspectives

GWAS have revealed many novel genetic risk variants in CVDs, making it an auspicious era for the better understanding of genetics. For the time being, we need to grasp the unexpected involvement of certain functional and mechanistic pathways identified in CVDs processes involving many QTs. These have to be extensively investigated in parallel with the pathology itself. Future GWAS must also detect low MAF and rare variants, and include GxG, GxE and well-designed candidate gene studies (in term of statistical power, homogeneity and exposure assessment) and involve subsequent transcriptomic and proteomic investigations. It is time to pause and think, instead of digging further down for more genetic loci with even smaller phenotypic effect.

Supplementary materials related to this article can be found online at doi:10.1016/j.cca.2011.05.035.

Disclosures

No conflicts of interest.

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II. Etude des interactions épistasiques dans la régulation des niveaux de pression artérielle (1/2):

Functional epistatic interaction between rs6046G>A in *F7* and rs5355C>T in *SELE* modifies systolic blood pressure levels.

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Bien que de nombreuses GWASs et études gène-candidat aient été effectuées, seul un nombre restreint de SNPs fonctionnels régulant la PA a été découvert. Ce phénomène peut être expliqué en partie par la présence d'éventuelles interactions épistasiques. Ainsi, notre objectif était d'étudier les interactions épistasiques de 10 SNPs appartenant à 10 gènes candidats de différentes voies métaboliques (PA, inflammation, métabolisme lipidique, adhésion cellulaire, coagulation sanguine).

Notre objectif était d'identifier une ou plusieurs interactions épistasiques fonctionnelles influençant la PA.

Trois mille six cent individus non apparentés de nationalité française ont constitué l'échantillon de phase 1 ou *'discovery set'*. Dans un premier temps, nous avons étudié l'éventuelle association des 10 SNPs avec les niveaux de PA. Ensuite, nous avons évalué les interactions épistasiques seulement entre les SNPs montrant des associations significatives. Nous avons également entrepris une phase de réplication des interactions épistasiques identifiées chez 4,662 participants Européens (*'replication set'*). De plus, nous avons recherché des associations avec l'expression de 10 gènes inflammatoires différemment exprimés selon le statut d'HTA (*LL37, DEFA1-3, FPR1, ICAM1, SELL, SELP, NAMPT* (*visfatin*)*, LEP, TNF* et *IL-6*) dans les PBMCs d'un sous-échantillon de 90 individus supposés sains.

Dans le *'discovery set'*, nous avons pu mettre en évidence 2 SNPs significativement associés à une diminution de PA c'est à dire, l'allèle T du rs5355C>T du gène *SELE* ($P=8,2x10^{-4}$, $\beta=-0,04$) et l'allèle A du rs6046G>A du gène *F7* ($P=5x10^{-3}$, $\beta=-0,08$). Par la suite, nous avons mis en

évidence que le rs5355C>T et le rs6046G>A interagissent aussi pour modifier les niveaux de PAD (P=0,047) et PAS (P=0,048). Nous avons constaté que l'allèle rs6046A inversait l'effet bénéfique de l'allèle rs5355T sur les niveaux de PA.

Plus intéressant encore, dans le *'replication set'*, nous avons trouvé des signes de réplication pour le rs6046G>A avec la PAS et la PAD ($P=8,45x10^{-4}$, $\beta=-0,03$ et $P=2,58x10^{-7}$, $\beta=-0,03$ respectivement) et pour l'interaction épistasique entre le rs5355C>T et le rs6046G>A avec la PAS (P=0,03).

Dans les PBMCs, l'allèle rs6046A de *F7* était positivement associé aux taux d'ARNm *NAMPT* ($P\leq9,1x10^{-5}$). L'augmentation des niveaux d'ARNm de *NAMPT* était positivement corrélée avec l'expression des gènes *ICAM1*, *SELL*, *FPR1*, *DEFA1-3* et *LL-37* ($P\leq5x10^{-3}$). Seuls les taux d'ARNm de *DEFA1-3* et de *LL-37* étaient positivement associés aux niveaux de PAS ($P\leq0,01$), expliquant 4% de sa variabilité phénotypique.

L'étude de la génétique de PA et d'HTA n'a jamais été facile. Ce domaine a été toujours affecté par une contradiction frappante entre sa forte héritabilité génétique et la réalité frustrante qu'aucun SNP clairement reproductible et fonctionnel pourrait être découvert,et les interactions épistasiques ont été acceptées en tant que cause de divergences à travers les études. La présente étude est la première montrant une interaction épistasique fonctionnelle et reproductible influençant la PAS. Cependant, notre étude a également présenté plusieurs limites. Alors que se concentrer sur les populations d'origine Européenne à l'avantage de minimiser les problèmes potentiels de stratification, nos conclusions ne peuvent être généralisées à d'autres groupes ethniques. Nous avons également été incapables d'investiguer les associations qui pourraient exister entre les SNPs et les niveaux plasmatiques des protéines des 10 gènes inflammatoires. De même, des investigations complémentaires cherchant l'association entre le rs5355C>T, le rs6046G>A et l'expression génique de *SELE* et *F7* dans les cellules endothéliales, seraient d'une grande valeur.

Les données les plus récentes indiquent qu'au delà des SNPs identifiés en PA, un grand nombre de variants génétiques communs, affectant la PA, restent à identifier. Une fois révélés, ils pourraient expliquer une partie importante de la variation phénotypique de la PA. L'ensemble de nos résultats confortent les observations précédentes et révèlent la nécessité de prendre en compte les interactions épistasiques et les mécanismes inflammatoires lors de l'étude de la génétique de la PA.

Mots clés : Pression artérielle, épistasie, polymorphisme génétique ponctuel, cellules mononuclées du sang périphérique.

Contribution : *Nous avons participé à la conception et au design de l'étude. Nous avons pris en charge les expériences de génotypage et de transcriptomique. Nous avons également rédigé la première version du manuscrit.*

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Functional Epistatic Interaction between rs6046G>A in *F7* and rs5355C>T in *SELE* Modifies Systolic Blood Pressure Levels

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Abstract

Background: Although numerous genetic studies have been performed, only 0.9% of blood pressure phenotypic variance has been elucidated. This phenomenon could be partially due to epistatic interactions. Our aim was to identify epistatic interaction(s) associated with blood pressure levels in a pre-planned two-phase approach.

Methods and Results: In a discovery cohort composed of 3,600 French individuals, we found rs6046A allele in *F7* associated with decreased blood pressure levels ($P \leq 3.7 \times 10^{-3}$) and rs5355T allele in *SELE* associated with decreased diastolic blood pressure levels ($P = 5 \times 10^{-3}$). Both variants interacted in order to influence blood pressure levels ($P \leq 0.048$). This interaction was replicated with systolic blood pressure in 4,620 additional European individuals ($P = 0.03$). Similarly, in this replication cohort, rs6046A was associated with decreased blood pressure levels ($P \leq 8.5 \times 10^{-4}$). Furthermore, in peripheral blood mononuclear cells of a subsample of 90 supposed healthy individuals, we found rs6046A positively associated with *NAMPT* mRNA levels ($P \leq 9.1 \times 10^{-5}$), suggesting an eventual involvement of *NAMPT* expression in blood pressure regulation. Confirming this hypothesis, further transcriptomic analyses showed that increased *NAMPT* mRNA levels were positively correlated with *ICAM1*, *SELL*, *FPR1*, *DEFA1-3*, and *LL-37* genes expression ($P \leq 5 \times 10^{-3}$). The last two mRNA levels were positively associated with systolic blood pressure levels ($P \leq 0.01$) and explained 4% of its phenotypic variation.

Conclusion: These findings reveal the importance of epistatic interactions in blood pressure genetics and give new insights for the role of inflammation in its complex regulation.

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Introduction

Blood pressure (BP) is a heritable trait with estimates indicating that 30–70% of its variance is attributed to genetics [1,2]. In family studies its heritability varies, according to measurement processes, from ≈31% [single-measure of systolic blood pressure (SBP) and diastolic blood pressure (DBP)], to ≈57% (long-term average of SBP and DBP phenotypes) and to ≈68% (24-hour profile of SBP and DBP) [3]. Both BP and essential hypertension (HTN) are considered polygenic traits [4]. Inflammation, blood coagulation cascade, cellular adhesion molecules and lipid metabolism appear to have significant roles [5].

The largest Genome-wide association study (GWAS) on BP including ≈200,000 individuals [6] reported 29 loci to be associated with SBP, DBP and/or essential HTN [6]. However, their genetic risk score explained only 0.9% of BP phenotypic variation [6], this representing the so-called 'dark matter' of genetic risk [7]. Despite the very large sample-size studies used for gene discovery, many common variants with small effects on BP may remain unidentified [8]. A large 'hidden heritability' of unknown nature may be explained by rare variants, structural large variants, epistatic [gene*gene (G*G)] and gene*environment (G*E) interactions [7]. We pointed out that epistatic interactions might also play an important role in discovering new genes [7]. This statement has been extensively reviewed in the last years and multi-locus methods have been developed to detect such interactions [7].

Epistatic interactions have been documented for susceptibility to cancer [9], morphology [10] and autoimmune conditions [11]. However, to date they have not been extensively studied in BP regulation. We hypothesize that the research of epistatic interactions among candidate single nucleotide polymorphisms (SNPs) represents a challenge in the investigation of disease-risk variants, as their application to high-dimensional genome-wide data

exhaustively including all SNPs combinations is not yet feasible [7]. In previous candidate gene studies, we showed interesting results concerning the identification of BP candidate SNPs [12–16]. However, these studies were conducted in limited-sized populations.

Therefore, in the present study, we investigated BP epistasis mechanisms in a pre-planned two-phase approach gathering 8,220 European individuals. The effect of 10 candidate SNPs and then, G*G interactions between significant SNPs were assessed in a discovery population of 3,600 individuals. Highlighted epistases were replicated in 4,620 additional European individuals. We further searched for association(s) with 10 inflammation-related genes in peripheral blood mononuclear cells (PBMCs) (*LL37*, *DEFA1-3*, *FPR1*, *ICAM1*, *SELL*, *SELP*, *NAMPT* (*visfatin*), *LEP*, *TNF* and *IL-6*) [17] of a subsample of 90 supposed healthy individuals. Finally, we sought to propose a possible molecular mechanism of action.

Materials and Methods

Ethics Statement

All participants involved in the present study were recruited in accordance with the latest version of the Declaration of Helsinki for Ethical Principles for Medical Research Involving Human Subjects and gave written informed consent. Genetic studies protocols were approved by the local ethics committees for the protection of subjects for biomedical research: 1) the *Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine*, Nancy, France, for populations recruited in the Center of Preventive Medicine. 2) the *Comité d'Ethique du Centre Hospitalier Universitaire de Cochin*, Paris, France, for ERA population. 3) The ethic committee of Belfast, Ireland, for population recruited in Ireland.

Study Populations

Discovery population. A sample of 2,971 unrelated individuals was recruited during free medical check-ups at the Center of Preventive Medicine of Vandœuvre-lès-Nancy in the East of France. They were Caucasians, born in France for three generations and their clinical and biological data were collected at baseline before any eventual drug prescription following consultation. They were selected on the basis of the following criteria: (1) no antihypertensive drug therapy at recruitment; (2) complete clinical and genotypic data available; (3) and BP levels ranging from normotensive to stage 2 HTN (for hypertensive individuals, data were gathered before the prescription of any medication).

As our purpose was to assess BP as a continuous trait, and in order to have a proper inter-individual variability, we included individuals from the ERA cohort (*Evolution de la Rigidité Artérielle*) in the discovery population. ERA participants were selected from a Parisian cohort that had a health check-up at the *'Investigations Préventives et Cliniques'* center. The details of this study have been previously presented [18]. Six hundred and twenty nine individuals randomly selected in ERA were incorporated in the discovery population. As no significant differences between minor allele frequencies (MAFs) of the investigated genetic variants and BP levels in these samples were found, we regrouped both discovery samples in order to perform our statistical analyses.

The corresponding samples were part of a human sample storage platform: the Biological Resources Bank 'Interactions Gène-Environnement en Physiopathologie CardioVacsulaire' (BRC IGE-PCV) in Nancy, East of France.

Replication population. We used a non-overlapping sample extracted from the BRC IGE-PCV. Altogether, 4,620 individuals with (1) no antihypertensive drug therapy at recruitment; (2) complete clinical and genotypic data for rs5355C>T in *SELE* and rs6046G>A in *F7* were available; (3) BP levels ranging from normotensive to stage 2 HTN; and (4) only European origins were analyzed (Ireland, French). Stage 3 HTN patients were also excluded in the replication population as they were treated with antihypertensive medication.

Clinical and Biological Data Collection

SBP and DBP were measured under constant temperature (19°C–21°C) and standardized conditions (supine position) using a manual sphygmomanometer (Colonne à mercure, Mercurius) by expert nurses [18]. The recorded values were the means of 3 readings with 20 min intervals. An adjustable BP cuff was used to correct errors due to variations in arm circumference [19]. HTN was defined as SBP≥140 mmHg or DBP≥90 mmHg as recommended in the Seventh Report of the Joint National Committee on the prevention, detection, evaluation, and treatment of high BP [20]. All individuals underwent complete medical examination including anthropometric and biochemical measurements collected with standardized methods as described elsewhere [17].

Genotyping Assays

We selected rs1799752Ins>del in *ACE*, *rs5882A>G in CETP*, rs1801133C>T in *MTHFR* rs662A>G in *PON1* and rs1800629G>A in *TNF* from the "Cardio-Vascular Disease 35" assay, a multilocus genotyping assay developed in collaboration with Roche Molecular Systems [12]. These genetic variants were candidate markers for cardiovascular disease (CVD) risk factors, specifically involved in the predisposition to essential HTN (rs1799752Ins>del in *ACE*), in the development of atherosclerotic plaques and in the progression of atherosclerosis (*rs5882A>G in CETP*, rs1801133C>T in *MTHFR* rs662A>G in *PON1* and rs1800629G>A in *TNF*) [12]. In addition, rs5355C>T in *SELE* [13,21], rs1800790G>A in *FGB* [14], rs6046G>A in *F7* [15], rs328C>G in *LPL* [16,22] were chose based on our previous published studies that found these SNPs associated with BP levels and/or HTN in European populations [12–16,21,22]. Finally, rs3025058T>Ins in *MMP3* was selected from an internal investigation showing a link between this genetic variant and BP levels.

A summary of investigated genetic variants (nearby gene, location, type and mutation) was shown in Supplementary Data S1.

Genomic DNA was extracted from peripheral blood samples using the salting out method [23]. Genotyping was performed using two methods in the discovery population. 1) A multilocus assay with an immobilized probe approach designed by Roche Molecular Systems, Pleasanton, California, USA [24]. After PCR amplification using pooled biotinylated primers and hybridization to sequence-specific oligonucleotide probes, two independent observers using proprietary Roche Molecular Systems image processing software performed genotype assignments. Among 2,971 individuals, discordant results (<3% of all scoring) were resolved by a third observer and if necessary, by a joint reading. 2) Evidence Investigator™ biochip designed by Randox Laboratories, Antrim, UK was used to genotype ERA participants. This genotyping assay is based on a combination of probe hybridization, ligation, PCR amplification and microarray hybridization. This unique design permits high assay multiplexing and ready discrimination between genotypes. For the validation of genotyping results, blinded replication analysis was performed on 50

common samples. Both genotyping methods gave matched results at 99% (data available on demand).

Replication population. Only rs5355C>T in *SELE* and rs6046G>A in *F7* were genotyped. Among all individuals; 2,059 were genotyped by Kbioscience company using the competitive allele specific PCR (KASP) chemistry coupled with a FRET-based genotyping system (http://www.kbioscience.co.uk/reagents/KASP/KASP.html). The remaining 2,561 individuals were genotyped by Roche multilocus assay as described previously.

PolyPhen Analysis of Nonsynonymous SNPs

The prediction of nonsynonymous SNPs possible impacts on their protein structures was performed using PolyPhen [25].

Peripheral Blood Mononuclear Cells Collection

Freshly drawn peripheral venous blood (10 ml) was collected into tubes containing EDTA (Vacutainer, Becton Dickinson) under fasting conditions. PBMCs were isolated by centrifuging on a density gradient of Ficoll as described previously and stored at -80°C until RNA extraction [26]. PBMCs bank with high recovery of lymphocytes (97.5%) was constituted as described elsewhere [26].

RNA Extraction and qRT-PCR Analysis

Using a microarray analysis [5]; we selected the top 10 inflammation-related genes (from a total of 182 genes) having a higher expression in PBMCs of hypertensive individuals when compared with normotensives. Total RNA was isolated from PBMCs by an automated isolation procedure (MagNa Pure LC instrument). mRNA quality and stability were carefully tested [26] and reverse transcribed as previously described [26]. Quantitative real-time PCR (qRT-PCR) was performed using LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with Master Plus SYBR Green I kit for all gene transcripts. *SELE* and *F7* were not quantified, as they were not expressed. Specific primers were designed using Primer Premier 3.0 software (Supplementary Data S1). All experiments were carried out in duplicates in a total reaction volume of 20 µl containing 0.5 mM of each specific primer. Negative and internal controls were included. All mRNA levels were normalized to the mRNA levels of *POL2RA*. The specificity of all PCR products was further verified by electrophoresis on 10% polyacrylamide gel (data available on demand). The clinical characteristics of the studied subsample were presented in Supplementary Data S1.

Statistical Analyses

Statistical analyses were performed using the SPSS® statistical software version 19.0 (SPSS, Inc, Chicago, Illinois). Polymorphisms with MAF deviating from Hardy-Weinberg equilibrium (HWE) were excluded from individual analyses. In order to determine the effect of the 10 selected genetic variants on SBP and DBP assuming additive models using the common wild type as the reference group; age, gender and body mass index (BMI)-adjusted linear regressions were performed for individual association analyses. Due to multiple testing, the significance level was set at $P \leq 5 \times 10^{-3}$ in the discovery and replication populations.

G*G interactions. Two-locus additive epistasis was defined as significant statistical interaction between two SNPs [27] and was determined when significant interaction existed on a linear additive model adjusted for age, gender and BMI. Epistatic interactions were only tested between individually significant associated SNPs. In both populations, Bonferroni correction for multiple testing was applied. The significance level was set at $P \leq 0.05$.

SNP-mRNA association analysis. Linear regressions were performed to assess the effect of SNPs previously associated to SBP and/or DBP in the first stage of our analyses on mRNA levels. In epistatic conditions, an interaction term was introduced in the model. The significance level was set at $P \leq 5 \times 10^{-3}$ due to multiple testing.

Pearson's correlation analyses. Pearson's correlation was used to test the correlation between all genes expression and *NAMPT* levels (values log-transformed). The significance level was set at $P \leq 5 \times 10^{-3}$ due to multiple testing.

Linear regression analysis between genes expression and BP levels. Linear regression models were used to further assess the association of *SELL*, *FPR1*, *ICAM1*, *DEFA1-3* and *LL-37* with mRNA levels with SBP and DBP after adjustment for age and gender. The significance level was set at $P \leq 0.01$ due to multiple testing.

URLs

Primer Premier 3.0 is available at: http://frodo.wi.mit.edu/primer3/.

Polyphen is available at: http://genetics.bwh.harvard.edu/pph2/.

Results

Table 1 presents the clinical characteristics of the studied populations. According to the Seventh Report of the Joint National Committee [28], 21.8% of participants had normal BP, 32% were pre-hypertensive and 46.2% had HTN stage 1 and 2 in the discovery population (Table 1). In the replication set, 34% had normal BP, 39.8% were pre-hypertensive and 26.2% were stage 1 and 2 hypertensive (Table 1). A higher frequency of HTN was observed in the discovery compared to the replication population (46.2% vs. 26.2% respectively), which is partly due to the presence of older individuals in the discovery set.

Table 2 shows genetic variants associated with BP traits. We found two SNPs, rs5355C>T in *SELE* and rs6046G>A in *F7* showing associations with SBP and/or DBP respectively in the

Table 1. Characteristics of studied individuals.

	Discovery population	Replication population
N (% women)	3,600 (47.4)	4,620 (43.3)
Age (years)	47.3±10.5	38.2±16.6
BMI (kg/m^2)	25.4±3.8	24.3±4.4
SBP (mmHg)	136.9±20.2	130.6±20.1
DBP (mmHg)	84.1±13.8	77.1±16
BP category (%)		
<120/80 mmHg	21.8	34
120–139/80–89 mmHg	32	39.8
≥140 and/or 90 mmHg	46.2	26.2
MAF (%)		
rs5355C>T	16	9
rs6046G>A	25	25

BMI: body mass index, BP: blood pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAF: minor allele frequency.
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discovery population ($P_{discovery} \leq 5\times10^{-3}$, Table 2). rs5355T allele in *SELE* was associated with decreased DBP levels ($P = 5\times10^{-3}$, $\beta = -0.04$, Table 2), whereas rs6046A allele in *F7* was associated with decreased SBP and DBP levels respectively ($P = 3.7\times10^{-3}$ and $P = 8.2\times10^{-4}$ respectively, Table 2). Both SNPs are nonsynonymous, introducing amino acid substitutions (Leu575Phe and Arg353Gln respectively). According to Polyphen, they were predicted to have a null effect on their corresponding protein structures. full individual association results with BP in the discovery and the replication population were shown in Supplementary Data S1.

In order to examine whether rs5355C>T in *SELE* and rs6046G>A in *F7* may also indirectly influence BP levels, we tested their G*G interaction (Table 3). Both SNPs interacted in order to influence SBP and DBP in the discovery population ($P = 0.048$ and $P = 0.047$ respectively, Table 3A). Table 3A shows BP variations according to rs5355T allele in *SELE* and rs6046G/A genotypes in *F7* when compared to rs5355C allele in *SELE*. We found that individuals carrying rs5355T allele in *SELE* and rs6046GG in *F7* had 6.5 mmHg and 8 mmHg decrease in SBP and DBP respectively when compared with carriers of rs5355C allele in *SELE* and rs6046GG genotype in *F7* (Table 3A). In contrast, individuals carrying rs5355T allele in *SELE* and one minor allele of rs6046G>A (rs6046GA) had 6.1 mmHg and 1.2 mmHg increase in SBP and DBP respectively when compared with carriers of rs5355C allele in *SELE*, rs6046GA genotype in *F7* (Table 3A). Furthermore, carriers of rs5355T allele in *SELE* and two minor alleles of rs6046G>A (rs6046AA) had higher BP levels when compared with those carrying rs5355C allele in *SELE* and rs6046AA genotype in *F7* (5.1 mmHg and 3.8 mmHg increase in SBP and DBP respectively) (Table 3A). We concluded that rs6046A might invert the BP-lowering effect of rs5355T on DBP and SBP.

In the replication population, rs6046G>A in *F7* was also associated with decreased SBP ($P_{replication} = 8.45\times10^{-4}$ and $P_{meta} = 2.03\times10^{-4}$) and DBP ($P_{replication} = 2.58\times10^{-7}$and $P_{meta} = 9.16\times10^{-4}$). In contrast, rs5355C>T was not associated with DBP ($P_{replication} = 0.86$). Most importantly, we found rs5355C>T in *SELE* and rs6046G>A in *F7* interacting in order to influence the SBP ($P_{replication} = 0.03$, Table 3B). Similar SBP variations according to rs5355T allele in *SELE* and rs6046G/A genotypes in *F7* were successfully found (Table 3B).

In conclusion, rs5355C>T in *SELE* interacted with rs6046G>A in *F7* in order to influence SBP in a total of 8,220 European individuals.

We investigated the eventual relation(s) between the epistatic interaction and the inflammation-related genes in a PBMCs model. rs5355C>T in *SELE* was not associated with any of the investigated transcripts. In contrast, rs6046A allele in *F7* was positively associated with *NAMPT* mRNA levels in both models (individual association and epistatic interaction models) ($P = 9.2\times10^{-5}$, $\beta = 0.489$ and $P = 1.1\times10^{-5}$, $\beta = 0.552$ respectively).

Increased *NAMPT* mRNA levels were positively correlated with *ICAM1* ($P < 1\times10^{-4}$ and $\beta = 0.576$, Table 4), *SELL* ($P = 5\times10^{-3}$ and $r = 0.308$, Table 4), *FPR1* ($P = 2\times10^{-4}$ and $r = 0.394$, Table 4), *LL-37* ($P = 4\times10^{-3}$ and $r = 0.452$, Table 4) and *DEFA1-3* ($P = 5\times10^{-3}$ and $r = 0.28$, Table 4) genes expression. In addition *ICAM1*, *SELL*, *FPR1* and *DEFA1-3* expressions were also correlated ($P \leq 5\times10^{-3}$, Table 4). Only *DEFA1-3* and *LL-37* mRNA levels were positively associated with SBP. We found that both mRNAs explained 4% of SBP phenotypic variation ($P = 3\times10^{-3}$, $\beta = 0.04$ and $P = 0.01$, $\beta = 0.03$ respectively).

Discussion

In the current study, we found rs6046A allele in *F7* associated with decreased BP levels ($P \leq 3.7\times10^{-3}$ and $P_{meta} \leq 2.03\times10^{-4}$). In the discovery cohort, rs5355T allele in *SELE* was also associated with decreased DBP ($P = 5\times10^{-3}$).

rs6046G>A in *F7* was shown to be associated with increased F7 plasmatic levels [15]. More interestingly, this SNP was reported to have a role in protection against myocardial infarction in two different studies performed on Italian populations [29,30]. rs5355C>T in *SELE* is located in chr.1q, a genomic region linked to BP related phenotypes in two independent linkage studies [31,32]. These findings were supported by observation of mouse and rat BP-related quantitative trait loci in regions homologous to the human 1q chromosomal locus [33].

Herein, we showed that in a total of 8,220 European individuals, rs5355C>T in *SELE* interacted with rs6046G>A in *F7* and the latter SNP in order to alter SBP ($P_{discovery} = 0.047$ and $P_{replication} = 0.03$ respectively, Table 3). The above interaction was differently associated with SBP variations according to rs6046G>A genotypes (Table 3). In fact, epistatic interactions are phenomena where the effect of a gene is modified by another one [34,35], thus although rs6046A allele in *F7* was associated with decreased BP levels, it interacted with rs5355T allele in *SELE* in order to influence SBP levels, resulting an increase in SBP mean values.

The non-replication of the association between rs5355C>T in *SELE* and DBP is not surprising as insignificant interaction effect on DBP between these two variants was found in the replication cohort. It is important to point out that, it has been postulated that epistatic interactions may identify genetic markers that are not captured by individual marker analysis and/or revealed by the combinatory effect of loci in other pathways [34,35]. This

Table 2. Genetic variants associated with blood pressure.

Chr	Gene	SNP ID	Discovery population		Replication population		P_{meta}	BP trait
			$P_{discovery}$	Beta* (mmHg)	$P_{replication}$	Beta* (mmHg)		
1q22-q25	*SELE*	rs5355C>T	5×10^{-3}	−0.04	0.86	–	0.09	DBP
13q34	*F7*	rs6046G>A	3.7×10^{-3}	−0.06	8.45×10^{-4}	−0.03	2.03×10^{-4}	SBP
			8.2×10^{-4}	−0.08	2.58×10^{-7}	−0.03	9.16×10^{-4}	DBP

*: Log10 transformed values.
Beta coefficients are shown for significant associations.
Chr: chromosome, SNP: single nucleotide polymorphism, MAF: minor allele frequency, Beta: coefficient in the linear regression model, BP: blood pressure, P_{meta}: P meta-analysis, SBP: systolic blood pressure, DBP: diastolic blood pressure.
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Table 3. Blood pressure variations according to rs5355T allele in *SELE* and rs6046G/A genotypes in *F7* when compared to rs5355C allele in *SELE*.

A-Discovery population	*SELE*					
		rs5355T	P*		rs5355T	P*
		SBP (mmHg)			DBP (mmHg)	
F7	rs6046GG	−6.5		rs6046GG	−8	
	rs6046GA	6.1	0.047	rs6046GA	1.2	0.048
	rs6046AA	5.1		rs6046AA	3.8	
B-Replication population	*SELE*					
		rs5355T	P*		rs5355T	P*
		SBP (mmHg)			DBP (mmHg)	
F7	rs6046GG	−6.5		rs6046GG	–	
	rs6046GA	2.2	0.03	rs6046GA	–	0.102
	rs6046AA	3		rs6046AA	–	

Only significant blood pressure variations are shown.
BP variations in individuals carrying rs5355T allele in *SELE* and rs6046GG in *F7* were compared with carriers of rs5355C allele in *SELE* and rs6046GG genotype in *F7*. BP variations in individuals carrying rs5355T allele in *SELE* and rs6046GA genotype in *F7* were compared with carriers of rs5355C allele in *SELE*, rs6046GA genotype in *F7*. BP variations in carriers of rs5355T allele in *SELE* and rs6046AA genotype in *F7* were compared with those carrying rs5355C allele in *SELE* and rs6046AA genotype in *F7*.
DBP: diastolic blood pressure, P*: p value for epistatic interaction model, SBP: systolic blood pressure, BP: blood pressure.
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postulate might explain why the two variants investigated here (and many others) were not reported among the top GWAS SNPs.

It was proven that blood coagulation factors enhance the inflammatory response leading to endothelial dysfunction accounting in part, for the vascular complications occurring in CVDs and their risk factors [36]. Thus, we searched for eventual relation(s) between the epistatic interaction and the inflammation-related genes in a PBMCs model. Numerous studies have revealed the importance of studying PBMCs in a strategy targeting the metabolic pathways of cardiovascular risk factors, such as HTN [5,37,38]. It has been recently demonstrated that PBMCs mRNA expression closely mimic the *in vivo* state and generate more physiologically relevant data concerning many health related traits [39]. The role of multiple metabolic pathways in HTN makes the study of PBMCs transcriptome important for the possible developing of diagnostic and prognostic tests [40], we assessed associations between rs5355C>T in *SELE* and rs6046G>A in *F7* with the inflammation-related genes expression. rs6046A allele in *F7* was associated with increased *NAMPT* mRNA levels ($P \leq 9.2 \times 10^{-5}$). *NAMPT* levels were also positively correlated with *ICAM1*, *SELL*, *FPR1*, *DEFA1-3* and *LL-37* genes expression ($P \leq 5 \times 10^{-3}$, Table 4). In addition *ICAM1*, *SELL*, *FPR1* and *DEFA1-3* expressions were also correlated ($P \leq 5 \times 10^{-3}$, Table 4). Only *DEFA1-3* and *LL-37* expressions were associated with SBP ($P = 3 \times 10^{-3}$ and $P = 0.01$ respectively) and explained 4% of its variation. Therefore, we suggest that the associations of *DEFA1-3* and *LL-37* mRNAs and SBP reflect the epistatic interaction and not the main effect of rs6046G>A in *F7*. Visfatin is a multifunctional protein that has been reported to be involved in innate immune system [41] and several other biological processes such as the cardiovascular system [42]. However, its role in BP was unclear. Supporting our results, three different *in vitro* studies have demonstrated that visfatin induced an endothelial dysfunction by increasing inflammatory and adhesion molecules expression such as ICAM1 [43–45]. In addition, in a previous study we have reported that gene expression of an antimicrobial peptide LL-37 in PBMCs was associated with altered BP levels [46]. The above findings support our epistatic and the *in vivo* results revealing an indirect link between *NAMPT* gene expression and BP through the expression of adhesion and innate immune system molecules.

Table 4. Pearson's correlations between *NAMPT*, *ICAM1*, *SELL*, *FPR1*, *DEFA1-3* and *LL-37* genes expression.

r P	*NAMPT*	*ICAM1*	*SELL*	*FPR1*	*DEFA1-3*	*LL-37*
NAMPT		0.6	0.3	0.4	0.3	0.5
ICAM1	$<1 \times 10^{-4}$		0.5	0.6	0.3	0.4
SELL	5×10^{-3}	$<1 \times 10^{-4}$		0.697	–	–
FPR1	2×10^{-4}	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$		–	0.3
DEFA1-3	5×10^{-3}	5×10^{-3}	–	–		0.9
LL-37	4×10^{-3}	1×10^{-3}	–	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$	

Only Significant correlations are shown ($P \leq 5 \times 10^{-3}$).
All genes expression were normalized to *POL2RA* mRNA levels.
r: Pearson's correlation coefficient, P: P-value.
doi:10.1371/journal.pone.0040777.t004

Strengths and Limitations

The genetics of BP has never been easy [47]. For many years, it has been dominated by the stark contrast between its high heritability and the frustrating reality that no clearly reproducible and functional genetic variant could be discovered [3], with epistatic interactions accepted as cause of discrepancies across the studies.

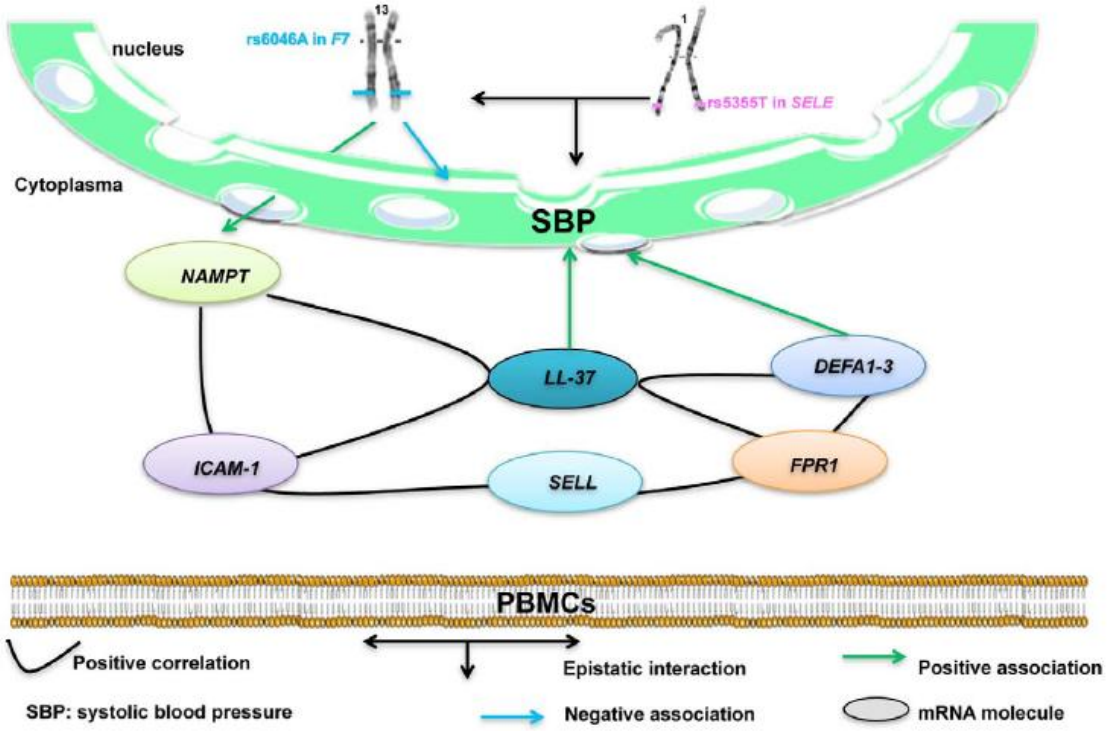

Figure 1. Summary of the study and hypothesis for rs5355C>T in *SELE* and rs6046G>A in *F7* interaction. rs6046A allele in *F7* was associated with decreased BP levels. rs5355C>T in *SELE* and rs6046G>A in *F7* interacted in order to alter SBP levels, rs6046A inverted the BP-lowering effect of rs5355T. rs6046A allele in *F7* was positively associated with increased *NAMPT* gene expression. *NAMPT* levels were positively correlated with *ICAM1*, *SELL*, *FPR1* and *DEFA1-3* genes expression. Only *DEFA1-3* and *LL-37* expressions were correlated and associated with SBP levels and explained 4% of its variation.
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The current study shows the first replicated epistatic interaction in the BP genetics field. This interaction between a coagulation factor gene (*F7*) and an adhesion molecule gene (*SELE*) is putatively functional through its link with five inflammation-related gene expression. Going in the same direction; it has been demonstrated that some blood coagulation factors can induce an endothelial dysfunction (*SELE* is a marker of endothelial dysfunction) through an inflammatory response accounting for the vascular complications occurring in CVDs and their risk factors [36]. Similarly, *NAMPT* expression was shown to increase the expression of inflammatory and adhesion molecules such as *ICAM1* [43–45]. Based on these findings we speculate a biological plausibility for the reported epistatic interaction. Supporting this statement, Tomaszewski et al [48] showed that a genetic risk score including SNPs from the fibroblast growth factor signaling pathway was able to explain a larger proportion of variation in HTN as compared with a genetic risk score including a similar number of SNPs based on the previous top SNPs from the GWAS [48]. This suggests that biological knowledge might support the reported epistatic interactions.

However, our study also had several limitations. Whereas focusing on European populations, our findings cannot be generalized to other ethnic groups. We also were unable to further investigate SNPs associations with plasmatic levels of the inflammation-related genes as the availability of biological materials was unfortunately limiting. Similarly, further studies looking at the SNPs association with *SELE* and *F7* expression in endothelial cells, would be of great value.

Conclusion

Our findings are summarized in Figure 1. In European populations, we confirmed that rs6046A in *F7* is associated with decreased BP. Furthermore, we found that rs5355C>T in *SELE* and rs6046G>A in *F7* interacted in order to alter SBP levels. In addition rs6046A allele in *F7* was positively associated with increased *NAMPT* gene expression, which was linked with BP through inflammatory mechanisms via the expression of adhesion and innate immune system molecules.

Perspectives

Even if additional investigations are needed, the present study highlighted the importance of taking into account candidate genes, GWAS and epistatic interactions in order to in deep investigate BP genetic regulation. One must also consider the functionality of relationships and G*E interactions that might be at the origin of the low until now predictive values of results in HTN. This integrative approach could better explain the missing heritability of this complex trait.

Supporting Information

Supplementary Data S1
(DOC)

Author Contributions

Conceived and designed the experiments: SVS SES. Performed the experiments: SES HAM CM. Analyzed the data: NCN MGS SES SVS. Contributed reagents/materials/analysis tools: PF JVL. Wrote the paper: SES SVS. Biological Resources Center (BRC): "Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire" (IGE-PCV) populations: SVS. ERA population: AB.

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III. Etude des interactions épistasiques dans la régulation des niveaux de pression artérielle (2/2):

Two epistatic interactions may be involved in blood pressure genetic regulation.

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BMC Medical Genetics, accepté avec modifications.

Dans la même logique que l'étude précédente, nous avons entrepris la recherche des interactions épistasiques de cent SNPs candidats de diverses voies métaboliques liées aux facteurs de RCV : la régulation de la PA, l'inflammation, le métabolisme lipidique, le métabolisme de l'homocystéine, l'adhésion cellulaire et la thrombose en rapport avec la PA chez 1,912 individus d'âge moyen (51,1±10,2) et d'origine française issus de notre CRB IGE-PCV. Ces sujets étaient soit non HT ne prenant donc aucune médication anti-hypertensive, soit HT, et dans ce cas, leurs données cliniques étaient déterminées avant l'administration de médication. Ce sont ces données sans effet de la thérapie qui ont été utilisées dans notre étude.

Nous avons génotypé 1,912 adultes français faisant partie de la cohorte STANISLAS par la technique du multiplex Roche « *discovery cohort* ». Ensuite, nous avons entrepris une phase de réplication des interactions épistasiques influençant en même temps la PAS et la PAD chez 1,755 participants européens (âge moyen de 42±10).

Nous avons mené des études d'association individuelle de chaque variant ayant une MAF supérieure ou égale à 2% et ayant un équilibre Hardy-Weinberg conforme.

Neuf SNPs ont montré une association significative avec la PAS et la PAD. Parmi eux: rs5742910Ins/Del et rs6046G>A du gène *F7* (P≤0,024) et rs1800469T>C au sein du gène *TGFB1* (P<0,001) sont potentiellement responsables d'une diminution de PA via la modification des taux protéiques de leurs protéines correspondantes. Le rs1800590T>G du gène *LPL* (P≤0,003) et le rs2228570G>A du gène *VDR* (P<0,001) avaient quant à eux une fonction potentielle d'altération de la structure protéique.

Nous avons également observé deux interactions épistasiques pour tous les traits de PA : rs1041163T>C dans *VCAM1* interagit avec rs1367117G>A dans *APOB* (P≤0,04) et rs3741240G>A dans *SCGB1A1* interagit avec rs1800590T>G dans *LPL* (P≤0,031). Ces interactions étaient de nouveau trouvées significatives avec la PAD de 1,754 participants dans la population de réplication (P≤0,05).

L'étude de la bibliographie nous a permis de proposer un mécanisme hypothétique concernant l'interaction entre le rs3741240G>A dans *SCGB1A1* et le rs1800590T>G dans *LPL*. Le SNP rs3741240 est un variant fonctionnel qui a été démontré capable d'inhiber la phospholipase A2 et de diminuer les taux plasmatiques des HDL-C (*high-density lipoprotein-cholesterol*). La *LPL* et la *SCGB1A1* pourraient influencer la PA via les taux d'HDL-C.

L'ensemble des résultats soutient l'hypothèse que des gènes appartenant aux mêmes ou à différentes voies métaboliques pourraient agir en synergie pour modifier les niveaux de PA.

Mots clés : Pression artérielle, épistasie, polymorphisme génétique, interactions.

Contribution : *Nous avons participé au design de l'étude et à l'interprétation des analyses statistiques et effectué la recherche bibliographique et bio-informatique. Nous avons également participé activement à la rédaction du premier manuscrit.*

Epistatic study reveals two interactions involved in Blood Pressure genetic regulation.

Short title: Epistasis and Blood Pressure

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Abstract

Background: Although numerous studies using candidate gene and genome-wide approaches have been performed, a small number of genetic variants regulating blood pressure have been identified. This phenomenon can partially be explained by possible gene-gene interactions: epistasis.

Methods: One hundred single nucleotide polymorphisms (SNPs) in 65 candidate genes were genotyped in 1,912 French unrelated adults in order to study their combined effects on blood pressure (BP) levels.

Results: Among 9 genetic variants significantly associated with systolic and diastolic BP, SNPs rs5742910 and rs6046 in *F7* and rs1800469 in *TGFB1* may be responsible for BP defects modifying their plasma protein levels, whereas rs1800590 in *LPL* and rs2228570 in *VDR*, possibly functional, may act via altering protein structure. Specific epistatic interactions were found for systolic and diastolic BP: *VCAM1* (rs1041163) * *APOB* (rs1367117), and *SCGB1A1* (rs3741240) * *LPL* (rs1800590). These two interactions were replicated with DBP in an independent population of 1,755 unrelated adults of European origin. *In silico* analyses yielded putative functional properties of the SNPs involved in these epistatic interactions.

Conclusions: These findings support the hypothesis that different pathways may act synergistically with genes in the same or different pathways to modify BP and could highlight novel pathophysiologic mechanisms underlying hypertension.

200 words

Key words: blood pressure, epistasis, single nucleotide polymorphism, epidemiology.

Background

Blood pressure (BP) is a continuous, consistent and modifiable risk factor for cardiovascular diseases (CVD) such as ischemic heart disease (IHD) and stroke which are major causes of mortality and morbidity worldwide [1]: population-based studies showed that 972 million adults were hypertensive in 2005 and it is predicted to increase by about 60% to 1.56 billion in 2025 [2].

Existing evidence suggests that BP heritability ranges between 30-60% [3]. Despite the identification of specific causal genes involved in the regulatory pathways of rare familial forms of hypertension (HT) [4], both BP and HT are still considered polygenic traits involving a large number of metabolic pathways [5].

The complex architecture of BP regulation may account for the numerous discrepancies found among studies of the genetic background of HT. Inflammation, blood coagulation cascade, cellular adhesion molecules and lipid metabolism all appear to have significant roles [6-8]. While aspects of BP regulation may be the product of an inflammation stimulus [6, 9], thrombin is also known to induce several intracellular pathways [7], to modulate vascular tone [7, 10] and to have pro-inflammatory effects [7]. Growing evidence also suggests that cellular adhesion molecules and apolipoproteins are closely related to HT [8, 11]. All of these factors may be involved in initiating and maintaining elevated BP levels [7]. Overall, however, the genetic factors associated with BP are poorly characterized and the effects of metabolic pathways highlighted by previous studies remain unclear.

In the Era of Genome-wide association studies (GWAS), researchers have attempted to identify susceptibility loci of BP [12-15] but limited progress has been made so far.

The largest GWAS published until now included ≈200,000 individuals and reported 28 loci associated with systolic BP (SBP), diastolic BP (DBP) and/or HT [12]. However, their risk score explained only 0.9% of BP phenotypic variance [12], this representing the so-called 'dark matter' of genetic risk. A possible explanation could be the significant genetic and phenotypic heterogeneity of populations studied as well as the modest effect size of risk alleles [16]. In addition, conventional GWAS are based on independent gene/single nucleotide polymorphism (SNP) effects on a single phenotype without taking into account the possibility of two different genes/SNPs interactions that may affect the same trait. Studying regulatory mechanisms exerted by a given genetic variant modulating the effect of

another i.e. epistatic interactions may be essential [17] in identifying the genes involved in BP regulation. Based on the above rationale [14, 15, 17], we hypothesize that research of epistatic interactions among candidate SNPs may represent a challenge in the search for disease-risk variants as the effect of one SNP may be altered or masked by the effects of another SNP. Furthermore, power to detect the first SNP is likely to be reduced and elucidation of the joint effects of two loci will be hindered by their interaction, unless explicitly examined. In fact, it is well-known that these interactions may identify genetic markers that are not captured by individual marker analysis and/or revealed by the combinatory effect of loci in other pathways [13, 18].

To our knowledge, no previous study has assessed the epistasis of cardiovascular candidate genes on BP regulation in European individuals. As BP is a polygenic trait, where different metabolic pathways are involved (inflammation, coagulation cascade, sodium reabsorption, cellular adhesion and lipid metabolism), we regrouped 100 previously published cardiovascular candidate SNPs in 65 genes to perform a well-powered two-phases statistical analysis in two large independent populations in order to assess eventual epistatic interactions having an effect on BP.

Methods

Ethics statement

The samples are part of a human sample storage platform: the Biological Resources Bank (BRC) "Interactions Gène-Environnement en Physiopathologie CardioVasculaire" (IGE-PCV) in Nancy, East of France. All subjects gave written informed consent and the project protocol was approved by the local ethics committee of each centre.

Study population

In phase 1, the discovery study enrolled 1,912 unrelated adults (51.13 ± 10.02 years, 51.3 % women) recruited during free medical check-ups at the Center of Preventive Medicine of Vandœuvre-lès-Nancy in the East of France. They were Caucasians, born in France for three generations and their clinical and biological data were collected at entrance before any eventual drug prescription following consultation. They were selected on the basis of the following criteria: (1) no antihypertensive drug therapy at recruitment and (2) complete clinical and genotypic data available. As our purpose was to assess BP as a continuous trait, in a case-only design, normotensive and hypertensive subjects were included (for hypertensive individuals, data were gathered before the prescription of any medication).

In order to replicate our epistatic results, a sample of 1,755 unrelated healthy Europeans (42.06 ± 9.89 years, 33% women) from the ApoEurope Project [19] was used: individuals from Crete (Greece); Belfast (Northern Ireland) and Lisbon (Portugal) and with no antihypertensive medication were included in phase 2.

Clinical and biological data collection

SBP and DBP were measured under constant temperature (19°C-21°C) and standardized conditions (supine position) using a manual sphygmomanometer (*Colonne à mercure,* Mercurius) by expert nurses. The recorded values were the means of 3 readings on 20 min intervals. An adjustable BP cuff was used to correct errors due to variations in arm circumference [20]. All individuals underwent complete medical examination including anthropometric and biochemical measurements collected with standardized methods [21].

Genotyping assays

Concerning the selection of assessed SNPs, 1/3 (35 SNPs in 15 loci) were chosen from the "Cardio-Vascular Disease 35" assay, a multi-locus genotyping assay developed in collaboration with Roche Molecular Systems [22]. These SNPs were candidate markers for CVD risk [22] specifically involved in the development and progression of atherosclerotic plaque. The 2/3 remaining were chosen based on the bibliography and on internal investigations performed predominantly in small to medium sized samples [7, 23] (Supplementary Table 1).

Genomic DNA was extracted from peripheral blood samples of the discovery set using the salting out method [24]. Genotyping was performed using a multilocus assay with an immobilized probe approach designed by Roche Molecular Systems, Pleasanton, California, USA [22, 25].

After PCR amplification using pooled biotinylated primers and hybridation to sequence-specific oligonucleotide probes, genotype assignments were performed by two independent observers using proprietary Roche Molecular Systems image processing software. Discordant results (< 3% of all scoring) were resolved by a third observer and if necessary, by a joint reading.

In order to replicate the significant epistatic interactions observed in the discovery set, SNPs of interest were genotyped in Kbioscience (http://www.kbioscience.co.uk) using the competitive allele specific PCR (KASP) chemistry coupled with a FRET-based genotyping system (http://www.kbioscience.co.uk/reagents /KASP/KASP.html) in the replication population (Supplementary Table 2).

PolyPhen analysis of nonsynonymous SNPs

The prediction of possible impact of nonsynonymous (ns) SNPs on the structure and function of their specific protein was performed using PolyPhen [26].

Statistical analyses

Statistical analyses were performed using the SPSS® statistical software version 16.0 (SPSS, Inc, Chicago, Illinois) and Plink v01.7 [27].

Polymorphisms with minor allele frequencies (MAF) less than 2% or deviating from Hardy-Weinberg equilibrium (HWE) were excluded from individual analyses. However, for epistatic interaction analyses we included all SNPs as low MAF may identify an epistatic phenomenon.

Separate models were constructed to determine the independent effect of the genetic variants on SBP and DBP assuming additive models using the minor allele as the reference group. Age, gender and body mass index (BMI)-adjusted linear regressions were performed for individual association analyses. Associations were considered significant when p-values adjusted for multiple testing (Bonferroni correction) ≤ 0.05.

The variants individually associated in our study were compared to previous findings in the literature, notably on previous GWAS, by imputation analyses using Plink [27]. SNPs with a correlation coefficient ≥ 80% were considered in linkage disequilibrium (LD).

Two-locus additive epistasis were defined as significant statistical interaction between two SNPs [28]. All possible 2x2 combinations between the 100 SNPs involved in the discovery study were tested on a linear additive model (p-value ≤ 0.05) adjusted for age, gender and BMI. Similar models were applied for the replication of significant epistatic interactions.

Values of SBP and DBP were log-transformed in order to normalise their distribution.

Results

Characteristics of the study populations are presented in Table 1.

Assessed genetic variants

All genes following MAF quality control were consistent with HWE (data not shown). The complete details of genetic variants including their nearby gene, location, type and polymorphism are listed in Supplementary Table 1: the analysis included 100 SNPs belonging to 65 cardiovascular candidate genes [7, 22, 23]. Among the included SNPs, 50 were nonsynonymous, and according to Polyphen [26], rs1800888, rs5742912, rs7412, rs5361 and rs2228570 were related to a modification in their corresponding protein structures (Supplementary Table 1).

Individual association analysis

Associations among genetic variants and BP traits are shown in Table 2. Nine SNPs in 8 genes including different metabolic pathways were significantly associated with SBP or DBP: *LPL* and *LDLR* (lipid metabolism), *F7* (coagulation factor), *AGT1R* (BP regulation), *CSF2*, *IL1B*, *TGFB1* and *VDR* (inflammatory pathways). Seven SNPs were associated with both SBP and DBP levels; rs5186 in *AGT1R* ($P \leq 8.34 \times 10^{-04}$), rs25882 in *CSF2* ($P \leq 3.83 \times 10^{-04}$), rs5742910 in *F7* ($P \leq 3.72 \times 10^{-04}$), rs1143634 in *IL1B* ($P \leq 0.049$), rs5742911 in *LDLR* ($P \leq 3.54 \times 10^{-02}$) rs1800469 in *TGFB1* ($P \leq 0.03643$) and rs2228570 in *VDR* ($P \leq 9.48 \times 10^{-04}$).

Our imputation analyses showed that any of these significant SNPs have been reported or were in LD ($r^2 < 80\%$) with genetic variants highlighted in previous GWAS [14, 15].

Epistatic interaction with BP

Epistatic interactions with BP are shown in Table 3.

Two epistases: *VCAM1* (rs1041163)**APOB* (rs1367117) and *SCGB1A1* (rs3741240)**LPL* (rs1800590) were observed for both SBP and DBP in the discovery set ($P \leq 0.04$ and $P \leq 0.03$ respectively) and then replicated for DBP only in phase 2 ($P = 0.045$ and $P = 0.05$ respectively). These interactions were putatively functional according to PolyPhen.

Discussion

Even at early stages, HT is a major cause of disability and death for millions of people worldwide [1]. Observational studies involving more than 1 million adults from 61 prospective studies have indicated that death from HT-associated diseases (both IHD and stroke) increases linearly from BP levels as low as 115/75 mmHg among middle age and elderly individuals [29]. Therefore, isolating genetic variations that influence BP may have major implications for public health; however, individual genes have so far only shown a modest effect [30]. It is therefore essential to study possible epistatic gene interactions in order to identify gene combinations that synergistically influence BP regulation and HT.

In the current study, in a large French population of 1,912 unrelated adults, 9 SNPs in 8 genes involved in various biological pathways including lipid metabolism (*LPL* and *LDLR*), coagulation (*F7*), BP regulation (*AGT1R*) and inflammatory pathways (*IL1B*, *CSF2*, *TGFB1,* and *VDR*) were associated with at least one of the 2 BP traits assessed. Also, 7 SNPs were associated with SBP and DBP levels: rs5186 in *AGT1R* ($P \leq 8.34 \times 10^{-04}$), rs25882 in *CSF2* ($P \leq 3.83 \times 10^{-04}$), rs5742910 in *F7* ($P \leq 3.72 \times 10^{-04}$), rs1143634 in *IL1B* ($P \leq 0.049$), rs5742911 in *LDLR* ($P \leq 3.54 \times 10^{-02}$) rs1800469 in *TGFB1* ($P \leq 0.03643$) and rs2228570 in *VDR* ($P \leq 9.48 \times 10^{-04}$).

Genetic variation may be responsible for hypertensive phenotypes by altering either the structure of encoded proteins or gene expression [14]. Through an *in silico* analysis (PolyPhen), 5 SNPs were found to have possible functional effects through altering the corresponding protein structure (rs1800888, rs5742912, rs7412, rs5361 and rs2228570). In contrast, rs1800469 in *TGFB1* has been reported to be associated with increased *TGFB1* gene transcription and plasma protein levels [31], suggesting altered gene expression [31, 32]. Similarly, rs5742910 and nsSNP rs6046 in *F7* have been previously reported to be associated with a 1/3 decrease in F7 plasma levels [7]. These 2 SNPs have also been previously associated with BP in a population derived from the BRC IGE-PCV [7]. Combined with previous data, our finding suggests that these 2 SNPs are associated with BP possibly due to modifications in gene expression and alterations in plasma protein levels.

Polymorphisms may also affect gene expression by disrupting microRNA binding (miRNA). For example, in the present study, rs5186 C allele of *AGTR1* disrupts binding of miR155 to its complementary *AGTR1* mRNA [33, 34] resulting in an overall increase in gene expression. A

molecular mechanism for miR155 interaction with its polymorphic target rs5186A (3' UTR of *AGTR1*) has also been proposed [34].

Additionally, it is reported that rs1800590 polymorphism of *LPL* affects lipoprotein lipase enzyme function [35, 36]. Similarly, the coding nsSNP rs2228570 in *VDR* introduces an amino acid substitution (Met1Thr) that may modify the structure of the encoded protein, according to Polyphen [26]. Thus, the influences of *LPL* and *VDR* polymorphisms on BP may be the result of modifications in their protein structure and resulting changes in protein activity.

Epistatic interactions may also play a pivotal role in the discrepancies found among genetic studies. There are numerous reports that these interactions may identify genetic markers that are not captured by individual marker analysis and/or revealed by the combinatory effect of loci in other pathways [13, 18]. Herein we observed two epistatic interactions common for all BP traits: *VCAM1* (rs1041163)**APOB* (rs1367117) and *SCGB1A1* (rs3741240)**LPL* (rs1800590) (Table 3).

A number of potential mechanisms mediating the observed genetic associations were identified with an *in silico* review.

The significant interaction between *SCGB1A1* (rs3741240) and *LPL* (rs1800590) was validated in the replication phase in an independent population. SNP rs3741240 is a functional SNP that has been shown to inhibit phospholipase A2 [37] and decrease plasma levels of high-density lipoprotein cholesterol (HDL-C) [38]. Taken together, *LPL* and *SCGB1A1* may interact through HDL-C to influence BP.

The present study also showed epistatic interactions between *VCAM1* (rs1041163)**APOB* (rs1367117) that was replicated in an independent European population. Whereas rs1041163 is a nonfunctional SNP, rs1367117 may result in an altered protein structure according to PolyPhen. Human studies have reported significant correlations between Apolipoprotein B (APOB), the main component of low-density lipoprotein cholesterol, and VCAM1 in the context of HT. Hubel et al [39] have reported significant correlations between soluble levels of APOB and VCAM1 in women with endothelial dysfunction and HT as a result of preeclampsia.

Nevertheless, previous candidate gene studies have revealed other epistatic interactions in BP phenotypes [18, 40] in non-European populations. In a cross-sectional study conducted in 402 middle aged and elderly Japanese, Misono et al [18] demonstrated that individuals

carrying a combination of *ADRB2* Gly/Gly and *NOS3* Glu/Glu genotypes had higher hypertensive risk. Moreover, Kohara et al [40] in a case-control study conducted in 9,700 subjects reported that epistatic interactions are associated with increased risk of HT. Combined with our findings, there is clear evidence that numerous epistatic interactions involving multiple physiological pathways influence BP levels and HT risk.

Strengths and limitations

The present study is strengthened by the *in* silico approach (PolyPhen) and previous biochemical and molecular studies that have provided biological evidence for our putative functional epistatic interactions. Another strong point is the validation of these interactions in a replication phase involving a large independent population, although not all SNPs could be genotyped in the replication phase due to limited resources. Nevertheless, to confirm our epistatic interactions reproducibility, reported SNPs should be tested in a GWAS with high resolution arrays and higher genome-wide covering in a large population.

Conclusions and perspectives

In summary, we identified (a) susceptibility genes associated with BP including *LPL* and *LDLR* (lipid metabolism), *F7* (coagulation factor), *AGT1R* (BP regulation), *TNF*, *IL1B*, *CSF2*, *TGFB1, LTA* and *VDR* (inflammation); (b) epistatic interactions between functional SNPs involved in lipid metabolism: *LPL* (rs1800590) and *APOB* (rs1367117), inflammation: *SCGB1A1* (rs3741240) and cellular adhesion: *VCAM1* (rs1041163); (c) we replicated these epistatic interactions in an independent population. Our results were not previously reported in the literature (previous GWAS studies) and they were not in LD with other SNPs identified in these studies concerning BP. It is important to emphasize that these findings may provide insight into novel pathophysiological mechanisms underlying HT and they may provide future therapeutic targets.

List of abbreviations used

APOB	apolipoprotein B
BMI	body mass index
BP	blood pressure
BRC	biological resources bank
CVD	cardiovascular disease
DBP	diastolic blood pressure
GWAS	genome-wide association study
HDL-C	high-density lipoprotein cholesterol
HT	hypertension
HWE	Hardy-Weinberg equilibrium
IGE-PCV	Interactions Gène-Environnement en Physiopathologie CardioVasculaire
IHD	ischemic heart disease
LD	linkage disequilibrium
MAF	minor allele frequency
miRNA	microRNA
SBP	systolic blood pressure
SNP	single nucleotide polymorphisms

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Authors' contribution (in alphabetical order):

Conception and design: MGS, MYT, NCN, SES, SVS

Acquisition of data: GS, SVS

Analysis of data: MGS, NCN, SES

Interpretation of data: MGS, NCN, SES, SVS

Drafting of the manuscript: MGS, NCN, SES, SVS

Revision of the manuscript for important intellectual content: GS, MYT

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Table 1: Mean Characteristics of studied individuals.

Characteristics	Discovery population (n = 1,912)			Replication population (n = 1,755)		
Age (years)	51.13	±	10.02	42.06	±	9.89
BMI (kg/m²)	26.01	±	3.75	26.23	±	4.11
SBP (mmHg)	142.86	±	21.47	128.62	±	17.69
DBP (mmHg)	87.49	±	14.26	81.15	±	10.93

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure

Table 2: Genetic variants associated with blood pressure.

Gene	OMIM accession number	SNP	MAF	p-value	p-value adjusted	beta	SE	Trait
AGTR 1	*106165	rs5186	28.933	$8.34x10^{-04}$	0.027	-0.051	0.032	SBP
				$6.96x10^{-03}$	0.041	-0.045	0.051	DBP
CSF2	*138960	rs25882	25.596	$4.67x10^{-06}$	$3.83x10^{-04}$	0.124	0.030	SBP
				$7.25x10^{-07}$	$5.94x10^{-05}$	0.148	0.048	DBP
F7	227500	rs5742910	15.024	$3.72x10^{-04}$	0.030	-0.028	0.041	SBP
				$3.34x10^{-04}$	0.027	-0.042	0.065	DBP
		rs6046	12.874	$5.33x10^{-04}$	0.044	-0.028	0.044	SBP
IL1B	*147720	rs1143634	19.397	$4.21x10^{-04}$	0.035	-0.044	0.035	SBP
				$5.92x10^{-04}$	0.049	-0.052	0.056	DBP
LDLR	606945	rs5742911	32.308	$4.31x10^{-04}$	0.035	0.06	0.029	SBP
				$1.17x10^{-04}$	0.010	0.071	0.047	DBP
LPL	609708	rs1800590	2.586	$3.46x10^{-04}$	0.028	0.076	0.088	SBP
TGFB1	*190180	rs1800469	29.048	$4.44x10^{-04}$	0.036	-0.026	0.030	SBP
				$9.77x10^{-06}$	$8.02x10^{-04}$	-0.038	0.047	DBP
VDR	*601769	rs2228570	40.139	$1.16x10^{-05}$	$9.48x10^{-04}$	-0.012	0.028	SBP
				$6.02x10^{-06}$	$4.93x10^{-04}$	-0.009	0.045	DBP

SNP: single nucleotide polymorphism, MAF: minor allele frequency, beta: regression coefficient corresponding to the minor allele of each SNP, SE: standard error, SBP: systolic blood pressure, DBP: diastolic blood pressure.

Table 3: Epistatic interactions between genetic variants associated with blood pressure

	Discovery Set (n = 1,912)		Replication set (n=1,755)	
	SBP	DBP	SBP	DBP
VCAM1 (rs1041163) * *APOB* (rs1367117)	0.04	0.027	-	0.045
SCGB1A1 (rs3741240) * *LPL* (rs1800590)	0.02	0.03	-	0.05

Supplementary Table 1: Summary of-investigated genetic variants.

Locus	Gene (OMIM accession number)	Chromosome	SNP ID	Position	Type	Mutation
Blood pressure regulation						
ACE	Angiotensin I converting enzyme (+106180)	17q23.3	rs1799752	17 :61565890-61565891	Intronic	Ins/del
ADD1	Adducin 1 (alpha) (*102680)	4p16.3	rs4961**	4 :2906707	NsSNP-exonic	Gly460Trp
ADRB2	Adrenergic, beta-2-, receptor, surface (+109690)	5q31-q32	rs1042713*	5 :148186633	NsSNP-exonic	Arg16Gly
			rs1042714*	5 :148186666	NsSNP-exonic	Gln27Glu
			rs1800888***	5 :148187078	NsSNP-exonic	Thr164Ile
AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8) (+106150)	1q42-q43	rs699*	1 :228912417	NsSNP-exonic	Met235Thr
AGTR1	Angiotensin II receptor, type 1 (*106165)	3q21-q25	rs5186	3 :148459988	3'UTR	1166 A/C
GNB3	Guanine nucleotide binding protein (G protein), beta polypeptide 3 (*139130)	12p13	rs5443	12 :6954875	Synonymous-exonic	Ser275Ser
NOS2	Nitric oxide synthase 2, inducible (*163730)	17q11.2-q12	rs1137933	17 :23130059	Synonymous-exonic	Asp346Asp
NOS3	Nitric oxide synthase 3 (endothelial cell) (+163729)	7q36	rs1799983*	7 :150696111	NsSNP-exonic	Glu298Asp
			rs1800779	7:150689943	Intronic	-922 A/G
NPPA	Natriuretic peptide (*108780)	1p36.21	rs5063	1:11907603	Intronic	664 G/A
			rs5065	1:11906068	Stop-exonic	2238 T/C

SCNN1A	Sodium channel, nonvoltage-gated 1 alpha (*600228)	12p13	rs5742912***	12 : 6328611	Synonymous-exonic	Trp493Arg
			rs2228576*	12 : 6327323	Synonymous-exonic	Thr663Ala
	Lipid metabolism					
ADRB3	Adrenergic, beta-3-, receptor (*109691)	8p12-p11.2	rs4994*	8 : 37942955	Synonymous-exonic	Trp64Arg
APOA4	Apolipoprotein A-IV4 (*107690)	11q23	rs675*	11:116691675	Synonymous-exonic	Thr347Ser
			rs5110*	11:116691634	Synonymous-exonic	Gln360His
APOB	Apolipoprotein B (including Ag(x) antigen) (+107730)	2p24-p23	rs1367117**	2 : 21117405	Synonymous-exonic	Thr71Ile
			rs5742904**	2 : 21082665	Synonymous-exonic	Arg3500Gln
APOC3	Apolipoprotein C-III (+107720)	11q23.1-q23.2	rs2542052	11:116699984	Upstream	-641 C/A
			rs2854117	11:116700142	Upstream	-482 C/T
			rs2854116	11:116700169	Upstream	-455 T/C
			rs4520	11:116701535	Synonymous-exonic	Gky34Gly
			rs5128	11:116703640	3'UTR	3175 C/G
			rs4225	11:116703671	3'UTR	3206 T/G
APOE	Apolipoprotein E (+107741)	19q13.2	rs7412***	19:45412079	NsSNP-exonic	Arg158Cys
			rs429358*	19:45411941	NsSNP-exonic	Cys112Arg
CETP	Cholesteryl ester transfert protein, plasma [+118470]	16q21	rs1800775	16:56995234	Upstream	-629 C/A
			rs5882*	16:57016092	NsSNP-exonic	Ile422Val
			rs23037**		NsSNP-exonic	Asp442Gly

			rs5742907	16:57016150	Intron-essential splice site	1 G/A
LDLR	Low density lipoprotein receptor (+606945)	19p13.3	rs5742911	3'UTR	19:11243445	1453A/G
LIPC	Lipase, hepatic (+151670)	15q21-q23	rs1800588	15:58723675	Intronic	-480 C/T
LPA	Lipoprotein, Lp(a) (+152200)	6q26	rs7770628			93 C/T
			rs1800769	6:161085267	5'UTR	121 G/A
			rs1800590	8:19796671	5'UTR	-93 T/G
LPL	Lipoprotein lipase (+609708)	8p22	rs1801177*	8:19805708	NsSNP-exonic	Asp9Asn
			rs268*	8:19813563	Stop-exonic	Asn291Ser
			rs328	8:19819724	NsSNP-exonic	Ser447Ter
PON1	Paraoxonase 1 (+168820)	7q21.3	rs854560*	7:94946067	NsSNP-exonic	Met55Leu
			rs662*	7:94937439	Stop-exonic	Gln192Arg
PON2	Paraoxonase 2 (*602447)	7q21.3	rs7493*	7:95034775	NsSNP-exonic	Ser311Cys
PPARG	Peroxisome proliferator-activated receptor gamma (*601487)	3p25	rs1801282*	3 : 12368125	NsSNP-exonic	Pro12Ala
Cellular adhesion						
ICAM1	Intercellular adhesion molecule 1 (*147840)	19p13.3-p13.2	rs1799969*	19:10394792	NsSNP-exonic	Gly241Arg
			rs5491*	19:10385540	NsSNP-exonic	Lys56Met
SELE	Selectin E (+131210)	1q22-q25	rs5361***	1:169701060	NsSNP-exonic	Ser149Arg
			rs5355**	1:169695870	NsSNP-	Leu554Phe

SELP	Selectin P (granule membrane protein 140kDa, antigen CD62) (+173610)	1q22-q25	rs6131*	1 :167847509	exonic NsSNP-exonic	Ser330Asn
			rs6133*	1 : 167831970	NsSNP-exonic	Val640Leu
VCAM1	Vascular cell adhesion molecule 1 (*192225)	1p32-p31	rs1041163	1:101183824	Upstream	-1594 T/C
Homocystein metabolism						
CBS	Cystathionine beta-synthase (*613381)	21q22.3	rs5742905**	21 : 43356253	NsSNP-exonic	Ile278Thr
MTHFR	Methylentetrahydrofolate reductase (NAD(P)H) (*607093)	1p36.3	rs1801133**	1:11856378	NsSNP-exonic	Ala222Val
Coagulation cascade						
F2	Coagulation factor II (thrombin) (*176930)	11p11	rs1799963	11:46761055	3'UTR	20210 G/A
F5	Coagulation factor V (proaccelerin, labile factor) (*612309)	1q23	rs6025**	1 : 167785673	NsSNP-exonic	Arg506Gln
F7	Coagulation factor VII (serum prothrombin conversion accelerator) (+227500)	13q34	rs5742910	13: 113759833-113759834	Upstream	-323 Del/ins
			rs6046*	13 : 112821160	NsSNP-exonic	Arg353Glu
FGB	Fibrinogen beta chain (*134830)	4q28	rs1800790	4:155483708	Upstream	-455 G/A
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor) (+192974)	5q23-q31	rs1062535	5:52351413	Synonymous-exonic	Thr275Thr
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) (+173470)	17q21.32	rs5918*	17 : 42715729	NsSNP-exonic	Leu33Pro
SERPINE1	Serpin peptidase inhibitor, calde E (Nexin plasminogen activator inhibitor type 1), member	7q21.3-q22	rs7242	7:100781445	3'UTR	11053 G/T
			rs1799768	7:100769707-	Upstream	(-675) 5G/4G

	1 (*173360)			10076970		
Inflammation						
C3	Complement component 3 (+120700)	19p13.3-p13.2	rs2230199*	19 : 6669387	NsSNP-exonic	Arg102Gly
C5	Complement component 5 (+120900)	9q33-q34	rs17611*	19 : 122809021	NsSNP-exonic	Ile802Val
CCL11	Chemokine (C-C motif) ligand 11 (*601156)	17q21.1-q21.2	rs4795895 rs1129844 *	17:32611446 17:32612894	Upstream NsSNP-exonic	-1328 G/A Ala23Thr
CCR2	Chemokine (C-C motif) receptor 2 (*601267)	3p21.31	rs1799864*	3:46399208	NsSNP-exonic	Val62Ile
CCR3	Chemokine (C-C motif) receptor 3 (*601268)		rs5742906**	3:46306765	NsSNP-exonic	Pro39Leu
CCR5	Chemokine (C-C motif) receptor 5 (*601373)	3p21.31	rs333	3:46414947-46414978	Frameshift coding-exonic	wt/Δ580-611
CD14	CD14 molecule (*158120)	5q31.1	rs1799987 rs2569190	3:46411935 5:140012916	Intronic 5'UTR	-2454 G/A -260 C/T
CSF2	Colony stimulating factor 2 (granulocyte-macrophage) (*138960)	5q31.1	rs25882*	5 : 131439359	NsSNP-exonic	Ile117Thr
CTLA4	Cytotoxic T-lymphocyte-associated protein 4 (+123890)	2q33	rs5742909 rs231775*	2:204732347 2:204732714	Upstream NsSNP-exonic	-318 C/A Thr17Ala
CXCL12	Chemokine, (C-X-C motif), ligand 12 (*600835)	10q11.1	rs1801157	10:44868257	3'UTR	800 G/A
GC	Group-specific component (vitamin D binding protein)	4q12-q13	rs7041*	4: 72837198	NsSNP-exonic	Glu416Asp

	(+139200)					
IL1A	Interleukin 1, alpha (*147760)	2q14	rs1800587	2:113542960	Upstream	-889 T/C
IL1B	Interleukin 1, beta (*147720)	2q14	rs1143634	2 :113306861	Synonymous -exonic	Phe105Phe
			rs16944	2:113594867	Upstream	-1418 C/T
IL4	Interleukin 4 (*147780)	5q31.1	rs2243250	5:132009154	Upstream	-590 C/T
			rs1805010**	16: 27355703	Synonymous -exonic	Ile50Val
IL4R	Interleukin 4 receptor (*147781)	16p12.1-p11.2	rs1805015**	16: 27373680	Synonymous -exonic	Ser478Pro
			rs1801275**	16:27374400	Synonymous -exonic	Gln576Arg
IL5RA	Interleukin 5 receptor, alpha (*147851)	3p26-p24	rs2290608	3:3151759	5'UTR	-80 G/A
IL6	Interleukin 6 (interferon, beta 2) (*147620)	7p21	rs1800795	7:22766645	Intronic	-174 G/C
			rs1800796	7:22766246	Intronic	-572 G/C
IL9	Interleukin 9 (*146931)	5q31.1	rs2069885*	5 :135256064	Synonymous -exonic	Thr113Met
IL10	Interleukin 10 (*124092)	1q31-q32	rs1800872	1:206946407	Upstream	-571 C/A
IL13	Interleukin 13 (*147683)	5q31	rs1295686	5:131995843	Intronic	4045 C/T
LTA	Lymphotoxin alpha (TNF superfamily, member 1) (+153440)	6p21.3	rs909253	6 :31540313	5'UTR	252 A/G
LTC4S	Leukotriene C4 synthase (+246530)	5q35	rs730012	5:179220638	3'UTR	-444 A/C
MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase) (+185250)	11q22.3	rs3025079			(-1171) 5A/6A

TCF7	Transcription factor 7 (T-cell specific, HMG-box) (*189908)	5q31.1	rs5742913*	**I.A.** 5: 133 451 183	Synonymous -exonic	Pro19Ter
TGFB1	Transforming growth factor, beta 1 (*190180)	19q13.1	rs1800469	19:41860296	Upstream	-509 C/T
TNF	Tumor necrosis factor (*191160)	6p21.3	rs1800629	6 :31651010	Upstream	-308 G/A
			rs361525	6 :31651080	Upstream	-238 G/A
			rs1041981*	6 :31648763	Synonymous -exonic	Thr26Asn
SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin) (*192020)	11q12.3-q13.1	rs3741240	11 :61943118	5'UTR	38 G/A
VDR	Vitamin D (1,25-dihydroxyvitamin D3) receptor (*601769)	12q13.11	rs1544410	12:48239835	INTRONIC	63980G>A
			rs2228570** *	12 :48272895	Synonymous -exonic	Met1Thr

The prediction of possible nsSNPs impact on the structure and function of its specific protein was performed using PolyPhen.

*: benign nsSNP.

**: possibly damaging nsSNP.

***: damaging nsSNP.

nsSNP.: non synonymous single nucleotide polymorphism

Supplementary Table 2: Minor allele frequency of the investigated SNPs in ApoEurope

SNP	GENE	MAF
rs1041163	*VCAM1*	20.2
rs1367117	*APOB*	19
rs1800590	*LPL*	1.9
rs3741240	*SCGB1A1*	31.8

Supplementary Table 3: Sample size and mean blood pressure levels (standard error) for epistasis interactions observed in the discovery cohort

Table S3A - *VCAM1 (rs1041163) * APOB (rs1367117)*

SBP		***rs1041163***			
		11	12	22	N TOT
rs1367117	11	141.47 ± 21.31 (487)	138.55 ± 22.08 (213)	152.55 ± 19.69 (30)	730
	12	137.27 ± 21.22 (468)	140.67 ± 21.27 (182)	146.15 ± 20.68 (28)	678
	22	139.78 ± 19.24 (111)	135.43 ± 24.96 (41)	139.83 ± 26.73 (8)	160
	N TOT	1066	436	66	1568

SBP: systolic blood pressure
Mean value ± SE
(sample size)

Table S3B - *SCGB1A1 (rs3741240) * LPL (rs1800590)*

DBP		***rs3741240***			
		11	12	22	N TOT
rs1800590	11	87.66 ± 14.31 (846)	87.14 ± 14.25 (739)	87.95 ± 14.22 (217)	1802
	12	86.85 ± 13.89 (46)	94.14 ± 11.41 (34)	88.04 ± 13.41 (11)	91
	22	(0)	99.30 ± 2.45 (4)	96.00 (1)	5
	N TOT	892	777	229	1898

DBP: diastolic blood pressure
Mean value ± SE
(sample size)

Table S3C - *SCGB1A1 (rs3741240) * LPL (rs1801177)*

DBP		***rs3741240***			
		11	12	22	N TOT
rs1801177	11	87.66 ± 14.36 (856)	87.32 ± 14.22 (751)	88.05 ± 14.19 (219)	1826
	12	86.55 ± 12.38 (36)	92.91 ± 12.23 (26)	85.65 ± 13.72 (9)	71
	22	(0)	(0)	96.00 (1)	1
	N TOT	892	777	229	1898

DBP: diastolic blood pressure
Mean value ± SE
(sample size)

Supplementary Table 4: Sample size and mean blood pressure levels (standard error) for epistasis interactions observed in the replication cohort

Table S4A - *VCAM1 (rs1041163) * APOB (rs1367117)*

DBP		***rs1041163***			
		11	12	22	N TOT
rs1367117	11	81.73 ± 0.47 (509)	80.80 ± 0.74 (241)	85.00 ± 2.07 (29)	779
	12	80.88 ± 0.49 (441)	80.55 ± 0.84 (183)	78.25 ± 2.69 (30)	654
	22	80.05 ± 1.11 (92)	83.71 ± 1.81 (46)	83.16 ± 3.19 (6)	144
	N TOT	1042	470	65	1577

DBP: diastolic blood pressure
Mean value ± SE
(sample size)

Table S4B - *SCGB1A1 (rs3741240) * LPL (rs1800590)*

DBP		***rs3741240***			
		11	12	22	N TOT
rs1800590	11	80.58 ± 0.41 (681)	81.62 ± 0.41 (684)	81.09 ± 0.90 (168)	1533
	12	83.60 ± 2.04 (29)	81.76 ± 1.94 (21)	86.31 ± 3.02 (11)	61
	22	60.00 (1)	78.75 ± 1.2 (2)	(0)	3
	N TOT	711	707	179	1597

DBP: diastolic blood pressure
Mean value ± SE
(sample size)

IV. Etude des interactions gène-obésité dans la régulation des niveaux de pression artérielle :

Obesity inverts the beneficial effect of newly identified rs7556897T>C on blood pressure regulation at childhood.

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PLoS ONE, Soumis.

Les facteurs environnementaux, y compris le stress, l'inactivité physique, l'excès d'alcool et l'obésité sont connus pour augmenter les niveaux de PA. En particulier, des associations positives entre l'IMC et la PA ont été rapportées dans de nombreuses études transversales et longitudinales. Cette relation a été confirmée par de nombreux essais cliniques montrant clairement que la réduction du poids corporel entraîne une diminution des niveaux de PA.

D'une façon similaire aux GWAS, les études de liaisons génétiques ont enrichi nos connaissances sur la génétique de la PA et de l'HTA. Malgré le fait que ces approches ont montré que tous les chromosomes humains contiennent des régions liées à ces deux traits, seul le bras long du chromosome 2 (chr2.q) est le plus répliqué dans la littérature (neuf études sur des populations Européennes et trois sur des populations non Européennes). Au meilleur de notre connaissance, aucune recherche approfondie sur le chr2q entier n'a encore été conduite. De même, les éventuelles interactions qui peuvent exister entre cette région génomique et le statut d'obésité pour influencer la PA n'ont jamais été évaluées. Nous avons donc étudié simultanément 14,674 SNP localisés dans le chr2q chez 631 enfants minces de la cohorte STANISLAS et chez 1,015 enfants obèses issus de la population OBE. Dans les deux populations, un gène particulier nommé *CCL20* a attiré notre attention. En effet, le rs7556897C>T de ce gène était le seul à être associé à la PAD simultanément dans

les deux populations mais avec un effet opposé : une diminution de la PAD chez les enfants minces ($P=6x10^{-3}$, β=-0,56) et une augmentation de cette pression chez les enfants obèses ($P=2x10^{-3}$, β=+0,78). L'ensemble des résultats était renforcé par le fait que ce SNP interagissait avec l'obésité dans les populations françaises ($P=9,79x10^{-5}$).

Une étape de validation transcriptomique a été ensuite menée dans 2 modèles cellulaires: les PBMCs d'un sous échantillon de cinquante neuf enfants minces choisis dans la cohorte STANISLAS, ainsi que les PBMCs et le tissu adipeux de six enfants en surpoids comparés à dix autres minces de la population pédiatrique grecque.

Nous avons trouvé que le rs7556897C>T est associé avec une augmentation du niveau d'expression de *CCL20* dans les PBMCs de la cohorte STANISLAS (P=0,002).

De plus, nous avons montré que l'IMC est associé avec une augmentation d'expression du gène *CCL20* dans les PBMCs ($P=4x10^{-3}$) et les tissu adipeux (P=0.032) des enfants en surpoids comparés aux minces de la population pédiatrique grecque. En conclusion, le variant rs7556897C>T et l'obésité sont associés avec une augmentation de l'expression du gène *CCL20* dans les PBMCs.

Ces résultats soulignent que durant l'enfance, l'obésité pourrait inverser l'effet bénéfique des gènes impliqués dans la régulation de PA via l'expression du gène *CCL20*, donnant ainsi de nouvelles perspectives pour prévenir la survenue d'HTA chez les individus adultes grâce à la supervision du poids et la prévention de l'obésité dès le plus jeune âge.

Mots clés : Obésité, inflammation, pression artérielle, polymorphisme génétique ponctuel, bras long du chromosome 2.

Contribution : *Nous avons participé à la conception et au design de l'étude et à l'interprétation des analyses statistiques. Nous avons effectué la totalité des expériences de génotypage et de transcriptomique. Nous avons également participé très activement à la rédaction du premier manuscrit.*

Obesity inverts the beneficial effect of newly identified rs7556897T>C on blood pressure regulation at childhood

Short title:rs7556897, obesity in childhood blood pressure

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Abstract

The relationship between high blood pressure (BP) and obesity is well established in children and adults but poorly understood. Identifying genetic variants influencingBP according to the obesity status at childhood using the most replicated region associated to this trait: the long harm of chromosome 2 (chr.2q)might have implications for public health.

Chr.2q-BP associations were investigated in 577 normal-weight and 1,015 obese French children. Further transcriptomic investigations were then conducted in both adipose tissue and peripheral blood mononuclear cells (PBMCs) of 6 overweight and 10 normal weight children and PBMCs of 58 normal weight children.

SNP rs7556897T>C, located between *SLC19A3* and *CCL20*, showed opposite effects on BP depending of the obesity status: it was negatively associated to diastolic BP in normal-weight (β=-0.012 ± 0.004, $P_{adjusted}$=0.006) and positively in obese (β=+2.178 ± 0.71, $P_{adjusted}$=0.002) children and a strong interaction between rs7556897T>C and obesity status was detected (β=3.49, P=9.79x10^{-5}).

Transcriptomic results highlighted specific to *CCL20* involvement of obesity/inflammation communicating pathways in BP regulation. *CCL20*, an adipochemokine, was only expressed in adipose tissue of overweight children and was 10.7x higher expressed in PBMCs of overweight children when compared to normal-weight children. Finally CCL20 mRNA levels were positively associated to rs7556897T>C in the PBMCs of the 58 normal weight children (β=0.43, P=0.002).

Childhood obesity abolishes the beneficial effect of rs7556897T>C on diastolic BP possibly through modulation of *CCL20* expression levels. Body mass index should be taken into account also in future genetic epidemiology studies of hypertension.

243 words

Key Words: Blood pressure; obesity; inflammation; SNPs; long arm of chromosome 2;*CCL20*

Introduction

Over the last 30 years, childhood obesity prevalence increased drastically [1] and this phenomenon rises concerns about its long-term adverse consequences on morbidity and mortality. Recent systematic reviews investigatedthe impact of overweight and childhood obesity on disease outcome in adulthood and found evidences for associations between childhood body mass index (BMI)andadult hypertension (HTN), type 2 diabetes, coronary heart disease and premature mortality[2,3].

Raised blood pressure (BP) is responsible of more deaths than any other cardiovascular risk factors (7.6 million per annum worldwide, 13.5% of the total)[4] and is at the crossroads of many cardiovascular-linked pathologies [5]. The BMI-effect on BP has been reported in cross-sectional [6,7], prospective studies [8,9] and clinical trials [10,11]which clearly showed that a reduction in body weight induces a decrease in BP levels.

BP is a heritable trait, approximately 30-70% of its variance is attributed to genetics [12],and its heritability varies according to the assessed phenotypes from ≈31% in single-measure of systolic blood pressure (SBP) and diastolic blood pressure (DBP), to ≈57% in long-term average and to ≈68% in 24-hour profile of SBP and DBP in familial studies [13].

The largest scale genome-wide association study (GWAS) conducted so far (≈200,000 individuals) reported 28 loci associated with SBP, DBP and/or HTN explaining only 0.9% of BP phenotypic variance [14]. This large 'missing heritability' phenomenon implies a 'dark matter' on the HTN genetic risk concealed by more complex than simple single nucleotide polymorphism (SNP)-trait relationships such as gene-environment interactions[15] that still remain to be in-depth studied.

More specifically, we postulate that investigating a chromosomic region repetitively linked to BP and/or HTN in regard to BMI at childhood, where most other environmental factors able to impair or enhance 'normal' gene effect (stress, smoking, alcohol excess)are minims, could contribute to make a step forward in the understanding of early BP genetic etiology and identify specific obesity-BP related SNPs. Despite that almost all chromosomes have been demonstrated to contain regions related to BP and HTN [16], the long arm of chromosome 2 (chr2.q) has been the most repetitively linked to BP and/or HTN [17-25]. We then studied all the 14,674 SNPs located in chr.2q genotyped in normal-weight and obese independent French paediatric populationsin order to test this postulate.

Results

Baseline characteristics of the study populations are presented in Table 1.

In 577 normal-weight children from the STANISLAS Family Study (SFS) and 1,015obese children from the French Obesity Cases (FOC) population, only the intergenic SNP rs7556897T>C located between *SLC19A3* and *CCL20* loci (Figure 1) showed opposite effect on DBP within the 14,674 SNPs genotyped in chr.2q: its C allele decreased DBP in SFS (β=-0.012 ± 0.004, $P_{adjusted}$=0.006) and increased DBP in FOC (β=+2.178 ± 0.71, $P_{adjusted}$=0.002). When combining both populations, the obesity status significantly interacted with rs7556897T>C (β=3.49, P=9.79x10^{-5}).

SLC19A3 was homogeneously expressed in adipose tissue of 6 overweight and 10 normal-weight Greek children while *CCL20* expression was only observed in obese (Figure 2A). This last gene was the only one expressed in PBMCs of the same population (Supplementary Data S1) and had an increased levels in overweight children compared to normal-weight (10.7x higher, P=0.004, Figure 2B). *CCL20* was also higher expressed in PBMCs than in adipose tissue (P=0.03, Figure 2C).

Putative functional associations between rs7556897T>C and *CCL20* expression was assessed in PBMCs of 58 normal-weight children from SFS and showed a positive association between its mRNA levels and the rs7556897C allele (β=+0.43, P=0.002).

Discussion

The study of BP genetics has never been easy [26]. Numerous studies have shown that increased BP levels during childhood strongly predict HTN in adults [27-29].

We found that rs7556897C allele was associated with decreased DBP levels in normal-weight children (-0.012 ± 0.004, $P_{adjusted}$=0.006). In contrast the same variant allele increased DBP levels in obese children (β=+2.178 ± 0.71, $P_{adjusted}$=0.002).

We further hypothesized and observedan rs7556897T>C*obesity interaction in the modulation of BP.

SNP rs7556897T>C lies within an intergenic region of 95,831 bp between *SLC19A3* and *CCL20* loci in chr.2q33-37 that harbors a CpG island (336 bp) with a high GC content (65.2%) in the 5'UTR of *SLC19A3* locus (Supplemental Figure 2). This genomic region has been previously linked with HTN in previous BP linkage studies conducted on Europeans: Caulfield et al [17] associated chr.2q33 with HTN in a study conducted on 3,599 British Caucasian siblings. Several BP quantitative trait loci from hypertensive rat models exhibit homology with our region of interest, including rat chromosome 9q22-q34 which is homologous to the human 2q locus [17-22]. However, rs7556897T>C has not been reported in previous GWAS performed in adults by Levy D et al [30], Newton-Cheh et al [31] or Ehret et al [13] even if the *CCL20* region was previously associated to BP[32]. Pooling large ranges of BMI could thus conceal these types of interplays. In fact, when we performed a meta-analysis of chr.2q including our two discovery populations, no significant genetic variant came out. Indeed, meta-analyses' P-values are resultants of each individual association weighted by the effect of each variant in a specific population. A pooled analysis of 2 opposite effects will thus give a null average effect. Therefore, the obesity status should be taken into account in BP genetic epidemiology studies.

This part of our investigation is strengthened by the fact that both normal weight and obese children are French, thus limiting the possibility that the observed association is due to population admixture but we acknowledge the lack of replication in larger paediatric obese populations. One major obstacle for doing so was the absence of available children cohorts fulfilling our study design regarding obesity status.

In adipose tissue of normal and overweight children, we found that, while *SLC19A3* was homogeneously expressed in both populations, *CCL20* was only expressed in overweight children. In accordance with our results, Duffaut et al previously reported that *CCL20* mRNA

levels were ≈7x higher in adipose tissue of obese compared to normal weight individuals [33].

CCL20 encodes a small cytokine (96 amino acid) belonging to the CC chemokine family, mainly expressed in lymphocytes [33] and referred also as adipochemokine. Therefore, we further hypothesized that rs7556897T>C could be an obesity-related locus influencing BP levels through inflammation pathway. Indeed, different pathways related to inflammation have been proposed to link obesity and HT[34].Consequently, *CCL20* mRNA levels were deeper investigated in PBMCs where it was higher expressed than in adipose tissue (P=0.03, Figure 2C), implying a more pronounced effect of obesity on *CCL20* gene expression in PBMCs. Interestingly, in this inflammation linked cell model, overweight children had 10.7x higher *CCL20* mRNA levels than normal-weight children (P=0.004, Figure 2B). Finally, rs7556897C allele was associated with increased *CCL20* mRNA levels. *SLC19A3* was not expressed in PBMCs (Supplementary Data S1).

Working on human primary cells in which mRNA profile expression closely mimics the in vivo state, as PBMCs, seems a well reliable model when studying complex phenotypes as they generate physiologically relevant data [35]. Indeed, PBMCs are representative cell models for studying inflammatory pathways predisposing to CVDs [36]. They contain mainly lymphocytes that interact with different tissues, thereby probably acting as 'sensors' differentiating individuals with CVDs risk [36,37]. The important role of inflammation in HTN makes the study of PBMCs transcriptome crucial for developing diagnostic and prognostic tests, as these circulating cells 'sense' the pro-inflammatory state [36,38].

As a summary of this part of our investigation, our results revealed an unknown obesity-related effect of rs7556897T>C on *CCL20* expression in PBMCs.

Indeed, our transcriptomic data gave additional evidence for a putative SNP rs7556897T>C-obesity regulation of BP through *CCL20* mRNA at childhood.

In conclusion, we showed that rs7556897T>C, due to its interaction with adiposity, was associated with BP but had opposite effect on BP according to the obesity status. Our data also showed that rs7556897T>C may play a regulatory role on BP through its effect on *CCL20* expression specific to obese adipose tissue. Therefore, this finding highlights the importance of weight management and obesity prevention in childhood and should be taken into account in public health strategies for HTN and in future studies on the field. Also of

importance, we have shown that combining a hypothesis-free approach and a postulate based on previous studies could be beneficial in the identification of novel functional genetic variants probably via gene-environment interactions deeper investigations.

Materials and Methods

Ethic statement

All the populations involved in the present study were recruited in accordance with the latest version of the Declaration of Helsinki for Ethical Principles for Medical Research Involving Human Subjects. All participants and their parents gave a written informed consent. Genetic studies protocols were approved by the local ethics committees for the protection of subjects for biomedical research: the ComitéConsultatif de Protection des Personnesdans la RechercheBiomédicale (CCPPRB).

Study population

The STANISLAS Family Study (SFS)

The SFS, as part of the Biological Resources Bank (BRC) "Interactions Gène-Environnement en PhysiopathologieCardioVasculaire" (IGE-PCV) in Nancy-France, is a 10-year longitudinal survey initiated in 1993 and involving 1,006 volunteer families (2 parents and at least 2 siblings). Individuals with chronic disorders (cardiovascular or cancer) or having a personal history of cardiovascular disease (CVD) were not included, in order to investigate the effects of genetic susceptibility factors on the variability of intermediate cardiovascular phenotypes in physiological conditions without the influence of any long term medication or disease [39,40]. In total, 577 normal-weight unrelated children (defined as BMI<90th percentile for age and gender according to a French cohort [41]) were included. Among them, 58 had PBMCs collection available for transcriptomic analyses.

The French Obesity Cases (FOC)

Unrelated obese children (defined as BMI>97th percentile for age and gender according to a French cohort [41]) were recruited from 449 nuclear families with at least one obese offspring at the Paediatric Endocrine Unit of Jeanne de Flandres Hospital, Lille or through National media campaign. In total, 1,015 children with available BP traits were involved in the present study.

The Greek paediatric cohort

Six unrelated overweight (90th <BMI< 97th percentile[41]) and 10 normal-weight children (BMI<90th percentile[41]) with PBMCs and adipocytes were selected from a Greek paediatric cohort.

Genotyping

SFS and FOC genotypes were generated using an Illumina Human CNV370-Duo array. We used 750 ng of genomic DNA following Illumina's protocol and BeadStation genotyping solutions (Illumina), based on the GenCall software application (Illumina) to automatically cluster, call genotypes, and assign confidence scores using the GenTrain clustering algorithm (Illumina). We discarded 2,552 SNPs that had extreme HW disequilibrium (P< 0.001), low genotyping calling rates (<95%) or low minor-allele frequencies (<1%). We retained 318,237 SNPs for analysis. Genomic control λ_{GC} was 1.01. Fourteen thousand and six hundred seventy three SNPs harboring chr.2q were used for the following analyses.

Blood pressure measurements

After 5 minutes of rest in the seated position, BP was measured under constant temperature (19°C-21°C), and standardized conditions (supine position) with a manual sphygmomanometer (Colonne à mercure, Mercurius) by expert nurses. A BP cuff appropriate to the size of child's upper right arm was used. The values reported for SBP and DBP were the means of 3 readings at each examination on 20 minutes intervals.

Peripheral blood mononuclear cells (PBMCs) and adipose tissue collections

In SFS, freshly drawn peripheral venous blood (10 ml) was collected into tubes containing EDTA (Vacutainer, Becton Dickinson) under fasting conditions (or after 6 or 12 hours of fast). PBMCs were then isolated by centrifuging on a density gradient of Ficoll and stored at -80°C until RNA extraction as described previously [42].

The adipose tissue was cut into pieces of 220 mg each, in order to obtain a large amount of RNA. Isolated RNA was stored at -80^0C until quantification of the target mRNAs.

RNA extraction and quantitative real time PCR analysis

Total RNA was isolated from PBMCs and adipose tissue by an automated isolation procedure (MagNa Pure LC instrument), mRNA quality and stability were carefully tested [42] and reverse transcribed as previously described [42].

Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with Master Plus SYBR Green I kit for *CCL20*, *SLC19A3* and *POLR2A* transcripts (supplemental material). *CCL20* mRNA levels were normalized to the mRNA levels of *POL2RA*. The specificity of all PCR products was further verified by electrophoresis on 10% polyacrylamide gel (data available on demand).

Statistical analyses

Chr.2q association study

We first carried an association study for SBP and DBP levels using linear regression under additive genetic model with one degree of freedom screening the chr.2q localization. We have studied 14,674 SNPs after adjustment for age, gender, z-score BMI and multiple testing using PLINK [43] separately in normal-weight (SFS) and obese (FOC) children in order to determine possible discrepancies existing between obesity status and variant(s) regulating BP in chr.2q.

Both normal-weight and obese populations were then combined in order to determine potential interactions with obesity status: in a similar regression model, the interaction term SNP*obesity status was introduced in the model.

Hardy-Weinberg equilibrium was set at $P< 0.001$ and was tested using the chi-square test.

rs7556897T>C-CCL20 mRNA association analysis

The association analyses between rs7556897T>C and *CCL20* mRNA levels was assessed in four independent populations using a linear regression model after adjustment for age, sex and BMI.The association between rs7556897T>C and mRNA *CCL20* observed in the SFS subsample was assessed *in silico* in 3 independent populations available online: SNP rs7556897T>C was genotyped in HapMap population. In contrast we did not found it in GenCord project and MuTHER study databases. Thus, we searched for imputed SNPs within <13Kb using PLINK [43]. Among 9 found (Supplementary Table S1), only rs2048054 was common between all genotyping arrays (our Illumina Human CNV370-Duo array, Geneva GenCord project and MuTHER Pilot arrays). Both rs7556897T>C and rs2048054 are

intergenic (*SLC19A3-CCL2*0), in LD blocks (D'=1, r^2=0.81) and the latter SNP is located 7765bp upstream of rs7556897T>C (Supplementary Data S3). InGenCord project [44], rs2048054 test of association with *CCL20* expression in EBV-LCLs, fibroblast and T-cell was carried out in 75 European individuals. In MuTHER study [45],rs2048054 association with *CCL20* expression in EBV-transformed LCLs, fat and skin tissues was carried out in 196 European women twins [45]. Adjusted P-value for 10,000 permutations was calculated for rs2048054-*CCL20*expression association in a linear regression model. Genevar software was used.

BMI-CCL20 mRNA levels in PBMCs and adipose tissue

For the study of BMI influence on *CCL20* mRNA, comparisons between groups were analysed by paired samples t-tests in different cell types. Differences were considered significant when P<0.05.

URLs

PLINK1.07 is available at: http://pngu.mgh.harvard.edu/~purcell/plink/

Genevaris available at: http://www.sanger.ac.uk/resources/software/genevar/

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Figure legends

Figure 1: Mapping of genotyped SNP rs7556897T>C located between SLC19A3 and CCL20 loci.

A- Schematic representation of chromosome 2. B- Schematic representation of 1.Mb region centered on the intergenic SNP rs7556897T>C located at 228 660 112 bp between Solute Carrier family 19, member 3 (SLC19A3) and Chemokine (C-C motif) ligand 20 loci. C- Schematic representation of 200Kb interest region rs7556897T>C flanked by SLC19A3 gene on the reverse strand and CCL20 gene on the forward strand. D- Schematic representation of 5Kb region centered on rs7556897T>C. E- Schematic representation of 100bp region centered on rs7556897T>C. On both sides of the AC073065.6 contig is depicted 100bp of the nucleotide sequence flanked rs7556897T>C. Analyzed genomic data are displayed using ensembl browser, available at:

http://www.ensembl.org/Homo_sapiens/Info/Index

Figure 2: CCL20 gene expression in PBMCs and adipose tissue of normal weight and overweight children.

A- qRT-PCR analysis of CCL20 mRNA showed increased expression in PBMCs of overweight children (N=6) compared to normal weight children (N=10). B- qRT-PCR analysis of CCL20 mRNA showed expression only in adipose tissue of overweight children (N=10) compared to normal weight (N=6). Normalized CCL20 mRNA levels are presented as means ± SD. MA: adipose tissue, PBMCs: peripheral blood mononuclear cells.

Table 1: Clinical characteristics of the studies populations

	STANISLAS Family Study	**French Obesity Cases**
N (M/F*)	577 (290/287)	1,015 (484/531)
Age (years)	11.939 ± 2.334	11.268 ± 3.228
BMI† (kg/m²)	17.22 ± 2.064	28.243 ± 6.507
SBP‡ (mmHg)	109.742 ± 9.7	113.91 ± 14.66
DBP§ (mmHg)	54.919 ± 10.098	68.44 ± 11.57

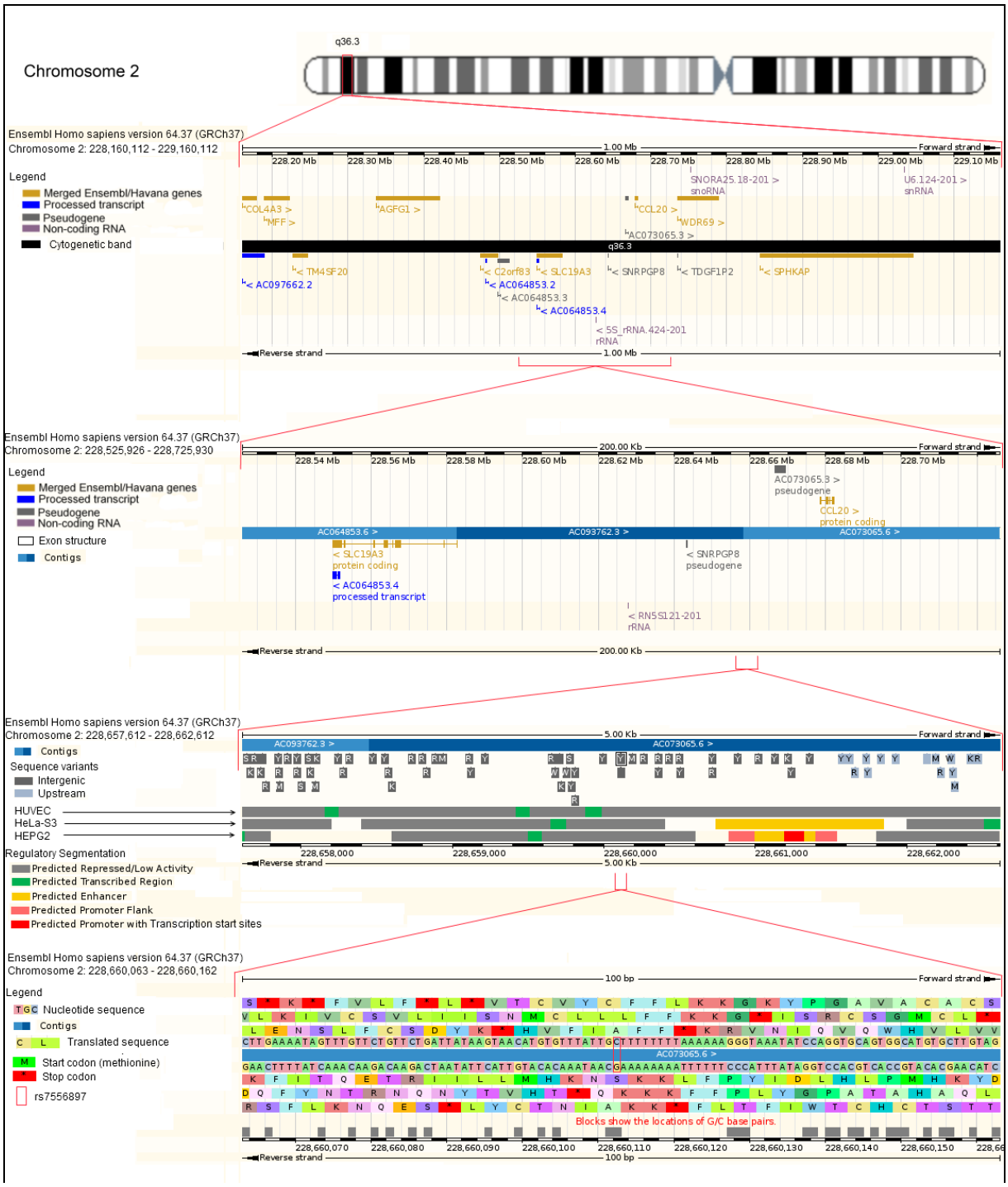
*M: male, F: female

†BMI: body mass index

‡SBP: systolic blood pressure

§DBP: diastolic blood pressure

Figure 1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Processed transcript
Pseudogene
Non-coding RNA
Cytogenetic band
Forward strand
Reverse strand
1.00 Mb
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U6.124-201 > snRNA
COL4A3 >
MFF >
AGFG1 >
CCL20 >
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TM4SF20
C2orf83
SLC19A3
SNRPGP8
TDGF1P2
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Upstream
HUVEC
HeLa-S3
HEPG2
Regulatory Segmentation
Predicted Repressed/Low Activity
Predicted Transcribed Region
Predicted Enhancer
Predicted Promoter Flank
Predicted Promoter with Transcription start sites
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Translated sequence
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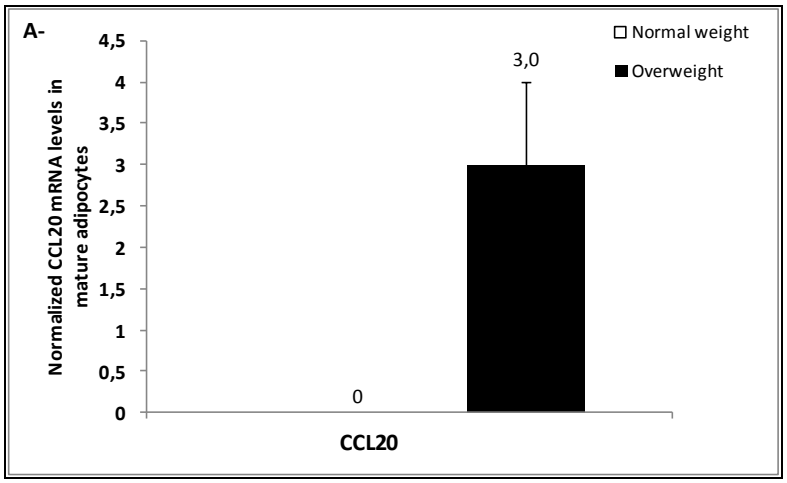
Stop codon
rs7556897
Blocks show the locations of G/C base pairs.

Figure 2.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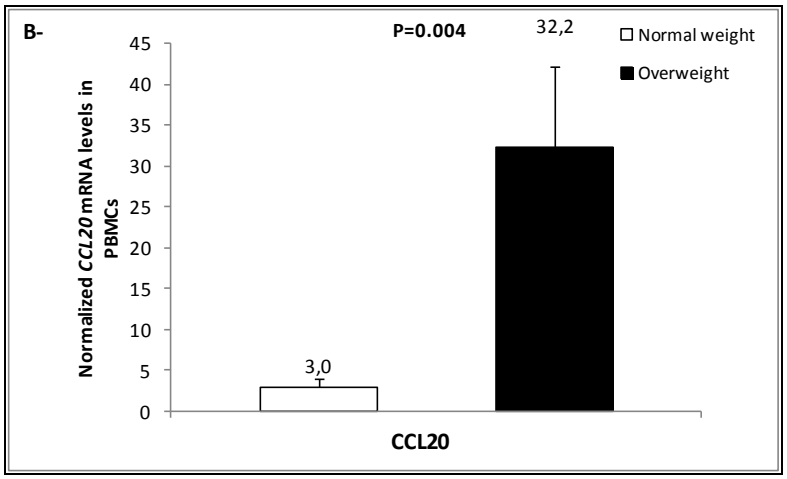
Normal weight
Overweight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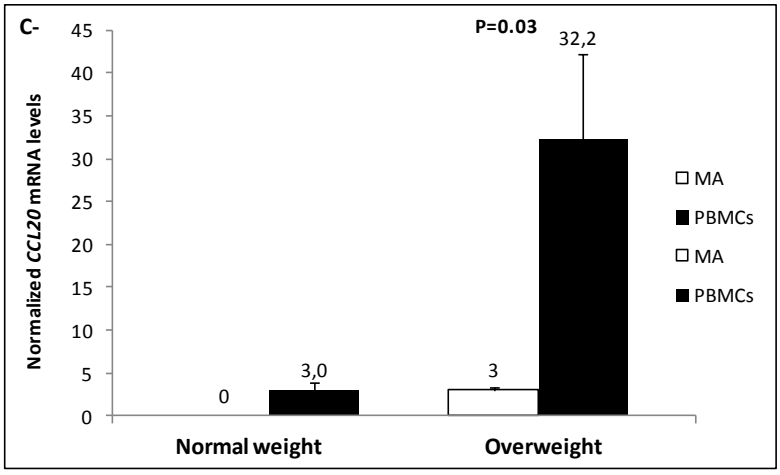
CCL20
Normal weight
Overweight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40
35
30
25
20
15
10
5
0
0
3,0
3
Normal weight
Overweight
MA
PBMCs
MA
PBMCs

Supporting information legends

Supplementary Data S1: *CCL20* and *SLC19A3* gene expression in PBMCs of normal weight children.

qRT-PCR for *CCL20* and *SLC19A3* mRNA levels. *CCL20* mRNA were expressed in PBMCs of normal weight SFS children. PBMCs: peripheral blood mononuclear cells.

Supplementary Data S2: Fine mapping of CpGisland found in the 5'UTR of *SLC19A3* locus using UCSC genome browser data.

A- The genomic region of chromosome 2 harboring CpG island is depicted in red (chromosome 2: 228582486-228582821). B- Schematic representation of 336bp region on the forward strand of chromosome 2 containing CpGisland with high GC content (65.2%) in 5'UTR of *SLC19A3* locus. Analyzed genomic data are displayed using the UCSC genome browser, available at:

http://genome.ucsc.edu/cgi-bin/hgGateway

Supplementary Data S3: *SLC19A3* gene expression in mature adipocytes of normal weight and overweight children.

qRT-PCR analysis of *SLC19A3* mRNA showed similar expression in mature adipocytes of overweight children (N=6) compared to normal weight children (N=10). Normalized *SLC19A3*mRNA levels are presented as means ± SD.

Supplementary Table S1: SNPs in L.D with rs7556897T>C

I.A.1. SNPs in LD with rs7556897T>C ($r^2 \geq 0.8$) were determined in the SFS and FOC populations using the PLINK Sofware.

*: rs2048054 was the only common SNP between our array and Geneva GenCord project and MuTHER Pilot arrays.

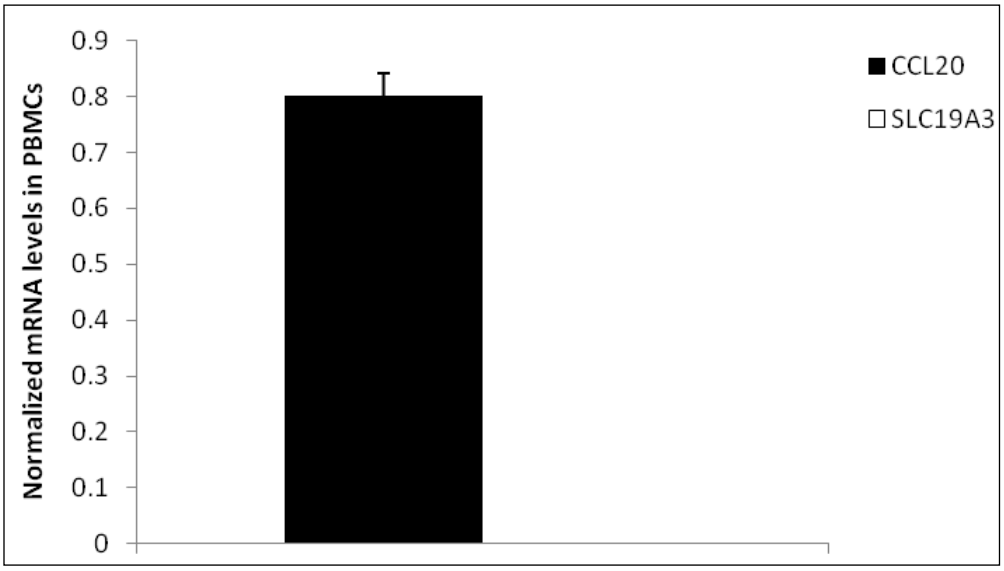
Chr: chromosome, MAF: minor allele frequency, P: p-value.

Supplementary Table S2: Primers sequences and qRT-PCR conditions.

CCL20: chemokine (C-C motif) ligand 20, SLC19A3: solute carrier family 19, member 3, POLR2A: polymerase (RNA) II.

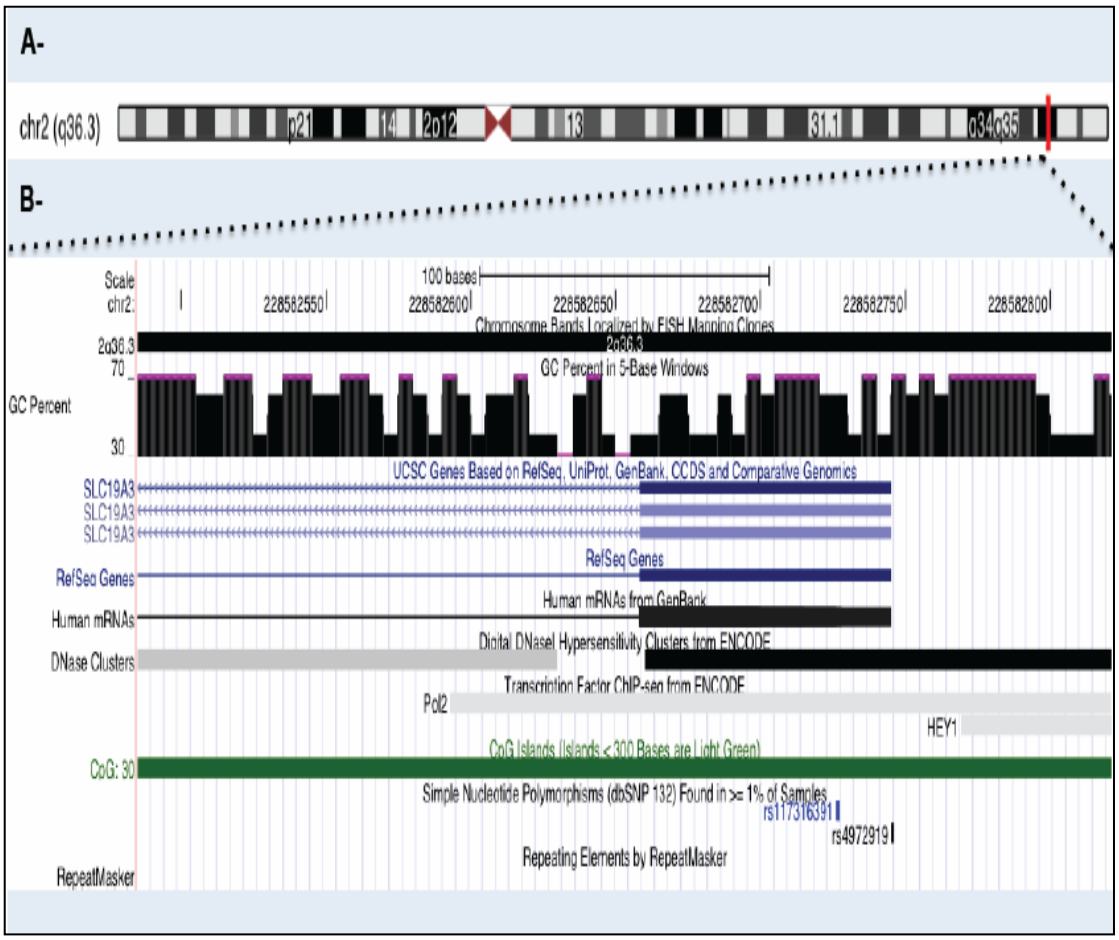
Specific primers were designed using Primer Premier 3.0 software [1] (Supplemental Table 2). All experiments were carried out in duplicate in a total reaction volume of 20 µl containing 0.5 mM of each specific primer, negative and internal controls were included. mRNA levels of *CCL20* and *SLC19A3* were normalized to the mRNA levels of *POL2RA*. The specificity of all PCR products was further verified by electrophoresis on 10% polyacrylamide gel.

Supplementary Data S1: *CCL20* and *SLC19A3* gene expression in PBMCs of normal weight children.



qRT-PCR for *CCL20* and *SLC19A3* mRNA levels. *CCL20* mRNA were expressed in PBMCs of normal weight SFS children. PBMCs: peripheral blood mononuclear cells.

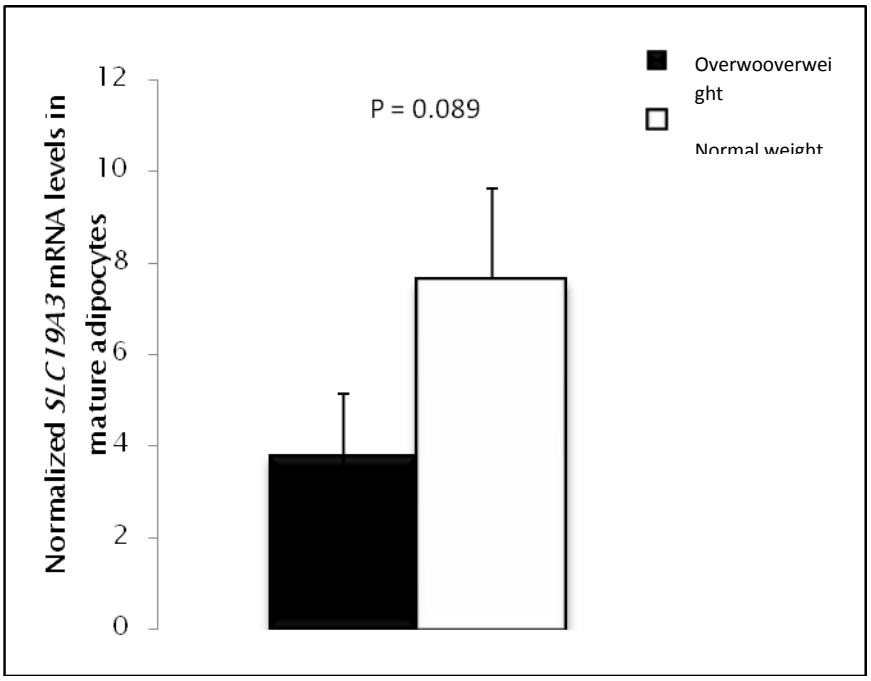
Supplementary Data S2: Fine mapping of CpG island found in the 5'UTR of *SLC19A3* locus using UCSC genome browser data.



A- The genomic region of chromosome 2 harboring CpG island is depicted in red (chromosome 2: 228582486-228582821). B- Schematic representation of 336bp region on the forward strand of chromosome 2 containing CpG island with high GC content (65.2%) in 5'UTR of *SLC19A3* locus. Analyzed genomic data are displayed using the UCSC genome browser, available at:

http://genome.ucsc.edu/cgi-bin/hgGateway

Supplementary Data S3: *SLC19A3* gene expression in mature adipocytes of normal weight and overweight children.



qRT-PCR analysis of *SLC19A3* mRNA showed similar expression in mature adipocytes of overweight children (N=6) compared to normal weight children (N=10). Normalized *SLC19A3* mRNA levels are presented as means ± SD.

Supplementary Table S1: SNPs in L.D with rs7556897T>C

SNP_rsID	Chr.	Position (bp)	MAF	Distance	r^2	D'	P
rs7556897T>C is associated with:	2	228368356	0.350	NA	NA	NA	NA
rs2396495	2	228355099	0.333	13kb	0.86	0.96	9.26e-22
rs2063021	2	228357138	0.400	11kb	0.81	1.00	1.13e-18
rs12694756	2	228357480	0.398	10kb	0.80	1.00	2.39e-18
rs2048054*	**2**	**228360591**	**0.400**	**7kb**	**0.81**	**1.00**	**1.13e-18**
rs13024444	2	228361408	0.400	6kb	0.81	1.00	1.13e-18
rs4973334	2	228362323	0.367	6kb	0.93	1.00	3.16e-22
rs4973341	2	228368606	0.328	250bp	0.93	1.00	2.91e-25
rs7593478	2	228369130	0.350	774bp	1.00	1.00	6.33e-28
rs11686438	2	228369373	0.347	1kb	1.00	1.00	1.73e-27

SNPs in LD with rs7556897T>C ($r^2 \geq 0.8$) were determined in the SFS and FOC populations using the PLINK Sofware.

*: rs2048054 was the only common SNP between our array and Geneva GenCord project and MuTHER Pilot arrays.

Chr: chromosome, MAF: minor allele frequency, P: p-value.

Supplementary Table S2: Primers sequences and qRT-PCR conditions.

Target transcripts	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing (°C, sec)	PCR product size (pb)
CCL20	GCGCAAATCCAAAACAG	CAAGTCCAGTGAGGCAC	60°C, 10sec	200
SLC19A3	TTCCTGGATTTACCCCAC	GTATGTCCAAACGGGGA	60°C, 8sec	154
POLR2A	CAGACCGGCTATAAGGT	GGTAGACCATGGGAGA	57°C, 5sec	123

CCL20: chemokine (C-C motif) ligand 20, SLC19A3: solute carrier family 19, member 3, POLR2A: polymerase (RNA) II.

Specific primers were designed using Primer Premier 3.0 software [1] (Supplemental Table 2). All experiments were carried out in duplicate in a total reaction volume of 20 µl containing 0.5 mM of each specific primer, negative and internal controls were included. mRNA levels of *CCL20* and *SLC19A3* were normalized to the mRNA levels of *POL2RA*. The specificity of all PCR products was further verified by electrophoresis on 10% polyacrylamide gel.

V. Etude de la relation du rs5030878T>C du gène *FPR1* avec les niveaux de pression artérielle et l'hypertension :

Human Formyl Peptide Receptor 1 C32T SNP Interacts With Age and is Associated With Blood Pressure Levels.

Said El Shamieh, Bernard Herbeth, Mohsen Azimi-Nezhad, Hamanou Benachour, Christine Masson, Sophie Visvikis-Siest.

Clin Chim Acta. 2012 Jan 18,413(1-2):34-8. Epub 2010 Dec 7.

Chez l'Homme, le gène *FPR1* code pour un récepteur de molécules chimio-attractantes, exprimé à la surface des neutrophiles, monocytes et macrophages. En dehors des leucocytes, son expression est détectée dans les cellules endocrines, les hépatocytes, les cellules musculaires lisses vasculaires et endothéliales. Il est présent en une seule copie de 6Kb de longueur sur le bras long du chromosome 19 (19q13.4), une région génomique qui a été liée aux niveaux de PA dans de nombreuses études de liaison génétique. Une étude intéressante a montré que le SNP rs5030878C>T du gène *FPR1*, situé à l'extrémité N-terminale de FPR1, est associé à la réponse inflammatoire, notamment à une augmentation du taux circulant de la protéine CRP. De même, au sein de notre laboratoire, il a été montré que ce même SNP est associé à une diminution du taux circulant de la molécule SELE. Compte tenu de ces observations, nous avons recherché une éventuelle association du SNP rs5030878C>T avec la PA et l'HTA chez des individus français.

Nous avons génotypé un total de 1,012 adultes d'âge moyen (entre 38,9±4,3 et 46,5±4,3), comprenant 491 individus supposés sains (avec 5 ans de suivi) ainsi que 521 hypertendus, pour le polymorphisme génétique rs5030878C>T par la technique de PCR-RFLP. Les individus supposés sains faisaient partie de la cohorte STANISLAS, et les HT appartenaient à un essai clinique.

A l'entrée, aucune association significative n'a été trouvée entre le SNP rs5030878C>T et les niveaux de PA chez les individus supposés sains. Cependant, 5 ans plus tard (T_{+5}), *FPR1* C32T était significativement associé aux niveaux de PAD et PAS (P=0,001 et P=0,009

respectivement), ainsi qu'avec leur variation longitudinale sur 5 ans (Δ) (P=0,025 et P=0,027 pour la PAD et la PAS, respectivement).

Afin d'expliquer les relations existantes entre le SNP rs5030878C>T et la PA à T_{+5}, nous avons séparé les individus supposés sains selon le génotype *FPR1* et l'âge moyen (45 ans) en deux groupes, «âge <45 ans» et «âge ≥45 ans».L'allèle rs5030878T interagissait avec l'âge pour influencer la PAD, PAS, ΔPAD et ΔPAS (P=0,014, P=0,008, P=0,015 et P=0,015 respectivement). Une plus forte augmentation des niveaux de PA a été signalée pour les individus sains ayant moins de 45 ans. Une même distribution allélique du rs5030878T a été mise en évidence après comparaison entre individus sains et HT (P>0,05).

Le SNP rs5030878T est associé à une augmentation des niveaux de PA, surtout chez les individus sains ayant moins de 45 ans. Une fois confirmés dans d'autres études, nos résultats suggèrent que l'interaction de ce polymorphisme génétique avec l'âge pourrait être utile à prendre en compte dans les études de régulation de PA.

Mots clés : Pression artérielle/ génétique, Hypertension artérielle/ génétique, *FPR1*, polymorphisme génétique ponctuel.

Contribution : *Nous avons participé à la conception et au design de l'étude et effectué la totalité des expériences de transcriptomique. Nous avons pris en charge les expériences de génotypage. Nous avons également rédigé la première version du manuscrit.*

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Human formyl peptide receptor 1 C32T SNP interacts with age and is associated with blood pressure levels

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ABSTRACT

Background: Human formyl peptide receptor 1 (FPR1) mediates inflammatory responses, recognized as important participants in the physiopathology of hypertension. Similarly, *FPR1* C32T SNP is associated with inflammation and BP related pathways. Therefore, the relationship between *FPR1* C32T SNP, BP and hypertension needs to be investigated.

Method: 1012 French middle-aged adults including 491 healthy individuals (5 years follow-up, T_{+0} and T_{+5}) and 521 hypertensive individuals were PCR-RFLP genotyped for *FPR1* C32T SNP (rs5030878).

Results: At entrance, there was no significant association between *FPR1* C32T SNP and blood pressure (BP) in healthy individuals. However, 5 years later, significant associations were found for DBP, SBP ($p<0.001$ and $p=0.009$ respectively) and for their 5 years changes (Δ) ($p=0.025$ and $p=0.027$ for DBP and SBP respectively). Significant interactions between *FPR1* C32T SNP and age on DBP, SBP, ΔDBP and ΔSBP were found ($p=0.014$, 0.008, 0.015 and 0.015 respectively). Consequently, stronger increase in BP was reported among healthy individuals aged less than 45 years. When normotensive individuals were compared to hypertensives ones, similar *FPR1* C32T genotypes and allele frequency distributions were found.

Conclusion: *FPR1* C32T SNP interacts with age, is associated with higher and a 5 years increase of BP levels in healthy individuals aged less than 45 years.

1. Introduction

Hypertension (HTN) is a continuous, consistent and modifiable risk factor for cardiovascular diseases (CVD) such as ischemic heart disease (IHD) and stroke. CVDs are a major cause of mortality and morbidity all around the world [1]. Population-based studies from different regions showed that 972 millions of adults worldwide were hypertensive in 2005, this number is predicted to increase by about 60% to a total of 1.56 billion in 2025 [2].

Multiple genetic determinants and environmental factors contribute to generate blood pressure (BP) levels [3]. Among environmental factors, we note high dietary intake of sodium and alcohol, obesity and stress [4]. Existing evidence suggests that genetic contribution is about 30 to 50% [3] and BP is a polygenic trait [5,6]. Moreover, numerous studies [7,8] have shown that tissue expression and plasma concentrations of inflammatory markers are associated with increased risk of HTN [7,8]. In addition, genome-wide analysis studies have reported that the long arm of chromosome 19 (19q) harbors candidate loci which have been linked to increased BP levels and related pathological phenotypes [9–13].

The human *formyl peptide receptor 1 (FPR1)* gene has retained some attention, via its involvement in inflammation [14] and BP related metabolic pathways [15]. *FPR1* is a 6-kb single copy gene clustering the long arm of the chromosome 19 (19q13.4) [16]. It encodes a 350 amino acid G protein-coupled receptor, which consists of seven hydrophobic transmembrane domains and expressed in phagocytic leukocytes, mainly neutrophils and monocytes [17]. Several SNPs were reported previously to be associated with inflammatory diseases such as aggressive peritonitis [18]. *FPR1* C32T (rs5030878), a non-synonymous coding SNP (Ile11Thr) was reported to be associated with increased C-reactive protein (CRP) levels [14], linearly related with BP [19]. In addition to CRP, our team has shown, in the STANISLAS cohort, that this SNP is also associated with the levels of E-selectin [15], a cellular adhesion molecule known for its involvement in BP regulation [20].

Based on the fact that *FPR1* C32T SNP is associated with CRP and E-selectin levels and clusters the long arm of chromosome 19 that harbors BP candidate loci, the purpose of this study was to determine whether this SNP might be associated with BP and HTN in 1012 French middle-aged adults, including 491 healthy individuals drawn from the STANISLAS cohort (5 years follow-up, T+0 and T+5) and 521 hypertensive individuals, enrolled in a clinical trial.

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2. Materials and methods

Appropriate institutional review board approval and Helsinki principles were followed for all human experimental investigations. In addition, each subject gave written informed consent to participate in this study.

2.1. Blood pressure study

For the present study, 491 (248 men and 243 women) unrelated apparently healthy individuals were selected from the STANISLAS cohort (5 years follow-up) [21] on the basis of the following criteria: (1) individuals were present at both, entrance (T_{+0}) and after 5 years follow-up (T_{+5}); (2) body mass index (BMI), triglyceride, HDL-cholesterol levels were available at both examinations (3) *FPR1* C32T genotypes were determined; (4) pregnant and breastfeeding women were also excluded in both visits.

2.2. Hypertension study

Hypertensive individuals were selected from 521 unrelated French individuals enrolled in a clinical trial (260 men and 261 women). The clinical inclusion criteria were: (1) French origin; (2) absence of antihypertensive medication, CVD, stroke; or diabetes; (3) after 5 minutes (min) of rest, the seated systolic blood pressure (SBP) was between 140 and 180 mm Hg or the diastolic blood pressure (DBP) between 90 and 110 mm Hg. Only individuals <56 years old were included in order to approach the sample age range of healthy individuals.

2.3. Clinical and biological data

All individuals underwent complete medical examination including weight and height measurements. BP was measured under constant temperature (19 °C to 21 °C) and standardized conditions (supine position) with a manual sphygmomanometer by expert nurses. The values reported for SBP and DBP were the means of 3 readings, each one taken within a 20 min interval. Classical biochemical measurements, including serum levels of triglycerides, HDL-cholesterol and glucose were performed with standardized methods and were recorded [21]. Hypertensive characteristics were obtained after a wash-out period for 3 weeks.

2.4. FPR1 Genetic analyses

Genomic DNA was extracted from peripheral blood cells using a salting-out method as described previously[22]. RFLP genotyping of the *FPR1* C32T polymorphism was done by amplifying a "205 pb" region located in the second exon of the *FPR1* gene (GENBANK access: 92357) followed by *BseLI* restriction enzyme digestion. The primers used were 5′-TGA GGA AAT GAC CAC GAC TGC A-3′ (forward primer) and 5′-TCC GGA ATC CAG CCA CCC AGA TC-3′ (reverse primer). All PCR reactions were performed using 200 ng of genomic DNA in a final volume of 50 µl containing 1.5 mM $MgCl_2$, 100 µM dNTPs, 0.5 µM of each primer and 1U of Taq DNA polymerase (Roche Diagnostic, Basel, Switzerland) with 1X Buffer. DNA was initially denatured at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 seconds (s), annealing was carried out at 58 °C for 1 min and extension at 72 °C for 30 s. A final extension at 72 °C for 7 min was then performed. RFLP analysis of the "205 bp" amplified product was performed by digestion of the PCR product for 1 h at 55 °C with *BseLI* restriction enzyme (Roche Diagnostic, Basel, Switzerland). After digestion, the presence of the C allele leads to the production of "125 pb" and "80 bp" fragments, while product with T allele remains undigested.

Blinded replication of the fragment analysis was performed with 10% of the selected samples on an automated high-performance liquid chromatography instrument (Wave system™; Transgenomics Inc. [NC, USA]), both sequencing and restriction analyses fully matched.

2.5. Statistical analysis

Statistical analyses were performed by using the SAS software package version 9.13 (SAS Institute, Inc., Cary, NC). To improve normality, triglyceride concentrations were log_{10}-transformed in the analyses. Performing a chi-square (χ^2), we verified that genotypes and allele frequencies in healthy and hypertensive individuals drawn from STANISLAS cohort and clinical trial respectively, were consistent with the Hardy–Weinberg equilibrium. For continuous variables, an analysis of variance (ANOVA) was performed for characteristic differences among the 2 groups of population. Categorical (C/C vs. C/T vs. T/T), recessive (C/C δ C/T vs. T/T), dominant (C/C vs. C/T δ T/T) and allelic effects (assigning 0, 1, 2 for the C/C, C/T, and T/T genotypes respectively) were evaluated using ANOVA test. The threshold for statistical significance was set at $p<0.05$ and power of analyse was more than 80%.

3. Results

Table 1 shows clinical, biochemical characteristics and *FPR1* C32T SNP genotypes for healthy and hypertensive individuals. Clinical and biochemical characteristics did not differ between men and women in healthy and hypertensive individuals. Over the 5 years follow-up, age and BMI increased in both men and women (for BMI, 1.2 kg/m^2 and 0.6 kg/m^2 respectively). When comparing both samples (healthy vs. hypertensive individuals), we have noted that BMI and BP parameters including DBP and SBP were higher in hypertensive compared to normotensive individuals ($p<0.01$). While *FPR1* C32T SNP genotypes (C/C, C/T and T/T) and alleles (C and T) frequency were consistent with Hardy–Weinberg equilibrium ($\chi^2=0.013$ and $p=0.715$), similar frequency distribution, without significant difference, was observed in both normotensive individuals (SBP<140 mm Hg or DBP<90 mm Hg and not using antihypertensive treatment) drawn from the STANISLAS cohort compared to hypertensive individuals enrolled in the clinical trial.

In order to determine whether the *FPR1* C32T SNP was associated with BP, two models of genetic inheritance were tested; recessive and categorical effects. Considering a recessive model of inheritance, Table 2 shows the relationship between *FPR1* C32T SNP and BP in healthy individuals. At T_{+0}, healthy individuals had same DBP and SBP according to *FPR1* C32T SNP ($p=0.242$ and $p=0.451$ respectively).

However, at T_{+5} both DBP and SBP were higher in individuals carrying T/T genotype as compared to C/T or C/C genotypes ($p<0.001$ and $p=0.009$ respectively). As shown in Table 2, individuals carrying T/T genotype had about 7 mm Hg increase in both DBP and SBP.

Similarly, for the 5 years changes (Δ) in DBP and SBP, an increase of about 4.3 and 4.8 mm Hg respectively was observed in *FPR1* T/T genotype carriers ($p=0.025$ and $p=0.027$ respectively.

By using a categorical model of inheritance, association term for *FPR1* C32T SNP and BP at T_{+5} remained significant for DBP, SBP and ΔDBP ($p<0.001$, $p=0.033$ and $p=0038$ respectively). For ΔSBP, nonsignificant results as compared to recessive model were found ($p=0.089$).

In order to explain the relationships between *FPR1* C32T SNP and increased BP at T_{+5}, we have separated normotensives according to *FPR1* genotype and median age (45 years) into two groups; «age<45 years»and« age ≥45 years». Fig. 1 shows the relationships between BP and *FPR1* genotype taking age into account. Stratification on age confirmed the previous significant associations between DBP, SBP, ΔDBP and ΔSBP ($p<0.001$, $p=0.008$, 0.022 and $p=0.041$ respectively). Moreover, significant interaction between *FPR1* C32T SNP and age on DBP, SBP, ΔDBP and ΔSBP is found when age was considered as dichotomized variable (p for interaction with age 0.014,

Table 1
Clinical, biochemical and *FPR1* C32T SNP characteristics of healthy and hypertensive individuals.

	Healthy individuals				Hypertensive individuals	
	Men ($n=248$)		Women ($n=243$)		Men ($n=260$)	Women ($n=261$)
	T_{+0}	T_{+5}	T_{+0}	T_{+5}		
Age[§‡] (years)	41.3 ± 4.1	46.1 ± 4.2	38.9 ± 4.3	43.5 ± 4.1	46.4 ± 7.9	46.5 ± 4.3
BMI[§‡] (kg/m^2)	24.5 ± 2.7	25.7 ± 3.1	23.4 ± 3.4	24 ± 3.7	27.3 ± 3.7	26.1 ± 4.9
DBP[‡] (mm Hg)	76.7 ± 9.1	76.6 ± 9.7	71.5 ± 9.7	71.4 ± 9.9	99.4 ± 3.8	98.6 ± 2.8
SBP[‡] (mm Hg)	126.1 ± 11.1	126.1 ± 12.6	119.3 ± 11.9	120.3 ± 13.6	155.7 ± 11.3	156.6 ± 10.1
Triglyceride (mmol/l)	1.35 ± 0.72	1.32 ± 0.76	0.96 ± 0.54	1.35 ± 0.57	1.74 ± 1.22	1.34 ± 0.76
HDL-cholesterol (mmol/l)	1.43 ± 0.41	1.45 ± 0.42	1.76 ± 0.5	1.82 ± 0.44	1.68 ± 0.40	1.72 ± 0.45
Glucose (mmol/l)	5.13 ± 0.46	5.26 ± 0.53	4.82 ± 0.47	4.91 ± 0.56	5.67 ± 0.82	5.27 ± 0.87
FPR1 Genotype						
C/C[†] (%)	53.6%[a]		55.2%[a]		56.2%	55.9%
C/T[†] (%)	39.5%[a]		39.5%[a]		37.3%	36.8%
T/T[†] (%)	6.9%[a]		5.3%[a]		6.5%	7.3%
T allele[†] (%)	26.0 %[a]				25.5%	

Values are arithmetic mean ± SD for clinical and biochemical characteristics and percentage for *FPR1* genotype and allele. BMI: body mass index, DBP: Diastolic Blood Pressure, SBP: Systolic Blood Pressure, T_{+0}: Entrance of healthy individuals, T_{+5}: Healthy individuals after 5 years follow-up.
§ $p<0.01$ in healthy individuals at T_{+5} compared to T_{+0}.
‡ $p<0.01$ in healthy compared to hypertensive individuals.
† $p>0.05$ in healthy compared to hypertensive individuals.
[a] Individuals with SBP < 160 mm Hg or DBP <95 mm Hg and not using antihypertensive treatment.

0.005, 0.015 and 0.015 respectively). As shown in Fig. 1, differences in DBP and SBP between C/C, C/T and T/T genotypes were higher in individuals having age <45 years as compared to older (age ≥ 45 - years): 11 vs. 4 mm Hg ($p<0.001$) and 12 vs. 2 mm Hg ($p=0.008$) for DBP and SBP respectively. Similarly, for ΔDBP and ΔSBP, 5.5 vs. 3 mm Hg ($p=0.022$) and 10 vs. 0 mm Hg ($p=0.041$) respectively were found in individuals aged less than 45 years as compared to older ones.

No interaction of *FPR1* C32T SNP with sex and BMI in both healthy and hypertensive individuals was found (data not shown). However, it is important to underline that healthy and overweight (BMI ≥ 25 kg/m^2) individuals carrying T/T genotype had 5 vs. 3.8 mm Hg in ΔDBP and ΔSBP ($p=0.017$ and $p=0.036$ respectively) as compared to non-obese ones (BMI <25 kg/m^2) (Supplementary Table 1).

4. Discussion

Even at early stages, HTN is a major cause for millions of disabilities and death throughout the world [1]. Observational studies involving more than 1 million adults from 61 prospective studies have indicated that death from HTN related diseases (both IHD and stroke) increases linearly from levels as low as 75 mm Hg and 115 mm Hg DBP and SBP among middle (40–49 years) and elderly (80–89 years) age individuals [23]. Therefore, isolating genetic variants at early middle age has major benefits on public health.

To the best of our knowledge, this is the first study reporting an association between BP and *FPR1* C32T SNP ($p<0.028$). More precisely, significant associations were observed for DBP, SBP, ΔDBP and ΔSBP in healthy individuals ($p<0.001$, $p=0.009$, $p=0.025$ and $p=0.027$ respectively).

It is noteworthy to mention that genome-wide analysis and candidate gene studies reported the importance of the long arm of chromosome 19 in BP regulation [9]. For example, in a genome-wide linkage study conducted on 2259 African individuals, Cooper et al [9] associated the 19q region to increased BP [9]. In European populations, the largest study was the one conducted by Levy D et al., [24]. Eight loci were shown to be associated with BP and/ or HTN [24]. In aggregate, the proportion of BP variation explained was only 1% after accounting for the major nongenetic determinants of BP (age, sex and BMI) [24]. Unsurprisingly, given the limited heritability explained, *FPR1* C32T SNP was not reported among the BP loci in the previously mentioned study. A recent meta-analysis reported the genetic variant of *Apolipoprotein E* (*ApoE*), *ApoE ε4* (chromosome 19q13.2) to be associated with an increased risk of developing HTN [25].

To date, little information is available on the mechanistic role of *FPR1* [17], in BP regulation. Otherwise, the chemoattractant fMLP (FPR1 canonical ligand) has its role [26]; [27]. Keitoku and his colleagues [27], showed this hydrophobic tripeptide producing transient tension changes in the human coronary arteries [27]. When binding, fMLP triggers reactive oxygen species generation by activating the NADPH oxidase enzyme [15], an important factor in hypertensive vascular damage [28]. Multiple domains are crucial for fMLP high-affinity binding, with a major determinant located in the first extracellular loop and its adjacent domains [29]. Even if the *FPR1* C32T SNP is not located in the gene region coding for the first extracellular loop, it results an interesting amino acid substitution (Ile11Thr) since it adds more hydrophobic residues to the extracellular N-terminal region. In addition, this amino acid substitution may add conformational changes enhancing the FPR1–fMLP binding. Kinetic and crystallographic assays are required for testing the above mentioned hypothesis.

A significant interaction between *FPR1* C32T SNP and age was observed in our healthy population. A more pronounced increase in BP was found among young T/T genotype carriers (age <45 years).

Table 2
Blood pressure according to *FPR1* C32T SNP in healthy individuals.

Healthy individuals					
Number	C/C	C/T	T/T	*P*	
	267	194	30	Model 1	Model 2
DBP (mm Hg)					
T_{+0}	74.4 ± 10.1	74.8 ± 9.3	76.7 ± 10.2	0.242	0.553
T_{+5}	74.0 ± 10	73.8 ± 9.6	81.0 ± 10	<0.001	<0.001
Δ	0.0 ± 11.2	−1.0 ± 10	4.3 ± 10.8	0.025	0.038
SBP (mm Hg)					
T_{+0}	122 .4 ± 12.1	123.3 ± 11.4	125.0 ± 12	0.451	0.743
T_{+5}	123.6 ± 13.2	123.6 ± 13.7	130.0 ± 16	0.009	0.033
Δ	0.2 ± 12.1	0.3 ± 11.1	5.0 ± 14	0.027	0.089

Values are arithmetic means ± SD for clinical characteristics.
Crude values were analyzed.
DBP: Diastolic Blood Pressure, T_{+0}: Entrance of healthy individuals, T_{+5}: Healthy individuals after 5 years follow-up, Δ: $T_{+5} - T_{+0}$, SBP: Systolic Blood Pressure.
Model 1: Genotype recessive effect, Model 2: Genotype categorical effect.

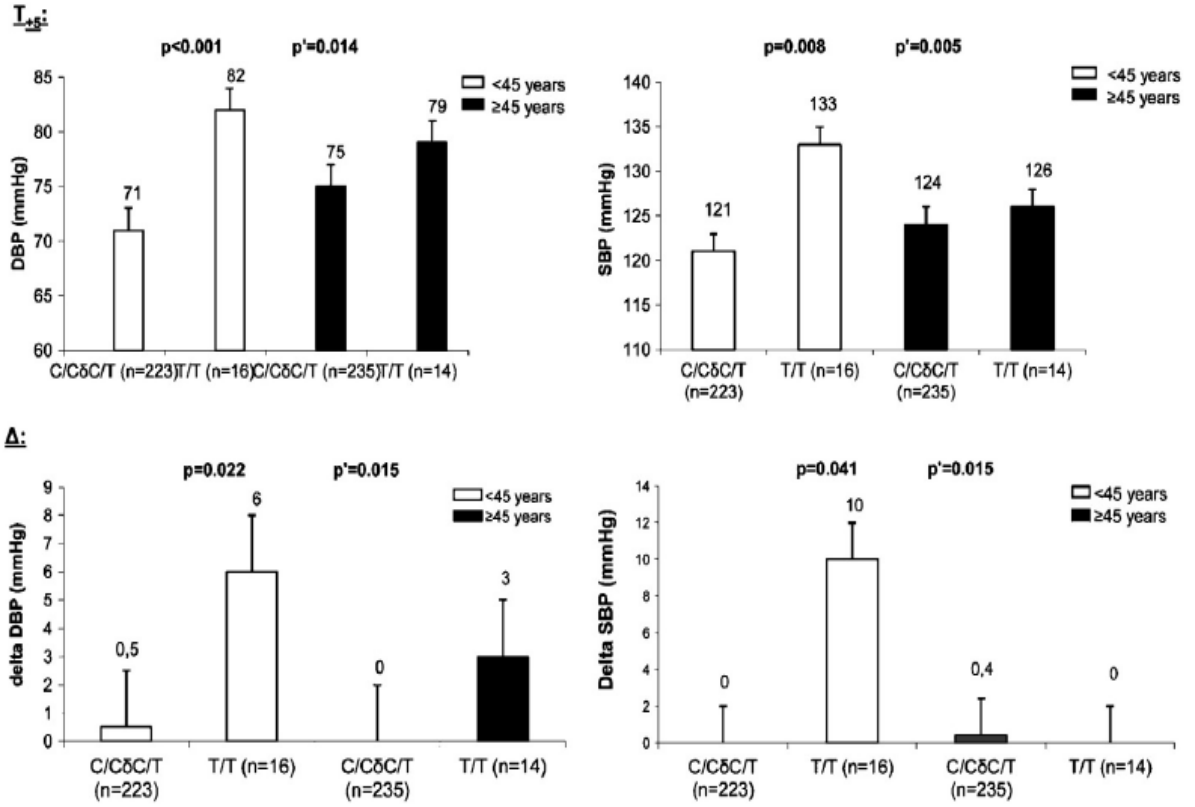

Fig. 1. Association between *FPR1* C32T SNP and blood pressure in healthy individuals according to age. Values are arithmetic means ± SD for clinical characteristics. Crude values were analyzed. p values correspond to the difference in BP levels among DBP, SBP and PP in normotensives classified according to 45 years age (median age) and *FPR1* genotypes. p = BP(T/T − C/C δ C/T) < 45 years vs. BP(T/T − C/C δ C/T) ≥ 45 years, p' = Interaction *FPR1* C32T SNP* age. DBP: Diastolic Blood Pressure, SBP: Systolic Blood Pressure, Δ: $T_{+5} - T_{+0}$.

Thus, an 11 vs. 4 mm Hg ($p<0.001$) and 12 vs. 2 mm Hg ($p=0.008$) increase for DBP and SBP respectively. Similarly, for ΔDBP and ΔSBP, 5.5 vs. 3 mm Hg ($p=0.022$) and 10 vs. 0 mm Hg ($p=0.041$) respective increases were found in individuals having age <45 years as compared to the older ones.

The present findings point in the same direction of previous candidate gene studies revealing the importance of gene–age interaction in increasing the cardiovascular risk (CVR) at younger age, for instance *Angiotensin converting enzyme* (*ACE*) and *ApoE* [30,31]. Among a large sample of 6714 participants, only younger smokers carrying *ACE D/D* genotype had an increased risk for mortality [30]. Similarly, *ApoE ε4/ε3* genotype was strongly related in younger men and women with the lipid metabolism [31]. Together, both studies with ours point to a quite important gene–age influence on CVR.

When comparing the association between *FPR1* C32T SNP and BP in healthy with hypertensive individuals, nonconclusive results were found in terms of relationship with HTN. While a significant association with BP was found in healthy individuals, similar allele frequencies in hypertensive compared to healthy individuals were found. This result could be explained by the fact that hypertensive individuals are not healthy; they have higher mean BMI and BP levels than healthy ones. Moreover, despite our efforts to approach the age of hypertensive individuals, healthy ones were younger than hypertensives. Finally, it is important also to underline that even if we did not observe an interaction with BMI, healthy and overweight individuals carrying T/T genotype had 5 vs. 3.8 mm Hg in ΔDBP and ΔSBP ($p=0.017$ and $p=0.036$ respectively) as compared to non-obese individuals.

Discrepancies in results were previously reported in BP regulation and HTN genetic studies even for the well known association between *ACE I/D* SNP and BP [6,32]. An explanation for the above discrepancies could be the fact that the effect of genetic polymorphisms varies with time, as do their interactions with environmental factors. These observations represent the major obstacles especially for case–control studies including subjects aged over 45 years, the age in which genetic contribution starts to decrease and that of environment to increase, making difficult to identify prognostic genetic markers.

4.1. Strengths and limitations

Our results are strengthened by several facts: (1) both healthy and hypertensive individuals are ethnically homogenous; therefore the observed associations are not due to population admixture and stratification. (2) Significant associations persisted for DBP and SBP after adjustment for confounding BP covariates such as sex, waist circumference and BMI (data available on demand).

Finally, some limitations should be mentioned: (1) As described previously, since FPR1 is a G protein-coupled receptor, we could not quantify its FPR1 plasmatic levels and therefore it is difficult to interpret the observed relationship and its biological significance. To the best of our knowledge, no data is available to date concerning the functional relevance of *FPR1* C32T SNP. (2) The significant associations rely on a small number of individuals carrying T/T genotype; larger samples are required to confirm these findings. (3) A possible linkage disequilibrium between *FPR1* C32T SNP and another genetic variant could not be excluded.

4.2. Conclusion and perspectives

FPR1 C32T SNP is associated with increased BP levels, especially in healthy individuals having <45 years. Additional correlation evidence

in larger samples and association studies across ethnic populations may be feasible in the near future. If confirmed in other studies, our findings suggest that this SNP and its interaction with age may be useful in BP regulation studies.

Supplementary materials related to this article can be found online at doi:10.1016/j.cca.2010.11.038.

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VI. Etude de la relation de l'allèle KL-VS du gène *KLOTHO* avec les niveaux de pression artérielle:

Klotho KL-VS genotype is involved in blood pressure regulation.

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Chez l'Homme, le gène *Klotho* subit un mécanisme d'épissage alternatif pour produire 2 isoformes protéiques (transmembranaire et secrétée) impliquées dans la régulation de la voie de signalisation de l'insuline, et dans le processus de vieillissement. *Klotho* est présent en une seule copie de 50Kb de longueur sur le bras long du chromosome 13 (13q12), une région génomique connue pour son association avec le risque cardiovasculaire (RCV) dans de nombreuses études de liaison génétique. L'allèle KL-VS du gène *Klotho* est caractérisé par 3 polymorphismes génétiques présents dans une région de 800pb, couvrant le deuxième exon et la séquence adjacente. Parmi eux, deux sont codants et résultent d'une substitution d'acides aminés, Phe352Val (rs9536314) et Cys370Ser (rs9527025) et 1 silencieux (rs9527026). Ces polymorphismes influencent le métabolisme de la protéine Klotho.L' allèle (KL-VS) a été décrit dans des populations non-européennes, comme étant associé aux facteurs de RCV , l'HTA, la consommation de cigarettes, les dyslipidémies, les diabètes, et l'obésité. L'ensemble de ces observations nous a mené à étudier le rôle que joue cet allèle dans le syndrome métabolique et son association avec l'HTA chez des individus français. Un deuxième objectif était d'étudier sa relation avec le traitement antihypertenseur et les paramètres de la rigidité artérielle et de l'artère carotide.

Nous avons génotypé un total de 1,023 adultes pour l'allèle KL-VS par PCR-RFLP. Les participants faisaient partie des deux cohortes françaises ERA et STANISLAS.

L'IMC, le tour de taille, le profil lipidique, la glycémie et les autres facteurs de RCV (hypercholestérolémie, diabète, HTA et syndrome métabolique) ne différaient pas significativement selon l'allèle KL-VS. En revanche, la PAS et la pression pulsée (PP) étaient significativement associées au génotype KL-VS/KL-VS (P=0,003 et P<0,001 pour la PAS et PP, respectivement) chez les hommes et les femmes. Les sujets ayant un génotype KL-VS/KL-VS avaient une PAS diminuée d'environ 8 et 11 mmHg. De même pour la PP, les sujets homozygotes KL-VS présentaient des valeurs inférieures de 9 et 8 mmHg par rapport aux homozygotes sauvages et aux hétérozygotes respectivement. Aucune association significative entre la PAD et le génotype KL-VS/KL-VS n'a été observée (P=0,603). De même, aucune interaction entre l'allèle KL-VS et le sexe n'était statistiquement significative. Par contre, l'allèle KL-VS interagit avec le traitement antihypertenseur pour influencer la PAS et la PP. A titre d'exemple, la différence de PP entre homozygotes sauvages et le génotype KL-VS/KL-VS était plus élevée chez les patients prenant des médicaments antihypertenseurs par rapport aux sujets non traités: 16,2 mmHg contre 4,6 mmHg (P=0,019 pour interaction).

En conclusion, nous avons montré que le génotype KL-VS/KL-VS est associé avec une baisse des niveaux de PAS et de PP et interagit avec un traitement antihypertenseur afin de diminuer la PA. Cette découverte pourrait conduire à identifier des sous-groupes d'adultes HT qui pourraient bénéficier d'un traitement antihypertenseur personnalisé.

Mots clés : Hypertension artérielle/ génétique, maladies cardiovasculaires, Allèle KL-VS, traitement antihypertenseur, polymorphisme génétique ponctuel.

Contribution : *Nous avons participé au design de l'étude. Nous avons pris en charge les expériences de génotypage. Nous avons également rédigé la première version du manuscrit.*

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Klotho KL-VS genotype is involved in blood pressure regulation☆,☆☆

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ABSTRACT

Background and aims: Genome-wide linkage analysis studies reported the importance of the long arm of chromosome 13 in systolic blood pressure regulation. Therefore, isolating a genetic variant related to this chromosomal region could be challenging. *Klotho* KL-VS allele is located on this chromosomal region and its relationships with cardio-vascular risk factors need extensive investigations. The aim of the present study is to examine whether the *klotho* KL-VS genotype is associated with cardio-vascular risk factors, more particularly hypertension, in two independent cohorts. A secondary objective was to investigate relationships with antihypertensive treatment, arterial stiffness and carotid artery parameters.

Methods and results: A total of 1023 French individuals were genotyped for *klotho* KL-VS. Participants were part of the French ERA and STANISLAS cohorts. In both cohorts, *klotho* KL-VS/KL-VS genotype was significantly associated with lower systolic blood pressure and pulse pressure when compared to homozygous and heterozygous more frequent (WT) allele carriers ($p=0.003$ and $p<0.001$ respectively). Antihypertensive treatment stratification confirmed the previous significant associations, while a significant interaction between *klotho* KL-VS genotype and antihypertensive treatment was also interestingly found (0.019 for p interaction).

Conclusion: *Klotho* KL-VS/KL-VS genotype may be associated with decreased cardio-vascular risk and may interact with antihypertensive treatment in order to reduce blood pressure. This finding could lead to identify subgroups of hypertensive adults who might benefit antihypertensive drug therapies.

1. Introduction

Multiple genetic determinants and environmental factors contribute to generate blood pressure (BP) final phenotype. Existing evidence suggests that genetic contribution is about 30 to 50% [1]. In agreement with this observation, linkage or association analysis has consistently shown that this pathology is not due to a single genetic variant [2]. Several studies have reported that polymorphisms of a number of candidate genes may influence BP [3]. Studies have focused on the molecular genetics of renin–angiotensin system to improve our understanding of this pathology [4].

With the completion of human genome project, researchers are having a growing enthusiasm for applying genome-wide mapping to determine susceptibility loci to BP [4,5]. This led investigators to map human BP loci using several linkage analysis studies. Moreover, genome-wide linkage analysis studies reported the importance of the long arm of chromosome 13 (13q) to increase CV risk via its association with postural systolic BP change, stroke and abnormal cardiac development [6–8]. Therefore, isolating a genetic variant related to this chromosomal region could be challenging.

Klotho is a 50Kb single copy gene located on chromosome 13q12 [9]. It encodes 350 amino acid protein with 86% of similarity with mice [9]. Subsequent human studies have identified a functional variant of *klotho* gene (MIM 604824), termed "KL-VS". This variant is prevalent in the general population with a frequency of 15.7%. It is characterized by six SNPs clustering 800-bp region, spanning the second exon and its flanking sequence. Of the three variations in exon 2, one is silent (rs9527026), and two code for amino acid substitutions, F352V (rs9536314) and C370S (rs9527025). The latter 2 SNPs were demonstrated to influence Klotho metabolism [10].

Several studies showed that KL-VS allele is increasing the CV risk [11,12]. However, these studies are relatively small, in terms of sample size and require replication. Therefore, the aim of the present study is to examine whether the *klotho* KL-VS genotype may influence CV risk, more particularly BP levels, in two independent cohorts of French origin. A secondary objective was to investigate relationships with antihypertensive treatment, arterial stiffness and carotid artery parameters.

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2. Materiels and methods

Appropriate institutional review board approval and Helsinki principles were followed for all human experimental investigations. In addition, each individual gave written informed consent to participate in this study.

2.1. Population samples

The STANISLAS cohort: a family based study, with a 10-years longitudinal follow-up. It was conducted since 1994 on 1006 families who were free of chronic or acute disease selected at the Center for Preventive Medicine of Vandoeuvre-lès-Nancy (east of France) [13]. In the present investigation, we present data from the second examination (1998–2000) obtained from a random sub-sample of 516 middle-aged men (mean age 46.9 ± 4.9 years; aged between 35 and 65 years). The research protocol was approved by the "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine" and each individual has given a written informed consent.

The ERA cohort: Individuals participating in the ERA (Evolution de la Rigidité Artérielle) study were selected from a Parisian cohort that had a health checkup at the IPC (Investigations Préventives et Cliniques) center, which is one of the medical centers of the French national health care system (Securité Sociale-CNAM). The details of this study have been presented previously [14]. The study population was composed of 345 men and 162 women aged between 25 and 88 years (mean age 57.5 ± 10 years for men individuals, and 59.45 ± 11.3 years for women individuals), who had a health checkup (1998–1999) at the IPC Center. All individuals signed an informed consent and the study protocol was approved by an ethics committee (Comité d'Ethique du Centre Hospitalier Universitaire de Cochin).

2.2. Biological and clinical parameters

Blood samples were collected after an overnight fast. Serum total cholesterol,HDL-cholesterol, triglyceride and glucose concentrations were measured by using commercially available kits (Merck, Darmstadt, Germany) on AU5021 apparatus (Olympus, Merck). Serum LDL cholesterol concentrations were estimated by using the Friedewald formula.

Participants answered a standardized questionnaire, which provided information about demographic background, occupation, medical history, personal habits, and drug use. Diabetes was defined as use of anti-diabetic agent or fasting glucose ≥ 7 mmol/l. Hypertension was defined as DBP ≥ 95 mm Hg or SBP ≥ 160 mm Hg. Hypercholesterolemia was defined as use of lipid lowering agent or cholesterol ≥ 6.5 mmol/l.

All individuals underwent a complete medical examination including weight and height measurements. Samples and data used for the STANISLAS cohort study are part of the Biological Resources Center (BRC) "Interactions Gène-Environnement en Physiopathologie CardioVasculaire" (IGE-PCV) in Nancy, France.

2.3. Blood pressure measurements

In both studied cohorts, blood pressure was measured with a manual sphygmomanometer under standardized conditions (room temperature comprises between 19 °C and 21 °C, the individual in the supine position). The values reported for systolic (SBP) and diastolic (DBP) blood pressure are the means of three readings at each examination. A part of investigated individuals were normotensive (40.9% of ERA cohort, and 12% for the STANISLAS cohort), and the others were hypertensive on antihypertensive medications.

45% of hypertensive individuals in ERA cohort were taking multitherapy. The distribution of drug classes was: diuretics (39%), beta blockers (37%), IEC (40%), ICal (20%) and other (17%). In the STANISLAS cohort, about 25% of treated hypertensive individuals were taking multitherapy, and the most used therapy was, as in the ERA cohort, IEC (38.7%).

2.4. Pulse wave velocity

Pulse wave velocity (PWV) measurements were available only for ERA cohort and were determined as the foot-to-foot velocity. The foot of the pressure wave was identified as the beginning point of the sharp systolic upstroke. Pulse transit time was determined as the average of 10 consecutive beats. The distance traveled by the pulse wave was measured over the body surface as the distance between the two recording sites. Aortic PWV was calculated as the ratio of distance to transit time with an automatic device (Complior, Colson) [14].

2.5. Ultrasonography

Ultrasound examinations were available only for ERA cohort and were performed by two trained ultrasonographers using the Aloka SSD-650, with a transducer frequency of 7.5 MHz. Acquisition, processing and storage of B-mode images were computer-assisted with of a software's version previously described (M'ATHS, Metris, France) [15].

The protocol involved scanning of the common carotid arteries (CCAs), the carotid bifurcations, and the origin (first 2 cm) of the internal carotid arteries. At the time of the examination, the near and far wall of these arterial segments were scanned longitudinally and transversally to assess the presence of plaques. The presence of plaques was defined as localized echo structures encroaching into the vessel lumen for which the distance between the media-adventitia interface and the internal side of the lesion was ≥ 1 mm. For intima-media thickness (IMT) and lumen diameter measurements, near and far walls of the right and the left CCAs 2 to 3 cm proximal to bifurcation, were imaged. In patients with carotid artery plaques, IMT measurements were realized in plaque-free segments of the CCAs. Details of the methodology used have been previously described [15].

2.6. Genotyping klotho for KL-VS polymorphism

Genomic DNA was isolated from peripheral blood cells using a salting-out method [16]. In order to define the basic population genetics of the KL-VS allele, we optimized a genotyping method based on PCR amplification of a specific polymorphic region of the of the *Klotho* gene followed by restriction enzyme digestion (PCR-RFLP). We used the primers 5′-AGGCTCATGCCAAAGTCTGG-3′ (forward) and 5′-GTTTCCATGATGAACTTTTTGAGG-3′ (reverse). Primers were designed form the genomic sequence of Klotho found in GENBANK (Gene ID: 9365). All PCR reactions 200 ng of genomic DNA in a final volume of 50 µl containing 1.5 mM $MgCl_2$, 100 µM dNTPs, 0.5 µM of each primer and 1U of Taq DNA polymerase (Roche Diagnostic, Basel, Switzerland) with 1X Buffer. DNA was initially denatured at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 seconds (s), annealing was carried out at 60 °C for 30 s and extension at 72 °C for 30 s. A final extension at 72 °C for 10 min was then performed. RFLP analysis of the amplified product was performed by digestion of the PCR product for 2 h at 55 °C with *Mae*III restriction enzyme (Roche Diagnostic, Basel, Switzerland). Digestion products were then analyzed on a 10% polyacrylamide gel. The KL-VS allele is characterized by diagnostic *Mae*III restriction fragments of 285-bp and 165-bp. Blinded replication of the fragment analysis was performed with 10% of the selected samples on an automated high-performance liquid chromatography instrument (Wave system™; Transgenomics Inc. [NC, USA], both genotyping analyses fully matched.

2.7. Statistical methods

Statistical analyses were performed by using the SAS software package version 9.13 (SAS Institute, Inc., Cary, NC). Triglyceride concentrations were log10-transformed in the analyses in order to improve normality. A chi-square test was performed to determine whether genotype frequencies were in Hardy–Weinberg equilibrium. For continuous variables, an analysis of variance was performed for characteristic differences among the 3 groups of population (men and women from ERA, and men from STANISLAS). When the analysis of variance was significant, a Student–Newman–Keuls test was used to detect which groups were statistically different from each other. The significance of differences among the 3 groups of population for the categorical variables was analyzed by using the chi-square test or the Fisher's exact. Likewise, an analysis of variance was performed for quantitative trait differences among the 3 groups of genotype (WT/WT, WT/KL-VS and KL-VS/KL-VS). When population groups were matched, group*genotype interaction was checked and data were adjusted for groups (ERA or STANISLAS, men or women). The threshold for statistical significance is set at $p<0.05$.

3. Results

Table 1 shows characteristics and *klotho* KL-VS polymorphism for individuals selected form ERA and STANISLAS cohorts (men and women from ERA and men from STANISLAS). When comparing both cohorts, BP parameters including DBP, SBP and PP were higher in individuals from ERA than from STANISLAS ($p<0.001$). As it would be expected, higher values for BMI, waist circumference, total cholesterol, triglycerides and fasting glucose were observed for individuals selected from ERA ($p<0.001$); this is mainly a consequence of the presence of older individuals in ERA. Based on these findings, it is obvious that CV risk such as hypercholesterolemia, diabetes, arterial hypertension and metabolic syndrome were more pronounced in ERA population than in STANISLAS. While genotype and allele frequency are consistent with Hardy–Weinberg equilibrium, similar distribution of KL-VS genotypic and allelic frequency, without significant difference, was observed in both cohorts ($p=0.702$ and $p=0.473$, respectively).

Analysis of sex and age adjusted variance indicates that BMI, waist circumference, lipid profile, glucose levels and CV risk factors (hypercholesterolemia, diabetes, hypertension and metabolic syndrome) did not significantly differ according to *klotho* KL-VS genotype (data not shown). In contrast, SBP and PP were significantly associated with *klotho* KL-VS/KL-VS genotype ($p=0.003$ and $p<0.001$ for SBP and PP, respectively) in both men and women (Table 2). Individuals with KL-VS/KL-VS genotype had about 8 and 11 mmHg less in SBP for men and women respectively. Similarly for PP, individuals being homozygous for *klotho* KL-VS allele present lower levels of 9 and 8 mmHg for men and women respectively when compared to heterozygous and WT homozygous. No significant associations for DBP with *klotho* KL-VS/KL-VS genotype were observed ($p=0.603$). As shown in Table 2, interaction term for sex * *klotho* KL-VS genotype was not statistically significant. Therefore, in the following step we regrouped men and women for the analyses.

Table 3 displays relationships between blood pressure and *klotho* KL-VS genotype taking into account the use of antihypertensive drugs. Antihypertensive treatment stratification confirmed the previous significant associations between SBP, PP and *klotho* KL-VS genotype, while a significant interaction between *klotho* KL-VS genotype and antihypertensive treatment was found for PP. As shown in Table 3, difference in PP between wt/wt and KL-VS/KL-VS genotypes was

Table 1
Blood pressure, KL-VS genotype and other characteristics of studied individuals from ERA and STANISLAS cohorts.

	ERA		STANISLAS	P
	Women	Men	Men	ANOVA
N	213	416	516	
Age (years)	60.1 ± 11.9^a	58.1 ± 10.2^b	46.9 ± 4.9^c	<0.001
Blood pressure				
Diastolic blood pressure (mm Hg)	82.8 ± 9.3^a	88.3 ± 10.7^b	77.0 ± 10.5^c	<0.001
Systolic blood pressure (mm Hg)	140.9 ± 21.9^a	142.6 ± 19.1^a	127.8 ± 13.1^b	<0.001
Pulse pressure (mm Hg)	58.2 ± 18.4^a	54.3 ± 14.6^b	50.8 ± 9.5^c	<0.001
Hypertension (%)[1]	52.5^a	58.2^b	12.0^c	<0.001
KL-VS genotype				
wt/wt (%)	67.6	70.2	72.1	0.702
wt/KL-VS (%)	28.6	26.7	25.6	
KL-VS/KL-VS (%)	3.8	3.1	2.3	
KL-VS allelic frequency (%)	18.1	16.5	15.1	0.473
Other characteristics				
BMI (kg/m^2)	25.6 ± 4.4^a	27.3 ± 4.2^b	26.1 ± 3.3^b	<0.001
Waist circumference (cm)	80.7 ± 11.4^a	95.4 ± 10.9^b	90.6 ± 9.1^c	<0.001
Fasting glucose (mmol/l)	5.34 ± 0.68^a	5.73 ± 1.22^b	5.33 ± 0.84^a	<0.001
Diabetes (%)[2]	5.2	6.9	2.9	0.020
Total cholesterol (mmol/l)	6.21 ± 0.91^a	5.86 ± 1.00^b	5.94 ± 1.05^b	<0.001
Hypercholesterolemia (%)[3]	49.5^a	42.6^a	34.9^b	<0.001
Triglyceride (mmol/l)	1.06 ± 0.61^a	1.44 ± 1.02^b	1.43 ± 0.94^b	<0.001
HDL-cholesterol (mmol/l)	1.91 ± 0.47^a	1.44 ± 0.38^b	1.42 ± 0.39^b	<0.001
LDL-cholesterol (mmol/l)	3.87 ± 0.93	3.76 ± 0.88	3.91 ± 1.02	0.073
Metabolic syndrome (IDF %)	24.7^a	39.1^b	18.6^a	<0.001

Values are arithmetic mean ± SD or proportion (%).
Values of triglyceride were log10 transformed prior ANOVA.
P values were for the ANOVA or Chi2 across the 3 groups.
[a,b,c] Means in the same row not sharing a common superscript letter are significantly different: $P<0.05$ (Student–Newman–Keuls test).
[1] DBP ≥ 95 mm Hg or SBP ≥ 160 mm Hg or antihypertensive treatment.
[2] Blood glucose ≥ 7 mmol/l (1.26 g/l) or diabetes treatment.
[3] Cholesterol level ≥ 6.5 mmol/l (2.50 g/l) or lipid-lowering treatment.
For BMI, waist circumference, fasting glucose, diabetes, total cholesterol, hypercholesterolemia and triglycerides: N = 192, 378 and 516 for the 3 groups (women ERA, men ERA and men STANISALS), respectively.
For HDL-cholesterol and metabolic syndrome frequency: N = 162, 345 and 516 for the 3 groups (women ERA, men ERA and men STANISLAS), respectively.
For LDL-cholesterol: N = 146, 330 and 486 for the 3 groups (women ERA, men ERA and men STANISLAS), respectively.

Table 2
Klotho KL-VS genotype and blood pressure in men and women.

	WT/WT		WT/KL-VS		KL-VS/KL-VS		P genotype	P Sex	P sex * genotype interaction
	Men	Women	Men	Women	Men	Women			
N	664	144	243	61	25	8			
Age (years)	51.6 ± 9.2	59.7 ± 10.8	52.5 ± 10.0	60.5 ± 11.3	53.9 ± 10.4	63.0 ± 9.4			
DBP (mmHg)	82.6 ± 12.4	81.1 ± 9.1	82.2 ± 10.9	79.6 ± 9.7	83.8 ± 10.0	78.1 ± 10.6	0.603	0.045	0.599
SBP (mmHg)	136.5 ± 17.7	136.5 ± 22.1	134.9 ± 18.1	131.7 ± 21.0	128.6 ± 10.6	125.6 ± 24.3	0.003	0.383	0.443
PP (mmHg)	53.8 ± 12.2	55.5 ± 19.1	52.7 ± 12.2	52.0 ± 17.1	44.8 ± 7.6	47.5 ± 16.1	<0.001	0.505	0.498

Values are age adjusted arithmetic mean ± SD, DBP: Diastolic Blood Pressure, SBP: Systolic Blood Pressure, PP: Pulse Pressure.
P for ANOVA after adjustment for age by using the whole group of men and women.

Table 3
Klotho KL-VS genotype and blood pressure according to use of antihypertensive agent in the whole group of men and women.

	WT/WT		WT/KL-VS		KL-VS/KL-VS		P genotype	P treatment	P treatment genotype interaction
	Untreated	Treated	Untreated	Treated	Untreated	Treated			
N	576	232	216	88	21	12			
Age (years)	50.3 ± 8.7	59.9 ± 9.6	51.3 ± 9.7	60.9 ± 10.1	51.6 ± 8.3	64.1 ± 10.3			
DBP (mmHg)	80.2 ± 12.4	85.2 ± 9.1	80.0 ± 10.9	83.9 ± 9.7	80.7 ± 10.0	84.7 ± 10.6	0.643	0.003	0.751
SBP (mmHg)	133.5 ± 17.7	142.6 ± 22.1	131.5 ± 18.1	140.7 ± 21.0	129.4 ± 10.6	125.7 ± 24.3	<0.001	0.021	0.085
PP (mmHg)	53.3 ± 12.2	57.3 ± 19.1	51.4 ± 12.2	56.8 ± 17.1	48.7 ± 7.6	41.1 ± 16.1	<0.001	0.713	0.019

Values are age and sex adjusted arithmetic mean ± SD, DBP: Diastolic Blood Pressure, SBP: Systolic Blood Pressure, PP: Pulse Pressure.
P for ANOVA after adjustment for age and sex by using the whole group of men and women.

higher in patients taking antihypertensive drugs in comparison to untreated individuals: 16.2 vs. 4.6 mm Hg (p for interaction term = 0.019). By using a recessive model of inheritance, interaction term for *klotho* KL-VS genotype and antihypertensive treatment was significant for SBP and PP (p = 0.027 and p = 0.007, respectively). Difference in SBP between the individuals carrying the wt allele and the homozygous KL-VS individuals was higher in patients taking antihypertensive drugs in comparison to untreated individuals: 16.3 vs. 3.5 mm Hg. For PP similar results as compared to categorical model were found.

We also investigated an eventual association between *klotho* KL-VS genotype, arterial stiffness and carotid artery parameters in ERA cohort. The analysis of PWV, IMT, media cross sectional area (MCSA), carotid radius/IMT, carotid diameter and presence of carotid plaques did not show any significant difference (Table 4).

4. Discussion

In the present study, we show that *klotho* KL-VS/KL-VS genotype is significantly associated with lower SBP and PP when compared to homozygous and heterozygous WT allele (p = 0.003 and p < 0.001 respectively). By comparison to Arking et al.[12] we had contradicting results, as they found a negative influence on SBP. Discrepancies between their study and ours could be referred to different ethnic origins. In fact, genetic variants found in Europeans can differ in their frequencies across populations [1,17]. Moreover, it is important to note that even among same ethnic populations difference can appear as environmental factors and lifestyles could be different [18].

The molecular mechanism by which KL-VS allele could modulate BP is still not clear. Animal experiments studies supported a role for *klotho* gene in protecting against endothelial dysfunction. Using rat models of atherosclerosis disease (OLETF), *klotho* gene delivery was demonstrated to ameliorate vascular endothelial function and reduce BP [19]. Based on this finding, we could suggest the hypotheses for a functional role for KL-VS allele in increasing klotho protein levels.

The fact that in the present study the *klotho* KL-VS/KL-VS genotype is significantly associated with lower SBP and PP suggests an implication of this genotype on arterial stiffness. Actually, it is well known that low SBP and low PP reflect in most of the cases low stiffness. Unfortunately, in the present study we had direct measurements of arterial stiffness only for the individuals of the ERA cohort but not for those of the STANISLAS cohort. No significant association was found between *klotho* KL-VS genotype, arterial stiffness and carotid artery parameters (Table 4). However, due to the small number of KL-VS/KL-VS genotype in the ERA study we cannot draw any solid conclusions about this question.

Our results were observed in 2 different sample populations with BP phenotype considered as a quantitative trait: (1) in young

Table 4
Klotho KL-VS genotype and arterial stiffness measurements in studied individuals from ERA cohort.

	WT/WT	WT/KL-VS	KL-VS/KL-VS	WT/WT	WT/KL-VS	KL-VS/KL-VS
	Men			Women		
N	292	111	13	144	61	8
PWV (m/s)	11.9 ± 2.6	11.8 ± 2.4	11.2 ± 2.7	10.9 ± 2.3	11.3 ± 2.4	11.7 ± 1.6
IMT (mm)	0.74 ± 0.11	0.76 ± 0.11	0.75 ± 0.11	0.73 ± 0.10	0.75 ± 0.12	0.80 ± 0.12
MCSA (mm^2)	15.6 ± 3.6	15.9 ± 3.5	16.0 ± 3.2	14.1 ± 2.9	14.2 ± 3.3	15.0 ± 3.1
Carotid Radius/IMT	4.2 ± 0.7	4.0 ± 0.6	4.1 ± 0.7	3.8 ± 0.5	3.8 ± 0.5	3.5 ± 0.4
Carotid Diameter (mm)	6.0 ± 0.7	6.0 ± 0.7	6.0 ± 0.7	5.4 ± 0.6	5.4 ± 0.6	5.3 ± 0.4
Carotid plaques (%)	33	39	45	17	25	0

Values are age adjusted arithmetic mean ± SD.
P for ANOVA after adjustment for age by using the whole group of men and women.
For Pulse wave velocity, intima media thickness, media cross-sectional area, R/H, diameter and carotid plaques, P value for the *Klotho* KL-VS genotype was >0.05.
PWV: pulse wave velocity, MCSA: media cross sectional area, IMT: intima-media thickness, Carotid Radius/IMT: carotid radius/intima-media thickness.

supposed healthy individuals (STANISLAS cohort) and (2) in older individuals from the ERA cohort. Associations were found in both young and older individuals, suggesting a continuous impact during lifetime or at least for the studied range. Our results are strengthened by several other facts: (1) both ERA and STANISLAS are ethnically homogenous cohorts; therefore the above significant associations are not due to population admixture and stratification. (2) Significant associations persisted for SBP and PP after adjustment for confounding BP covariates such as age and sex.

Our further investigations showed that this genotype was related to BP levels by interacting with antihypertensive treatment. Given the hypothesis that BP response to antihypertensive treatment is characterized by marked inter-individual variation, isolating candidate SNPs, has been a challenge [20]. While clinical trials are mainly used to directly assess BP responsiveness to therapies, they present limitations as they rarely take into account inter-individual response and consequently the complexity of BP.

Only few candidate genes have been identified to influence antihypertensive drugs response and analysis of their genetic variation has the potential to improve our understanding of antihypertensive drug determinants in order to individualize drug selection. To date, several studies have investigated associations between genetic variations and response to antihypertensive drugs. Some consistent results for differences in response to BP treatment are previously reported. For example, the adducin gene family is now considered as a candidate gene family to predict drug response [21–24]. A silent SNP (T>C, Ile (131)) in the *ADD1* gene was reported to influence BP response to antihypertensive drugs in order to modify individual response in essential hypertension [22]. In addition, rs1541582 SNP of *ADD2* gene was identified as a candidate genetic variation which interacts with beta blockers family in order to decrease the SBP [23]. Here we report that *klotho* gene could also be a potential candidate gene for pharmacogenomic studies.

Although of importance, our present study has some limitations: (1) even the important number of studied individuals (1,023 individuals), we reported a relative limited number of homozygous for KL-VS allele having low SBP, PP (33 individuals) and receiving antihypertensive treatment (12 individuals). It is a consequence of the low KL-VS genotype frequency (about 3%), therefore caution is need when dealing with the present results. (2) The second limitation is that our study has not a real pharmacogenetic design, as we did not study the same patients before and after treatment. However, the high significant interaction results we obtained for PP support our hypothesis even if we are conscious that it is essential to validate our findings in well-designed specific pharmacogenomic studies. (3) Unfortunately, it was almost impossible to isolate among antihypertensive drugs a specific class interacting the most with *klotho* KL-VS genotype.

5. Conclusion

Our results indicate that *klotho* KL-VS genotype was associated to lower CV risk. We have shown that *klotho* KL-VS genotype is associated with lower SBP and PP levels and interacts with antihypertensive treatment in order to decrease BP. This finding could lead to identify subgroups of hypertensive adults who might benefit antihypertensive drug therapies based on a personalized treatment.

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VII. Etude de la relation de l'haplotype CAT1/2 du gène *CAT* avec les niveaux de pression artérielle

Relationship between catalase rs769214 SNP and arterial aging.

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Atherosclerosis, Accepté avec modifications.

Bien que de nombreux facteurs de risque conventionnels aient été associés au développement du vieillissement artériel, les MCV demeurent la première cause de décès chez les personnes âgées. L'identification de nouveaux facteurs de risque pourrait s'avérer prometteuse pour la lutte contre ce problème majeur de santé publique. En effet, Le stress oxydatif et particulièrement la catalase (CAT), une enzyme antioxydante, joue un rôle important dans la physiopathologie de la cellule endothéliale en réponse au stress oxydant et par conséquent dans le vieillissement artériel.

Le but de cette étude était d'examiner les relations qui pourraient exister entre l'haplotype CAT1/2 du gène *CAT* et les phénotypes de vieillissement artériel (le diamètre interne de l'artère carotide, l'épaisseur de l'intima-média, la présence de plaques d'athérome) dans deux populations françaises (STANISLAS et ERA).

Afin de réaliser notre objectif, 624 individus français d'âge moyen (53±12 ans) ont été inclus dans l'étude. La PA, l'épaisseur de l'intima-média, le diamètre carotidien et le nombre de plaques d'athérome ont été mesurés. De plus, le génotypage du rs769214 du gène *CAT* (SNP représentant de l'haplotype) a été effectué par PCR en temps réel, avec la technique des sondes d'hybridations.

Aucune association entre l'haplotype CAT1/2 du gène *CAT* et les niveaux de PA n'a été observée. Les individus porteurs de l'haplotype CAT2 possédaient un diamètre carotidien moyen plus élevé avec l'âge (P=0,003) et/ou avec une PAS≥ 140 mmHg (P=0,018) par rapport aux porteurs de l'haplotype CAT1. Dans la population ERA, l'haplotype CAT2 était associé à un plus grand nombre de plaques d'athérome (P=0,011). L'haplotype CAT2 exerçait un effet

sur le vieillissement artériel même après ajustement à l'âge, la PAS, le sexe, la consommation de tabac, les taux de hs-CRP, l'IMC et le diabète. En outre, l'haplotype CAT2 pourrait être considéré comme facteur de risque indépendant du vieillissement artériel.

La présente étude a mis en évidence un haplotype du gène *CAT* qui pourrait influencer le vieillissement artériel. De même, l'ensemble des résultats a souligné l'impact bénéfique de cet haplotype sur le diamètre interne de l'artère carotide, et le nombre moyen de plaques d'athéromes.

Mots clés : Catalase, vieillissement artériel, hypertension, polymorphisme génétique ponctuel.

Contribution: Nous avons participé aux différentes réunions préparatoires concernant le design de l'étude, et à la rédaction de cet article.

Relationship between catalase rs769214 SNP and arterial aging

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Tables 4

Figures: 1

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Abstract

Background: Although many conventional factors have been associated with the development of arterial aging, cardiovascular diseases remain the first cause of death in old age. Therefore, identification of new risk factors may prove promising for monitoring this serious health problem. Oxidative stress and particularly catalase (CAT), an antioxidant enzyme, play an important role in endothelial cell pathophysiology, in shear stress response and ultimately in arterial aging.

Objective: Examine the relationships between CAT haplotypes and phenotypes of arterial aging (carotid artery internal diameter, intima-media thickness, presence of atheromatous plaques) in two French cohorts.

Methods and Resultats: 564 middle-aged French individuals (mean age 53±12 years) from two cohorts (ERA and STANISLAS cohorts) were included in the study. Blood pressure, intima-media thickness, carotid diameter and number of atheromatous plaques were measured. Catalase rs769214 SNP genotyping was performed. We identified a CAT haplotype that influences arterial aging. Individuals carrying the CAT2 haplotype had a higher mean carotid diameter with aging and/or with a SBP ≥ 140 mmHg and were associated with a greater number of atheromatous plaques than CAT1 haplotypes carriers. This CAT2 haplotype appeared as an independent risk factor of arterial aging, similarly to previously identified factors such as age, systolic blood pressure, sex, tobacco use, hs-CRP, BMI and diabetes.

Conclusion: The present study highlights the roles of CAT haplotypes in arterial aging and underlines the beneficial impact of the CAT1 haplotype on mean carotid diameter and atheromatous plaque number as well as on potential associated diseases.

Key words: atherosclerosis, carotid arteries, genetic polymorphism, blood pressure, ageing

1. **Introduction**

Arterial aging, characterized by arterio-atherosclerosis, involves a number of conventional risk factors [1]. Despite large scale epidemiological studies, nearly half the burden of atherosclerotic cardiovascular disease remains unexplained by these factors, although modification of risk factors (when possible) is associated with improved outcome [2].

Oxidative stress plays an important role in endothelial cell pathophysiology [3],[4] as well as in various aspects of the vascular response to stretch or shear stress [5],[6], and in vascular aging [7]. In the same manner, age-associated atherosclerosis has been induced in catalase activity-deficient mice [7]. In contrast, overexpression of catalase in a murine model leads to a delayed onset of atherosclerosis [8],[9], to lower blood pressure (BP) [4] and an increase in life span [10].

Catalase (*CAT*) is a 33.14Kb single copy gene located on chromosome 11p13. It encodes a 526-amino acid protein with 89% similarity with mice. Subsequent human studies have identified a haplotype of the *CAT* gene presenting different allelic frequencies according to ethnic origin [11]. This haplotype is characterized by three single-nucleotide polymorphisms (SNPs) [rs769214 (-844 G/A), rs7943316 (-89A/T) and rs1049982 (-20T/C)] clustering an 824-bp region spanning the catalase promoter region. The rs769214A allele has been reported to create a PAX6 binding site on the CAT gene promoter that modifies CAT transcription [12].

We have previously reported that the allele A of rs769214 in the *CAT* gene promoter is associated with a defect in renutrition of an elderly malnourished population because of an insulin-resistant state [12],[13], 21827848). In a longitudinal association study of a Japanese population, the same SNP was found to predict the risk of hypertension (HTN) progression [14]. Furthermore, an association was found between this SNP and essential HTN in Caucasian and Chinese populations, but not in African Americans [15],[16]. In a larger genetic study targeting a Caucasian population, T allele carriers of the rs1049982 presented a lower risk of HTN as well as lower blood pressure values [17]. Recently, Wang et al. demonstrated that subjects bearing the [-844A, -89T, -20C] haplotype presented a higher susceptibility to essential HTN and a precocious onset for HTN when compared with those carrying the [-844G, -89A, -20T] haplotype of the *CAT* gene [18].

Our hypothesis was therefore that the CAT haplotype may be implicated in the pace of age-related arterial alterations.

Objective: Examine the relationships between CAT haplotypes and phenotypes of arterial aging (carotid artery internal diameter, intima-media thickness, presence of atheromatous plaques) in two cohorts of middle-aged French individuals (ERA and STANISLAS cohorts) respectively recruited in two French centres (Paris and Nancy) performing regular health check-ups.

2. Methods

2.1 Study population

In the present investigation, data obtained from 564 middle-aged French individuals (mean age 53±12 years) are presented.

Among this population, 379 individuals were part of the ERA cohort: individuals participating in the ERA (Evolution de la Rigidité Artérielle) study were selected from a Parisian cohort that had a health check-up at the IPC (*Investigations Préventives et Cliniques*) centre, which is one of the medical centres of the French national health care system (Securité Sociale-CNAM). The details of this study have been presented previously [19]. In this cohort, 353 patients had been explored for carotid diameter measurements and 378 for carotid plaque number. All individuals signed an informed consent form and the study protocol was approved by the institution's ethics committee (*Comité d'Ethique du Centre Hospitalier Universitaire de Cochi*n).

The remaining 185 individuals were part of the STANISLAS cohort: a family based study, of which members were free of chronic or acute disease and were selected at the Centre for Preventive Medicine of Vandoeuvre-lès-Nancy (east of France) [20]. Individuals included in this study had been explored for carotid diameter measurements. The research protocol was approved by the "*Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine*" and each individual gave a written informed consent.

2.2 Clinical and biological data

All individuals underwent complete medical examination including weight and height measurements. Blood samples were collected according to the manufacturer's recommendations for assays. Plasma fasting glucose, serum total cholesterol, HDL-cholesterol, triglyceride and CRP high-sensitive concentrations were measured using commercially available kits (Merck, Darmstadt, Germany) on an AU5021 apparatus (Olympus, Merck).

2.3 BP measurements:

In the two cohorts, BP was measured with a manual sphygmomanometer under standardized conditions (room temperature between 19°C and 21°C and the individual in supine position). The values reported for systolic blood pressure (SBP) and diastolic blood pressure (DBP) are the means of three readings at each examination. Pulse pressure was calculated as the difference between DBP and SBP.

2.4 Intima-Media Thickness (IMT), carotid diameter and atheromatous plaque number measurements:

For the STANISLAS cohort, the ultrasound investigation was performed using a B-mode ultrasound imager (HITACHI EUB 565), Software lotec system (Data Processing Co., Paris, France). The right and left carotid arteries were examined with a 7.5 MHz probe, according to a standardized protocol. Measurements were taken at 3 cm proximal to the carotid bifurcations in the far wall and on a segment of at least 1 cm of the longitudinal length free from any atheromatous plaque. The average of the two right and the left measurements was used to calculate the IMT and the mean value of the two measurements was used for the analysis. All measurements were performed by the same physician throughout the study.

For the ERA cohort, ultrasound examinations were performed by 2 trained ultrasonographers using the Aloka SSD-650, with a transducer frequency of 7.5 MHz. Acquisition, processing and storage of B-mode images were computer-assisted with the new version of a software previously described (M'ATHS, Metris, France). The protocol involved scanning of the common carotid arteries, the carotid bifurcations, and the origin (first 2 cm) of the internal carotid arteries. At the time of the examination, the near and far walls of these arterial segments were scanned longitudinally and transversally to assess the presence of plaques. The presence of plaques was defined as localized echo-structures encroaching into the vessel lumen for which the distance between the media-adventitia interface and the internal side of the lesion was $\geq$1 mm. For intima-media thickness and lumen diameter measurements, near and far walls of the right and the left common carotid arteries 2 to 3 cm proximal to bifurcation were imaged. In patients with carotid artery plaques, intima-media thickness measurements were performed in plaque-free segments of the common carotid arteries. Details of the methodology used have been previously described [21].

2.5 Catalase genotyping

DNA extraction and catalase genotyping were performed as previously described [13]. In the present study, CAT1 haplotype included homozygous carriers of CATH1 [-844G, -89A, -20T], whereas CAT2 haplotype included heterozygous carriers (CATH1-CATH2) and CATH2 homozygous [-844A, -89T, -20C] [12].

2.6 Statistical analysis

Descriptive values are expressed as mean ± SD for the table. Significant differences were assessed using either the Wilcoxon Rank-Sum Test or chi-square (χ^2) test, as appropriate. When performing a chi-square (χ^2) test, we verified that rs769214 genotypes and allele frequencies were consistent with the Hardy-Weinberg equilibrium.

According to the univariate analyses (Pearson correlation), the variables associated at P <0.05 in carotid diameter or carotid plaque number were subsequently included in the multivariate analysis. The interaction between CAT haplotypes and age on SBP or carotid diameter was tested with the multivariate model by including the interaction term in the model, followed by the Tukey-Kramer multiple-test for assessing the influence of classes of age and SBP. The threshold for statistical significance was set at P<0.05. Statistical analyses were performed using the NCSS software.

3. Results

3.1 Anthropometric, biological and arterial parameters at inclusion in the entire study population and according to catalase haplotypes

The mean age of the study population was 53 years. Glucose, cholesterol, HDL-cholesterol, triglycerides and hs-CRP concentrations as well as SBP and DBP values are reported in Table 1. Mean carotid IMT and mean carotid diameter were respectively 0.69 ± 0.11 mm and 5.66 ± 0.72mm. Individuals with CAT1 haplotype were not different from those carrying the CAT2 haplotype with regard to anthropometric (weight, BMI) and biological parameters (glucose, cholesterol, HDL-cholesterol, triglycerides, hs-CRP). In addition, *CAT* haplotype was not associated with mean carotid IMT and diameter at inclusion (P=0.54 and 0.80 respectively).

3.2 Mean carotid diameter according to age classes and catalase haplotypes

Mean carotid diameter was compared between individuals according to *CAT* haplotype and three age classes (Figure 1A). Mean carotid diameter did not differ according to age (p=0.12, Figure 1) but was dependent on *CAT* haplotype (p=0.004, Figure 1). *CAT* haplotype interacted with age by influencing mean carotid diameter (p=0.003), notably showing an increase in mean carotid diameter among older *CAT2* haplotype carriers (age≥58 years). In contrast, individuals carrying the *CAT1* haplotype did show any significant difference in mean carotid diameter (Figure 1A). These differences persisted after adjustment for sex, SBP, hs-CRP and BMI.

3.3 Mean carotid diameter according to SBP classes and catalase haplotypes

Mean carotid diameter was also compared beween individuals according to *CAT* haplotype and two SBP classes (Figure 1B). Mean carotid diameter did not differ according to SBP levels (p=0.41) but was dependent on catalase haplotype (p=0.02). *CAT* haplotype interacted with SBP in influencing mean carotid diameter (p=0.018), revealing that individuals with SBP levels >140 mmHg and carrying the CAT2 haplotype had a higher mean carotid diameter when compared with those having normal SBP levels. These differences persisted after adjustment for age, gender, hs-CRP and BMI.

These date thereby indicate that elevated age and high blood pressure may lead to higher mean carotid diameter in *CAT2* haplotype carriers.

3.4 Multivariate regression analysis of mean carotid diameter

A univariate regression analysis assessing mean carotid diameter was first performed prior to conducting the multivariate analysis. Only age, sex, BMI, SBP and hs-CRP parameters were statistically correlated (P<0.05) with mean carotid diameter (data not shown).

A step by step multivariate regression analysis which included catalase haplotype*age interaction, catalase haplotype and the significant parameters of the univariate regression analysis led to the model presented in Table 2. This model explained 42% of mean carotid diameter.

3.5 Carotid plaques at inclusion in the ERA population and according to catalase haplotypes.

We also investigated an eventual association between CAT2 haplotype and the presence and number of carotid plaques in the ERA cohort (mean age= 58 ± 10). The CAT2 haplotype was associated with an increased presence and number of carotid plaques (P=0.015 and P=0.011 respectively, Table 3) whereby ERA individuals carrying the CAT2 haplotype had twice as many carotid plaques as CAT1 haplotype carriers (Table3).

3.6 Univariate and multivariate regression analysis of carotid plaque number

A univariate regression analysis was conducted with regard to the number of carotid plaques. Age, sex, history of high cholesterol, tobacco use and catalase haplotypes were statistically correlated with carotid plaque number (data not shown).

A step by step multivariate regression analysis which included the significant parameters in the univariate regression analysis led to the model presented in Table 4.This model showed that CAT2 was an independent risk factor of carotid plaque number and explained 14 % of their number.

4. Discussion

In the present study, we identified that, with aging, individuals carrying the CAT2 haplotype had a higher mean carotid diameter than CAT1 haplotype carriers. In addition, individuals carrying the CAT2 haplotype had a higher frequency and greater number of atheromatous plaques. These data suggest that the CAT2 haplotype influences the pace of arterial aging.

The implication of catalase in oxidative stress and vascular alterations has already been reported [3]. In the present investigation, our hypothesis particularly addressed the implication of the catalase rs769214 SNP in vascular aging. The study population presented "normal" levels of serum glucose, cholesterol, HDL-cholesterol and triglycerides with very low hs-CRP levels showing that this 53-year-old population appeared not to be at high cardiovascular risk. However, the BMI values were relatively higher than 25 Kg/m^2 showing a population growing increasingly corpulent with aging, a phenomenon also observed worldwide and likely linked to changes in lifestyle and diet. This finding only pertained to the mean BMI value since both mean SBP and DBP values were in keeping with French guideline levels [22]. Mean carotid IMT was also higher than 0.6 mm although this population was older than 50 years old. Considering the value of the mean carotid IMT observed, our population would be classified at low risk for myocardial infarction and stroke [23]. However, mean carotid diameter observed in our population was lower from the mean carotid diameter reported for the entire French population as a whole, which is established at about 6.5 mm: this is likely consequent to the atherosclerotic process which increases with aging [1]. No difference was observed in terms of biological, anthropometric or functional cardiovascular parameters between the two haplotypes. However, we did explore the link between CAT2 haplotype and an indicator of vascular aging, namely mean carotid artery diameter, in three age classes and two classes of SBP. While mean carotid diameter did not differ according to age and SBP, it did vary however according to catalase haplotype ($p=0.0037$). Indeed, the interaction analysis between age and catalase haplotype revealed that older individuals (age≥58 years) carrying the CAT2 haplotype had a higher mean carotid diameter when compared with younger individuals (≥45 age <58 and age <45 years). In contrast, individuals carrying the CAT1 haplotype did not show any significant changes in their mean carotid diameter. In addition, the interaction between catalase haplotype and SBP classes was statistically significant demonstrating that carriers of the CAT2 haplotype and presenting a SBP ≥140 mmHg showed a higher mean carotid diameter than those with lower SBP levels even after age adjustment. Furthermore, under multivariate analysis, CAT2 haplotype was found to be an independent risk factor for mean carotid diameter.

To complete this analysis, an additional indicator of arterial aging was assessed in our study, namely carotid plaque number. Herein, both the presence and number of carotid plaques were higher in

CAT2 haplotype carriers. In addition, multivariate regression analysis led to identify CAT2 haplotype as an independent risk factor of carotid plaques, as was age, sex, hypertension, diabetes, high cholesterol history and tobacco smoking. Appearance of these carotid plaques is the result of a slow, evolving process over a period of many years. Hence, the above data would suggest that CAT2 haplotype may induce a faster development of age-related arterial alterations (e.g. increase in carotid diameter and plaque number). The present findings also support previous data underlining the main role of the promoter of the catalase gene in HTA and in the aging process. Nevertheless, this is the first study to establish a direct link between CAT haplotypes, HTA and arterial aging. CAT haplotypes have recently been associated with different promoter catalase activities [12],[18]. According to these latter studies, CATH2 promoter activity, which is associated with a greater catalase activity, did not change upon appearance of oxidative stress conditions. By contrast, CATH1 promoter showed a higher transcriptional activity under high oxidative stress conditions thus conferring a potential adaptive mechanism [11].

Vascular aging and HTN are associated with an increase in reactive species as suggested by numerous data [7],[24]. Under these conditions of accumulated reactive species, we hypothesize that CATH1/CATH1 patients (CAT1 in this study) may have protection compared to CATH2/CATH2 or CATH1/CATH2 patients (CAT2 in this study), probably by a potential adaptation with an increase in catalase activity in extreme conditions [11],[18]. This protection could delay the onset, of arterial aging as reported both in the present study and previously described in a murine model [8],[9]. This phenomenon may also delay the development of hypertension as observed herein. This study thus highlights the role of haplotypes in arterial aging via the adaptation to lifestyle modifications. It establishes the beneficial impact of the CAT1 haplotype on both mean carotid diameter and on atheromatous plaque number, along with a likely favourable impact on associated diseases such as metabolic disorders.

4.1 Strengths and limitations

Our results are strengthened by several facts: (1) This is the first investigation to show that *CAT* haplotype interacts with age and SBP in influencing the increase in mean carotid diameter, a typical manifestation of arterial aging [1]. In addition, this haplotype was associated with increased carotid plaque abundance. (2) Study individuals were ethnically homogenous (French individuals); therefore the observed associations are not due to population admixture and stratification. (3) Significant associations persisted after adjustment for confounding covariates such as age, sex, hs-CRP, BMI and tobacco use.

The main limitations of this study are its cross-sectional character and the relatively limited number of phenotypes of arterial aging.

4.2 Perspectives

Additional association evidence in larger and older population samples as well as association studies across other ethnic populations may be feasible in the near future. If confirmed in other studies, our findings suggest that this *CAT* haplotype and its interaction could be useful in human vascular aging studies.

Acknowledgments

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Table 1:

Anthropometric, biological and arterial parameters according to CAT haplotypes

	All	CAT1 haplotype	CAT2 haplotype
N	538	62 (12%)	476 (88%)
Age (years)	52 ± 12	53 ± 12	52 ± 12
Women (%)	37	39	37
Height (cm)	168 ± 9	169 ± 9	168 ± 9
Weight (kg)	73 ± 16	75 ± 14	73 ± 16
BMI (kg/m2)	26 ± 4	26 ± 4	26 ± 4
Glucose (mmol/L)	5.42 ± 0.90	5.28 ± 0.55	5.44 ± 0.93
Cholesterol (mmol/L)	5.85 ± 1.05	5.92 ± 0.98	5.84 ± 1.05
HDL-cholesterol (mmol/L)	1.51 ± 0.45	1.52 ± 0.49	1.51 ± 0.45
Triglycerides (mmol/L)	1.23 ± 0.89	1.31 ± 0.79	1.22 ± 0.91
Hs-CRP (mg/L)	2.80 ± 8.48	2.81 ± 3.16	2.80 ± 8.96
SBP (mmHg)	134 ± 19	133 ± 17	134 ± 20
DBP (mmHg)	82 ± 12	82 ± 11	82 ± 12
PP (mmHg)	52 ± 14	51 ± 13	52 ± 14
Mean carotid IMT (mm)	0.69 ± 0.11	0.70 ± 0.11	0.69 ± 0.11
Mean carotid diameter (mm)	5.66 ± 0.72	5.64 ± 0.51	5.67 ± 0.74

Values are expressed as arithmetic mean ± SD. Crude values were analyzed. BMI: body mass index, hs-CRP: high sensitive C reactive protein, SBP: systolic blood pressure, DBP: diastolic blood pressure, PP: pulse pressure, IMT: intima media thickness.

Table 2 :

Multivariate regression analysis of mean carotid diameter

	Coefficient ± SEM	**R^2**	**P**
Age (years)	-0.026 ± 0.014	0.5%	0.069
BMI (Kg/m^2)	0.022 ± 0.008	1.4%	0.004
Hs-CRP (mg/L)	0.011 ± 0.004	1.5%	0.002
CAT2 (yes)	-0.912 ± 0.396	0.9%	0.022
Age x CAT2	0.017 ± 0.008	0.9%	0.032
SBP (mmHg)	0.007 ± 0.002	1.8%	0.0008
Women (yes)	-0.649 ± 0.068	14.6%	<0.0001
Model	42.2%		

R^2 when this independent variable is removed

Table 3:

Carotid plaques in the ERA population and according to haplotypes

	All patients		CAT 1	CAT 2		
N	378		44	334		
Age (y)	58±10		58±11	58±10		
Women (%)	33		38	32		
Hypertension (%)	69	67	69			
Treated (%)	70	74	69			
Tobacco (%)	20	19	20			
Diabetes (%)	4	4	4			
high cholesterol (%)	39	40	39			
Carotid plaques:						
yes (%)	3	2	16	34	(P=0.015)	
number	0.51±0.87		0.20±0.51		0.55±0.90	(P=0.011)

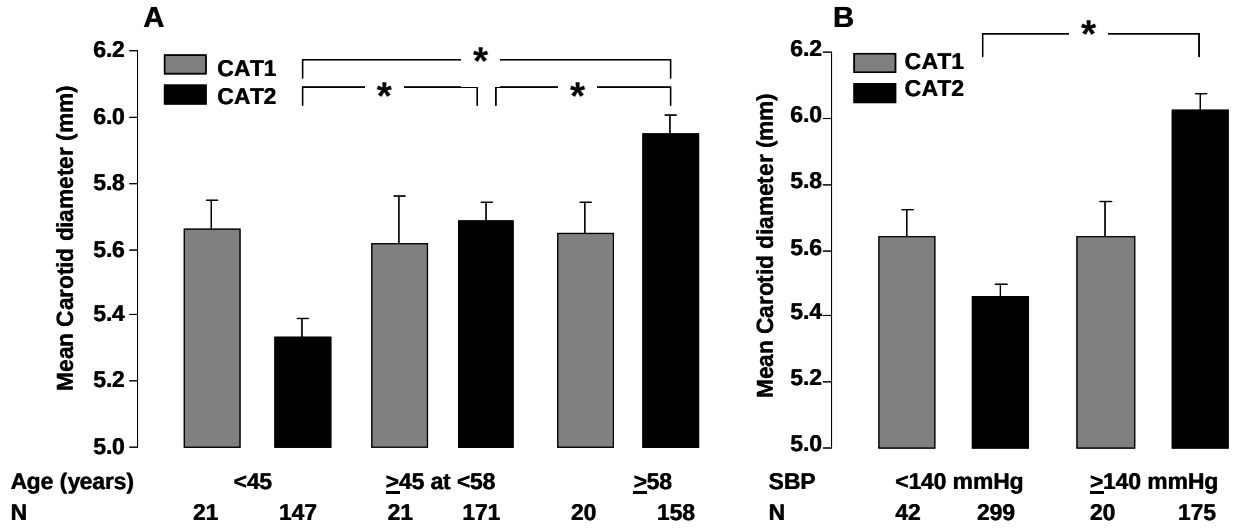
Values are expressed as arithmetic mean ± SD. Crude values were analyzed. P vs CAT1 haplotype.

Table 4: Multivariate regression analysis of carotid plaque number:

	Coefficient ± SEM	R^2	P
Age (years)	0.0218 ± 0.0041	6.6%	<0.0001
CAT2 (yes)	0.3418 ± 0.1304	1.6%	0.009
High cholesterol history (yes)	0.2019 ± 0.0875	1.2%	0.02
Women (yes)	-0.3385 ± 0.0922	3.1%	0.0003
Tobacco (yes)	0.2657 ± 0.1048	1.5%	0.012
Model 14.1%			

R^2 when this independent variable is removed

Figure 1: Mean carotid diameter according to age or SBP classes and catalase haplotypes



Values are adjusted as mean ± SEM.

* P<0.001, Tukey-Kramer Multiple-Comparison test.

N: number of individuals.

VIII. Etude pangenomique des concentrations plasmatiques de l'haptoglobine:

A genome-wide association study identifies rs2000999 as a strong genetic determinant of circulating Haptoglobin levels.

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Six cent trente un enfants de la cohorte STANISLAS ont constitué l'échantillon de phase 1 ou 'discovery set'. Nous y avons effectué une GWAS et identifié une forte association de variants, présents dans un block en déséquilibre de liaison de 218Kb incluant le gène Haptoglobin (*HP*) et le gène Haptoglobin Related Protein (*HPR*) sur le chromosome 16, avec les taux d'haptoglobine.

Seuls les variants rs2000999G>A (β=-0,123, $P=6,32x10^{-13}$) et rs10492825A>G (β=-0,093, $P=5,50\times10^{-08}$) atteignaient le seuil 'genome-wide significant'. Leur proximité physique (en déséquilibre de liaison, r^2=0,48–HapMap CEU release 27) pouvait laisser présumer que nous ne visualisions qu'un unique signal, et c'est ce que nous avons vérifié par une régression conditionnée de chaque variant sur l'autre. Lors de cette régression conditionnée, nous avons également testé tous les variants présents dans la zone de recombinaison de ce locus.

Au terme de cette procédure, seul le rs2000999G>A du gène *HPR* conservait une signification élevée : P ajusté sur rs10492825A>G = $1,9x10^{-7}$. Il était donc le seul à avoir un effet indépendant sur la variabilité interindividuelle de l'haptoglobine. Nous avons ensuite sélectionné 656 familles nucléaires (2,680 sujets) dans la cohorte STANISLAS pour lesquelles les variants rs2000999G>A et rs10492825A>G avaient été génotypés et nous avons évalué

l'héritabilité génétique de l'haptoglobine. La variance polygénique de ce trait dans cet échantillon était de 24,9% et un ajustement avec le rs2000999A>G diminuait ce résultat de 10,5% alors qu'un ajustement sur le rs10492825A>G ne changeait rien. Le variant rs2000999 participait donc à hauteur de 42% à l'héritabilité génétique globale de l'haptoglobine.

L'association du rs2000999 a été répliquée et confirmée lors d'une phase 2 dans un échantillon familial de la cohorte STANISLAS, et les cohortes GENDAI et OBE (N=4,579 , $P_{réplication}=3,5x10^{-41}$, $P_{méta-analyse}=8,09x10^{-59}$). L'éventuelle fonctionnalité de rs2000999 a encore été démontrée par son association spécifique avec les niveaux d'ARNm du gène *HP* ($\beta= 0,23 \pm 0,08$, P= 0,007).

Le rs2000999A>G a été très récemment associé au cholestérol total et au LDL-C dans des populations adultes : 4,200 sujets issus du consortium '*European Special Populations Research Network*' et plus de 100,000 sujets européens et non européens réunis dans une large méta-analyse ayant permis de confirmer 95 variants génétiques fortement associés aux lipides. Nous avons réaffirmé cette association dans 5 cohortes pédiatriques réunissant 8,946 enfants ($P_{cholestérol\ total}=0,002$, $P_{LDL}=8x10^{-4}$).

Compte tenu de l'ensemble des résultats, le génotypage du rs2000999A>G devrait être pris en compte lors de la mesure des concentrations plasmatiques d'haptoglobine. Une étape clef qui aura des conséquences diagnostiques/thérapeutiques et/ou préventives.

Mots clés : Haptoglobine, Inflammation, études pan-génomiques, maladies infectieuses, maladies cardiovasculaires.

Contribution : *Nous avons effectué une analyse transcriptomique et bio-informatique en utilisant la base de données '1000 génomes' pour cartographier le gène HP de manière plus fine. Nous avons participé à la correction des différentes versions du manuscrit.*

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PLoS one

A Genome-Wide Association Study Identifies rs2000999 as a Strong Genetic Determinant of Circulating Haptoglobin Levels

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Abstract

Haptoglobin is an acute phase inflammatory marker. Its main function is to bind hemoglobin released from erythrocytes to aid its elimination, and thereby haptoglobin prevents the generation of reactive oxygen species in the blood. Haptoglobin levels have been repeatedly associated with a variety of inflammation-linked infectious and non-infectious diseases, including malaria, tuberculosis, human immunodeficiency virus, hepatitis C, diabetes, carotid atherosclerosis, and acute myocardial infarction. However, a comprehensive genetic assessment of the inter-individual variability of circulating haptoglobin levels has not been conducted so far. We used a genome-wide association study initially conducted in 631 French children followed by a replication in three additional European sample sets and we identified a common single nucleotide polymorphism (SNP), rs2000999 located in the *Haptoglobin* gene (*HP*) as a strong genetic predictor of circulating Haptoglobin levels ($P_{overall} = 8.1 \times 10^{-59}$), explaining 45.4% of its genetic variability (11.8% of Hp global variance). The functional relevance of rs2000999 was further demonstrated by its specific association with *HP* mRNA levels ($\beta = 0.23 \pm 0.08$, $P = 0.007$). Finally, SNP rs2000999 was associated with decreased total and low-density lipoprotein cholesterol in 8,789 European children ($P_{total\ cholesterol} = 0.002$ and $P_{LDL} = 0.0008$). Given the central position of haptoglobin in many inflammation-related metabolic pathways, the relevance of rs2000999 genotyping when evaluating haptoglobin concentration should be further investigated in order to improve its diagnostic/therapeutic and/or prevention impact.

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Introduction

Human haptoglobin (Hp) is an acute phase inflammatory glycoprotein essentially synthesized by the liver and up-regulated by cytokines [1]. Hp is polymorphic with two co-dominant alleles, *Hp1* and *Hp2* encoded by the *Haptoglobin* (*HP*) gene located in chromosome 16 and resulting in three common isoforms: Hp1-1, Hp2-2 and Hp2-1 (called *HP* 'common polymorphism') [2]. In normal physiological conditions, Hp protein concentration in blood ranges between 0.3 and 2.0 g/L in adults [3] but significant fall in its level during the first decade of life [4]. The main property of Hp is to scavenge circulating hemoglobin (Hb) released by hemolysis or normal red blood cells turnover [5]. The resulting circulating Hp-Hb complexes are eliminated by Kupffer's cell(s) in the liver, preventing the generation of reactive oxygen species [2,6]. Therefore, Hp plays an important role in preventing renal damage and iron loss that can occur following an intravascular hemolysis. Hp is also able to bind apolipoprotein (Apo) A-I [7] to protect the Apo A-I effector domain of lecithin-cholesterol acyltransferase against oxidative stress, and Hp consequently modulates the high-density lipoprotein (HDL) function [8]. Furthermore, Hp can bind Apo E and the resulting complexes influence cholesterol esterification [9]. These functional characteristics confer to Hp a major role in the reverse transport of cholesterol between peripheral cells and the liver for degradation.

Hp levels and *HP* rs72294371 'common polymorphism' (Data S1) have been consistently associated with inflammatory-linked infectious [10,11] and non-communicable diseases [11,12]. Malaria caused by *Plasmodium falciparum*, which is associated with extensive intravascular hemolysis, decreases Hp to undetectable levels as the Hb-scavenging system is saturated [13].[13] In malaria-endemic areas, hypohaptoglobinemia has been proposed as an indirect biochemical indicator of malaria [14]. *HP* 'common polymorphism' should be considered at diagnosis of tuberculosis. Eisaev and colleagues [15] described an increased recurrence of pulmonary tuberculosis with worse prognosis in Hp2-2 Caucasians. Furthermore, this *HP* 'common polymorphism' contributes to mortality and viral load in Human immunodeficiency virus (HIV) infection [16]. Hp2-2 HIV carriers have a more pronounced viral replication rate and a worse prognosis compared to Hp1-1 or Hp2-1 HIV carriers [16,17]. Hepatitis C infection has also been associated with low serum Hp concentrations [18] and an overrepresentation of the Hp1-1 phenotype has been associated with high risk for chronic hepatitis C [19,20]. The *HP* 'common polymorphism' has also an effect on various other infectious diseases [21,22,23,24].

In Type 2 diabetes patients, the Hp2-2 phenotype has been suggested to confer greater risk of cardiovascular events [12,25,26] and of carotid atherosclerosis [27]. Moreover, high Hp level is a risk factor for acute myocardial infarction, stroke and heart failure [28,29]. Therefore, the routine measurement of Hp level has been suggested to be incorporated in daily medical practice to evaluate cardiovascular risk [29].

Despite these findings, the basis of Hp level inter-individual variability is still unknown. To identify genetic variants modulating physiological levels of Hp, we analyzed genome-wide association study (GWAS) data generated in European children for whom no age-related disease may influence Hp concentrations. We also assessed the association between the identified variants and cardiovascular risk factors (total, HDL and low density lipoprotein-cholesterol, Apolipoproteins A1 and B).

Results

Table 1 shows the phenotypic characteristics of the studied populations. Hp levels in children were low, in accordance with its reference distribution [4].

Patterns of family correlations for serum Hp concentrations were assessed following both unadjusted values (Table 2). Model 1, which was not adjusted, did not show any family correlation. Model 2, which took into account age and body mass index (BMI) as covariates showed significant correlations for all the various pairs of relatives. Model 3, which hypothesized no effect of gender on family correlations, showed significant father-mother, father-son and son-son correlations (Table 2).

We then assessed the components of variance attributable to additive genetic effects, shared household effects and residual environmental factors (including assay variability) in 656 nuclear bi-parental families (2,680 individuals) from the STANISLAS Family Study (SFS) cohort (Table 3). Model 2, which included the three components after adjustment for age and BMI gave a better description of the variance decomposition than model 1 which was

Table 1. Phenotypic characteristics of the studied populations.

Population	Subsample	Sample size (% female)	Age (years) [95% CI]	BMI (kg/m^2) [95% CI]	Haptoglobin (g/L) [95% CI]	LDL-cholesterol (mmol/L) [95% CI]	Total cholesterol (mmol/L) [95% CI]
SFS	Discovery cohort: Phase 1 vs Hp	631 (50.9%)	11.93 [11.76–12.11]	17.66 [17.49–17.84]	0.65 [0.62–0.68]	2.99 [2.94–3.05]	4.79 [4.74–4.85]
	Families: Phase 2 vs Hp and lipids	2,957 (49.2%)	29.84 [29.38–30.30]	22.66 [22.52–22.81]	0.95 [0.94–0.97]	3.43 [3.40–3.46]	5.30 [5.26–5.34]
Obese children	Replication cohort: Phase 2 vs Hp and lipids	1,015 (52.3%)	11.07 [10.86–11.27]	28.24 [27.84–28.64]	1.18 [1.15–1.22]	2.74 [2.70–2.79]	4.47 [4.42–4.52]
GENDAI	Replication cohort: Phase 2 vs Hp and lipids	419 (53.1%)	11.16 [11.10–11.23]	19.76 [19.46–20.07]	0.81 [0.76–0.85]	3.12 [3.06–3.17]	4.79 [4.73–4.86]
NFBC1986	Replication cohort: Phase 2 vs lipids	5,310 (50.8%)	16	21.27 [21.17–21.37]	NA	2.54 [2.52–2.56]	4.27 [4.24–4.29]
VERONA cohort	Replication cohort: Phase 2 vs lipids	401 (43.4%)	10.90 [10.75–11.04]	18.01 [17.78–18.23]	NA	2.41 [2.40–2.47]	4.21 [4.15–4.28]

CI: confidence interval; BMI: body mass index; LDL: low-density lipoprotein.
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Table 2. Estimates of familial correlations ± standard error for serum haptoglobin concentration (656 familles/2680 individuals).

	Model 1	Model 2	Model 3	Model 4
Adjustment for	None	Age & BMI	Age & BMI	Age, BMI & rs2000999 allelic frequency
Father - Mother (FM)	0.126±0.038	0.111±0.038**	0.111±0.038**	0.156±0.038***
Father - Son (FS)	0.277±0.037	0.264±0.037***	0.230±0.021***	0.206±0.022***
Father - Daughter (FD)	0.277±0.036	0.265±0.037***	[0.230]	[0.206]
Mother - Son (MS)	0.227±0.036	0.204±0.037***	[0.230]	[0.206]
Mother - Daughter (MD)	0.184±0.039	0.188±0.040***	[0.230]	[0.206]
Son - Son (SS)	0.213±0.060	0.186±0.064**	0.274±0.036***	0.239±0.036***
Son - Daughter (SD)	0.273±0.047	0.271±0.047***	[0.274]	[0.239]
Daughter - Daughter (DD)	0.355±0.067	0.386±0.064***	[0.274]	[0.239]
$Log_e L^2$ (Logarithm of likelihood function)	1458.83	1338.20	1342.20	1215.13
Alternate model		Model 1	Model 2	Model 3
χ^2 (df)		241.26(8)	8.00(5)	254.14(4)
P		***	NS	***

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$: compared to zero.
Model 1 estimated all eight correlations without adjustment.
Model 2 estimated all eight correlations with adjustment for age and BMI,
Model 3 estimated three correlations with adjustment for age and BMI with no gender effect on parent or offspring correlations: FS = MS = MD = FD and SS = SD = DD.
Model 4 estimated three correlations with adjustment for age, BMI and rs2000999 allelic frequency with no gender effect on parent or offspring correlations: FS = MS = MD = FD and SS = SD = DD.
Values in brackets were constrained to be equal to a preceding value according to the hypotheses of the model.
BMI: body mass index.
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not adjusted. Hp genetic variance represented 26% ($P<0.001$) of the total variance. Shared (*i.e.* within families) and random environmental variances were 11.6 and 62.4% respectively (Table 3).

Our GWAS based on 631 unrelated children of the SFS cohort showed strongest association signal for Hp levels in a 218-kb linkage disequilibrium (LD) block on chromosome 16 that includes the *HP* gene (Figure 1). Using the square-root transformed Hp measurement adjusted for gender, age and z-BMI under the additive model, we identified in this region two significant association signals 90-kb apart: rs2000999 (with A as allele effect: $\beta = -0.123$, standard error [SE] = 0.017, $P = 6.32 \times 10^{-13}$; Table 4) and rs10492825 (with C as effect allele: $\beta = -0.0876$, SE = 0.016, $P = 5.50 \times 10^{-08}$; Table 4). Both SNPs rs2000999 and rs10492825 display moderate LD ($r^2 = 0.48$, HapMap CEU release #27). In order to assess the redundancy between these two signals, we ran conditional regression analyses for both SNPs adjusted for each other and found that rs2000999 alone drove the association observed at the *HP* locus (rs2000999: $P_{rs10492825\ adjusted} = 1.95 \times 10^{-7}$, rs10492825: $P_{rs2000999\ adjusted} = 0.91$, Data S2, Table S1).

We confirmed the association between SNP rs2000999 and circulating Hp levels in three additional independent European cohorts: the GENDAI study of Greek children, a subset of obese children from the East of France ($N_{total} = 1{,}434$), and a familial subset ($N_{total} = 2{,}957$) of the SFS cohort ($P_{replication} = 3.49 \times 10^{-41}$, $P_{overall} = 8.09 \times 10^{-59}$; Table 4).

Table 3. Variance components of serum haptoglobin concentrations (656 families/2680 individuals, rs2000999 & rs4788597 as allelic frequency).

	Model 1	Model 2	Model 3
Adjustment for	None	Age & BMI	Age, BMI & rs2000999 allelic frequency
Polygenic variance: σ^2_G	0.0478±0.0120***, 24.9%	0.0460±0.0109***, 26.0%	0.0226±0.0101*, 14.2%
Household variance: σ^2_H	0.0250±0.0065***, 13.0%	0.0206±0.0059***, 11.6%	0.0233±0.0053***, 14.7%
Residual variance: σ^2_E	0.1194±0.0078***, 62.1%	0.1103±0.0072***, 62.4%	0.1130±0.0069***, 71.1%
$Log_e L^2$ (Logarithm of likelihood function)	−968.90	−1074.58	−1200.04
Alternate model	-	Model 1	Model 2
χ^2 (df)	-	211.36 (8)	250.92 (4)
P	-	***	***

*$P \leq 0.05$,
**$P \leq 0.01$,
*** $P \leq 0.001$.
BMI: body mass index.
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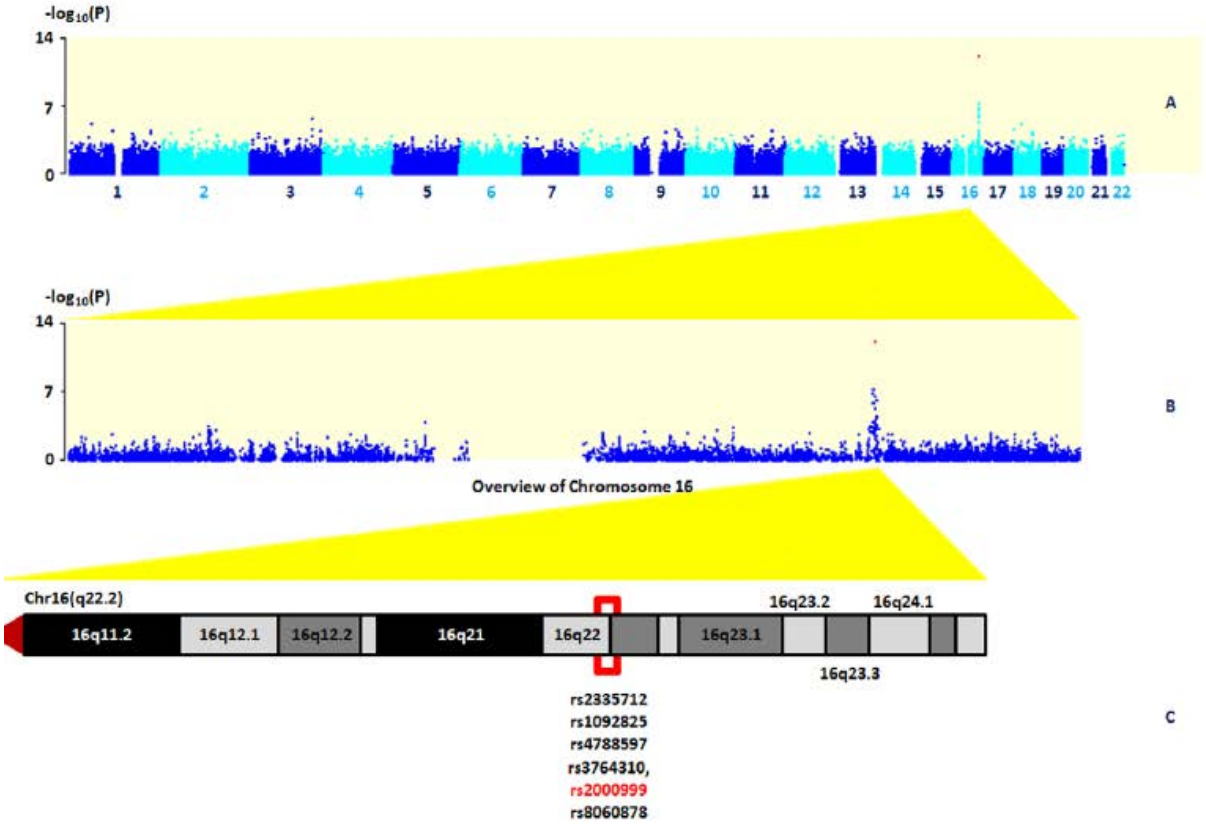

gure 1. Manhattan plot of the GWAS of the discovery cohort comprising 631 children. A, A Manhattan plot showing the $-\log_{10}(P$ lues) of SNPs from the association analysis of the 631 SFS children from stage 1. **B**, An overview of the $-\log_{10}(P$ values) of Chromosome 16. **C**, The nomic region of the 618 LD block displayed in UCSC Genome Browser.
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Accounting for the rs2000999 allelic frequency, the pattern of milial correlation (Table 2, model 4) decreased from 0.230 to 206 and from 0.274 to 0.239 for sibling and child-parent spectively, whereas the adequacy of the model was significantly proved. Additional adjustment for rs2000999 for the compo-nts of variance attributable to additive genetic effects, shared ousehold effects and residual environmental factors (Table 3, odel 3) significantly improved the likelihood function and the oportion of phenotypic variability accounted for by genetic effects decreased (26.0% to 14.2%, in comparison to model 2). Moreover, the component attributable to household factors increased (11.6% to 14.7%). We thus determined that rs2000999 is the major genetic determinant of Hp levels accounting for 11.8% of Hp global variance and 45.4% of the genetic variance of this trait.

In order to assess the degree of independence of rs2000999 from the *HP* rs72294371 'common polymorphism' (Data S1), we genotyped the latter in the GWAS first stage children (SFS cohort)

Table 4. Discovery and replication of rs2000999 association data for Hp levels.

				Haptoglobin (g/L)						
Population	Stage	N	MAF	GG	GA	AA	β	Standard error	raw P	P adjusted with rs10492825
SFS - unrelated children	Discovery	631	0.21	0.736±0.406	0.532±0.321	0.361±0.180	−0.123	0.017	6.32×10^{-13}	1.95×10^{-07}
SFS - families	Replication	2,957	0.20	1.056±0.467	0.819±0.445	0.549±0.330	−0.138	0.007	1.17×10^{-75}	
Obese children	Replication	1,015	0.22	1.261±0.492	1.064±0.455	1.002±0.534	−0.088	0.013	1.20×10^{-11}	
GENDAI	Replication	419	0.25	0.811±0.450	0.813±0.558	0.731±0.319	−0.021	0.021	0.306	
Replication sample	Replication	4,391	0.22						3.49×10^{-41}	
Overall meta-analysis	Replication	5,022							8.09×10^{-59}	

N: sample size; MAF: Minor Allele Frequency; β:beta coefficient for the effect allele A.
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by using a PCR-based method and a gel reading. Only a subset of 341 out of 631 samples was successfully genotyped after independent readings by two readers. In this sample set, we found no evidence for LD between *HP* 'common polymorphism' and rs2000999 ($r^2 = 0.135$) nor with the other SNPs that were genotyped by the Illumina array within the 218 kb LD block that includes both *HP* 'common polymorphism' and rs2000999 ($0.001 < r^2 < 0.137$; $N = 31$ SNPs). Furthermore, the *HP* 'common polymorphism' (minor allele frequency [MAF] = 0.46) and rs2000999 (MAF = 0.20) were both highly associated with Hp levels, as expected ($P = 4 \times 10^{-7}$ and $P = 1 \times 10^{-7}$, respectively). When both variants were included in the same regression model, we found that they significantly and independently contributed to the increased Hp levels ($P_{HP\ rs72294371\ 'common\ polymorphism'} = 0.001$ and $P_{rs2000999} = 5 \times 10^{-5}$) indicating that the association with rs2000999 would be novel and not redundant with the *HP* 'common polymorphism'. However, it is noteworthy that despite strong efforts, we did not succeed by far in genotyping all samples. We used two other technologies: a pre-designed TaqMan copy number assay (Applied Biosystems) and a PCR-based method with another design than previously used. Unfortunately, we did not find a good concordance (<70%) between the three methods. We conclude that given the state of art, we cannot definitively conclude that the present signal of association is not related to the *HP* 'common polymorphism' genotype.

In order to validate our main results, we secondary assessed the effect of SNP rs2000999 on *HP* gene expression in subcutaneous adipose tissue sample from 194 non-obese subjects ascertained from the Swedish SibPair cohort (Data S2). We found a significant contribution of rs2000999 to *HP* expression ($\beta = 0.23 \pm 0.08$; $P = 0.007$; $P_{Bayesian} = 0.006$).

We finally assessed by additive model the effect of SNP rs2000999 on total, HDL and low-density lipoprotein (LDL) cholesterol, Apolipoproteins A1 and B in five independent European pediatric cohorts totaling 8,789 children. Total cholesterol was ln-transformed and we normalized the LDL cholesterol by computing the square root. All measurements were adjusted for gender, age (excepting the NFBC1986) and z-score BMI. Our data showed that rs2000999, with A as allele effect, was associated with total cholesterol ($\beta = -0.011$, SE = 0.003, $P = 0.002$; Table 5) and LDL-cholesterol ($\beta = -0.017$, SE = 0.004, $P = 0.0008$; Table 5). The association with HDL-cholesterol and Apolipoproteins A1 and B are displayed in Table S2.

Discussion

We first determined in 656 nuclear families that 26% of the Hp plasma level variance was under genetic control. Then, using a GWAS in 631 children from the same population and replicating in three independent populations, we identified rs2000999 as the major genetic determinant of Hp levels. This genetic variant alone explained 45.4% of the genetic variance of this trait (11.8% of Hp global variance). SNP rs2000999 is located in the intronic region of *HP* gene, in a region previously believed to be the *HPR* gene (encoding the haptoglobin-related protein) which shares more than 90% nucleotide sequence homology with *HP* [30]. It is 17 kb apart a duplication of 59 α chain amino acid residues resulting to an intragenic duplication of 1.7 kb and which is known as the *HP* 'common polymorphism' [31].

Our study shows that SNP rs2000999 also modulated expression levels of the Hp mRNA in human adipose tissue suggesting that this SNP (or a SNP in very strong LD with this one) is indeed functional. It is noteworthy that SNP rs2000999 has been previously reported to associate with total cholesterol in 4,200 adults from the EUROSPAN consortium [32] and with both total and LDL-cholesterol in 100,000 adults of European and non-European ancestry [33]. Interestingly, we confirmed the effect of this SNP on these lipid traits in European children.

Increased plasma levels of several inflammatory markers correlate with higher incidence and prognosis of various cardiovascular diseases [34,35,36,37]. Hp level measurement has been recently shown to improve the predictive information for major cardiovascular events [29]. As rs2000999 is also associated with lipid levels, this marker links inflammation and cardiovascular risk. It is noteworthy that the impact of rs2000999 association on lipids occurs early in life and is consistent with previous findings that the precursors of cardiovascular diseases originate in childhood [38,39].

Interestingly, the effect of rs2000999 on Hp levels is more important in our discovery cohort which includes healthy children having low Hp concentration (0.65 g/L±0.39). As shown in the analyses for other diseases [40], the statistical power of GWAS can be increased in healthy homogeneous controls.

In addition, the effect of aging and of the environment is minimized in children. Then, by using healthy pediatric populations, we were able to assess more accurately the effect of the SNP rs2000999 on Hp levels.

We tried to assess the degree of independence between rs2000999 and the *HP* 'common polymorphism'. Three different methods were evaluated to genotype the *HP* 'common polymorphism' in our whole GWAS sample set. Unfortunately, we found no concordance between the three methods, which underlie a major difficulty to carry out an accurate genotyping of this polymorphism. Even if this difficulty was not clearly discussed and not published to our knowledge, it is admitted in the scientific field

Table 5. Association of rs2000999 with lipid traits.

			Total cholesterol			LDL-cholesterol		
	N	MAF	β	Standard error	p-value	β	Standard error	p-value
SFS children	1,644	0.202	−0.003	0.008	0.73	0.0004	0.010	0.967
Obese Children	1,015	0.216	−0.008	0.011	0.439	−0.009	0.013	0.507
GENDAI	419	0.253	−0.023	0.012	0.055	−0.031	0.013	0.022
NFBC1986	5,310	0.186	−0.013	0.004	0.002	−0.022	0.006	8.96E-05
Verona Cohort	401	0.200	−0.011	0.014	0.446	−0.011	0.016	0.515
Overall meta-analysis	8,789		−0.011	0.003	0.002	−0.017	0.004	0.0008

N: sample size; MAF: Minor Allele Frequency; β:beta coefficient for the effect allele A.
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and it should also be present in the clinical diagnosis setting. In contrast, SNP rs2000999 can be accurately and easily genotyped.

Our findings should be further replicated in non-European adults, especially in those affected by infectious diseases. More generally, rs2000999 should be assessed in cohorts of patients affected by the large variety diseases associated with Hp levels. It is not a trivial task, as Hp is a trait that has been infrequently measured in cohorts used for genetic studies. Given the major effect of rs2000999 on Hp gene expression and on Hp levels, Mendelian randomization approach would be of interest to test the causative effect of this SNP on infectious and non-communicable phenotypes in order to assess its clinical relevance.

Materials and Methods

Ethics Statement

All the populations involved in the present study were recruited in accordance with the latest version of the Declaration of Helsinki for Ethical Principles for Medical Research Involving Human Subjects. All participants and their parents gave a written informed consent. Genetic 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).

Study populations

The STANISLAS Family Study (SFS). The SFS is a 10-year longitudinal survey involving 1,006 volunteer families of European ancestry whose members were free of chronic disease (cardiovascular or cancer) with recruitment taking place from 1993–95 [41]. The SFS samples and data are part of the Biological Resources Centre (BRC) "Interactions Gène-Environnement en Physiopathologie CardioVasculaire" (IGE-PCV) in Nancy, France. Genome-wide genotyping was performed on a subset of 631 unrelated children (mean age 11.93 years [11.76–12.11]) constituting the discovery cohort [42] after screening for latent population substructure (Data S2). The 2,957 remaining individuals after quality control were analysed in the replication studies (mean age 29.84 [29.38–30.30]). Hp levels, BMI and the cardiovascular risk traits including total, high density lipoprotein (HDL) and low density lipoprotein (LDL)-cholesterol (calculated by the Friedewald formula [43]), Apolipoprotein A1 and B were available for all participants.

Obese Children. We studied obese children (defined as BMI>97th percentile for age and sex according to a French cohort [44]) ascertained from 449 nuclear families with at least one obese offspring, recruited in the Paediatric Endocrine Unit of Jeanne de Flandres Hospital of Lille, France or through a national media campaign. We analyzed 1,015 children (mean age 11.07 years [10.86–11.27]) for whom Hp, BMI, total, HDL and LDL-cholesterol, Apolipoprotein A1 and B measurements were available.

The GeNe and Diet Attica Investigation (GENDAI). The GENDAI pediatric cohort was recruited from children living in the Attica region of Greece [45]. From November 2005 to June 2006, 1,138 peri-adolescent children were recruited from randomly selected elementary schools of Attica. We analyzed 419 children (mean age 11.16 years [11.10–11.23]) for whom Hp, BMI, total, HDL and LDL-cholesterol, Apolipoprotein A1 and B measurements were available.

The Northern Finland 1986 Birth Cohort (NFBC 1986). The NFBC1986 is a prospective birth cohort including all Finnish mothers of European ancestry with children whose expected date of birth fell between July 1, 1985 and June 30, 1986 in the two northernmost provinces in Finland [46]. Clinical examination at 15–16 years follow-up was conducted between August 2001 and June 2002. All cohort members living in Finland with known address (n = 9,215) were invited, and 6,798 participated (74%). We analyzed 5,310 adolescents successfully genotyped in the NFBC1986 cohort for whom BMI, total, HDL and LDL-cholesterol, Apolipoprotein A1 and B measurements were available.

The Verona cohort. The Verona cohort consists of Italian children recruited from the general population of Verona, Italy, whose families were randomly chosen from the registry office database of the town, and contacted by post. We analysed 401 children (mean age 10.90 years [10.75–11.04]) successfully genotyped for whom at least BMI, total, HDL and LDL-cholesterol, Apolipoprotein A1 and B measurements were available.

The SibPair cohort. The SibPair cohort comprises 154 nuclear families (732 subjects) from Sweden, each containing an obesity-discordant sib pair (at least 10 kg/m^2 difference in BMI). Gene expression and genetic variation were analysed in 194 non-obese subjects from the SibPair cohort.

Genotyping

Genomewide genotypes were generated for the 631 unrelated SFS children using the Illumina Human CNV370-Duo array [42]. Briefly,750 ng of genomic DNA was processed using Illumina's protocol for the BeadStation genotyping platform (Illumina), followed by GenCall software analysis(Illumina) to automatically cluster, call genotypes, and assign confidence scores using the GenTrain clustering algorithm (Illumina). We discarded a total of 2,552 SNPs due to the following reasons: extreme Hardy-Weinberg disequilibrium (P<0.001), low genotyping call rates (<95%) or low minor-allele frequencies (<1%). We retained 318,237 SNPs for analysis. Genomic control λ_{GC} was 1.01.

We used the Applied Biosystems SNPlex^{TM} technology to replicate the association of genome-wide significant genetic variants in the SFS replication set, obese children and GENDAI, NFBC1986 and Verona cohorts.

SNPlex is based on the Oligonucleotide Ligation Assay (OLA) combined with multiplex PCR target amplification and was carried out as per the manufacturer's instructions (http://www.appliedbiosystems.com). Allelic discrimination was performed by capillary electrophoresis analysis using an Applied Biosystems 3730xl DNA Analyzer and GeneMapper 3.7 software. Genotyping call rate was above 95% in all populations studied and genetic variants were in HW equilibrium (p>0.001).

We used a PCR-based method [47] to genotype for the *HP* 'common polymorphism' in the 631 children of the discovery cohort (SFS cohort) in order to determine any linkage disequilibrium with regard to genome-wide significant variants identified in the analysis. Only genotypes that were concordant following a double blind genotyping call by two independent readers were retained for statistical analyses (N = 341). Two additional genotyping methods for *HP* 'common polymorphism' were used in order to validate the above-method: a custom TaqMan copy number assay (Applied Biosystems) following the manufacturer's recommendations and another PCR-based method using the following oligonucleotide primers : 5′-CTCTCCTTTCTCCC-TTCCTGTC-3′ and 5′-TTTATCCACTGCTTCTCATTGT-3′. We didn't obtain correspondence between the banding patterns and the Hp genotypes.

Haptoglobin measurement

Blood samples were collected between 8:00 and 9:00 am or 11:30 and 12:30 pm by venipeuncture after overnight fasting. Hp

protein levels were measured in blood plasma samples by high sensitivity immunophotometry analyses using the BN™II Siemens analyzer (Siemens, Marburg, Germany) and Siemens reagents and following the manufacturer's instructions.

Lipids measurements

Total cholesterol, HDL-cholesterol and apolipoproteins A1 and B were assayed using enzymatic methods (AU640 [Olympus, Watford, UK]) and LDL-cholesterol was calculated using the Friedewald formula [43].

Statistical analyses

Heritability estimate of Hp levels in the SFS. Intra-familial correlations were estimated by using maximum likelihood techniques [48] with and without adjustment for covariates. This statistical approach allowed adjustment for covariates within models, simultaneously and separately for fathers, mothers, sons and daughters. The significance of various familial correlations, or sex and generation differences in correlations, was tested using the log-likelihood ratio test. Correlations were computed under two sets of hypotheses: gender effects on correlations for parents and children and no gender effect for all correlations.

Variance component analysis was applied in order to assess the relative contributions of genetic, common household factors and individual specific environment in familial aggregation of serum haptoglobin concentrations. The variable used to estimate variance component was adjusted for age and BMI, separately for fathers, mothers, sons and daughters. The analysis was conducted by using a multivariate normal model for pedigree analysis as described by Lange and colleagues [49,50]. with the software FISHER, which also performed tests of goodness-of-fit of the underlying multinormal distribution. The general model assumed that the studied trait was the result of the sum of three independent random components: a polygenic component (G) representing additive genetic factors, household factors common to individuals within a family (H) and unmeasured environmental factors particular to an individual (including measurement error) (E). These three components were assumed to be normally distributed with mean equal to 0 and variance equal to σ^2G, σ^2H and σ^2E, respectively.

The hypothesis of no polygenic component or no household effect was checked by comparing a model including σ^2G, σ^2H and σ^2E with a model including only σ^2H and σ^2E or σ^2G and σ^2E, respectively. In addition, possible effects of covariates (age and BMI) and genome-wide significant variants' allelic frequency on these variance components were tested.

Comparison of nested models was based on the likelihood ratio criteria. Eventually, the best parsimonious model was selected. The percentage contributions of the three components, additive genetic factors (heritability), household factors and residual environmental, to residual phenotypic variance (after adjustment for covariates) were determined.

Genome-wide association and replication analyses. We carried out genome-wide association and replication analyses on Hp levels using linear mixed regression models under the additive genetic model with one degree of freedom, adjusting for age, gender and BMI and using PLINK [51]. The summary statistics were combined in the meta-analyses (Data S2), using the inverse normal method with equal weight for each population. In this method, P values of each study are transformed into their inverse normal z score and the weighted sum, over all studies, is compared to a normal N (0, 1), provided the sum of squared weights equals 1. The estimates of variants effects on Hp and their standard errors for each separate analysis were combined in the meta-analysis using the weighted inverse normal method, and the overall effect and its confidence interval were estimated using the inverse variance method implemented in the 'meta.summaries' function of the R RMETA package (http://cran.r-project.org/web/packages/rmeta/index.html). No major heterogeneity in effects was observed ($P<0.02$). The same mixed model and the same software were used to analyse the association of genetic variants with lipid traits.

Gene-expression investigation. To investigate the effect of genome-wide significant variants on gene expression (Data S2), we used data from 194 non-obese individuals from the SibPair cohort [52]. Gene expression data for *HP* was measured in subcutaneous adipose tissue [53] from 347 siblings using the Affymetrix Human U133 Plus 2.0 platform (208470_s_at and 208471_at, respectively). DNA was isolated from peripheral blood and genotypes were generated using Illumina 610-Quad arrays.

We used a linear mixed model (Pinheiro and Bates, 2000) to assess association of SNPs with gene expression. Log-transformed expression level was regressed on the random-effect term, which accommodates the family pedigree structure, and on the fixed-effect terms i.e. sex, age, BMI level and the SNP of interest (recoded as 0 = AA; 1 = AG; 2 = GG according to an additive model). Analysis was carried out using the R function lmer() (package *lme4*) with p-values obtained from the *t*-statistic.

Significance of the fixed effects was further investigated in the Bayesian set-up using the R function mcmcsamp() (package *lme4*) that generates Monte Carlo Markov Chain samples from the posterior distribution of the parameters of a linear mixed model. The prior on the fixed effects parameters is taken to be locally uniform while the prior on the variance-covariance matrices of the random effects is taken to be the locally non-informative prior. Based on 100,000 samples drawn from the posterior distribution, we calculated the smallest p such that the $(1-p)$ credible interval does not contain the value 0. This parameter was finally used to assess the p-value obtained from the *t*-statistic: if smaller than p, it was considered anticonservative and its value discarded.

Supporting Information

Data S1 *HP* rs72294371 'common polymorphism' flanking sequence. Source: 1000Genome. (http://browser.1000genomes.org/Homo_sapiens/Variation/Summary?r=16:72090747-72091766;v=rs72294371;vdb=variation;vf=13544249).
(DOC)

Data S2 Supplementary Methods. 1. Screening of latent population substructure. 2. Conditional analysis. 3. Meta-analysis. 4. Gene-expression analysis.
(DOC)

Table S1 Conditional regression within the recombination hotspot around rs2000999. UNADJ: unadjusted p-values, SNP: single nucleotide polymorphism, add: additive model.
(XLS)

Table S2 Association of rs2000999 with HDL-cholesterol and Apolipoproteins A1 and B. N: sample size; MAF: Minor Allele Frequency; β: beta coefficient for the effect allele A.
(DOC)

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Author Contributions

Conceived and designed the experiments: PF NCN SVS. Performed the experiments: RMGR AB NBN AD MF LB SES AW PJ LS LC. Analyzed the data: NCN BH CL YL. Contributed reagents/materials/analysis tools: PF SVS. Wrote the paper: PF NCN AB NBN GS MF AW GVD SVS. Study supervision: PF NCN SVS. Literature search: PF NCN AB NBN GS MRL SES MGS JRD SVS. Population providers: PF AM CM PJ LS LC PE MRJ GVD SVS AR. Haptoglobin measurements: GS RMGR SVS. Common polymorphism genotyping: AB NBN AD. Functionality studies/ Gene-expression investigation: MF LB SES AW. Microarray expression profiling of adipose tissue: PJ LS LC. Data interpretation: PF NCN AB NBN MRL MGS DM JRD SVS. Equally contributed as first authors: PF NCN. Equal corresponding authors: PF SVS.

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IX. Biomarqueurs génétiques d'hypertension artérielle et futurs défis suite à l'intégration de l'épigénomique.

Hypertension genetic biomarkers and future challenges integrating epigenomics

Said El Shamieh and Sophie Visvikis-Siest.

Clinica Chimica Acta, Sous Presse.

Malgré de récents progrès techniques visant à élucider la composante génétique de l'HTA, les biomarqueurs découverts n'ont qu'un faible effet et par conséquent, ils expliquent une infime partie de sa variance phénotypique (*missing heritability*).

Peu de données bibliographiques sont disponibles pour affirmer une régulation épigénétique de l'HTA, en effet aucun biomarqueur épigénétique n'a été encore publié.

Dans la présente revue, nous discutons les principales approches utilisées pour étudier la régulation génétique et épigénétique de l'HTA essentielle exclusivement, les biomarqueurs identifiés, leur utilité clinique ainsi que certains défis à surmonter.

En outre, nous proposons une nouvelle catégorie de biomarqueurs génétiques-épigénétiques fonctionnels, les eMethSNPs, et nous fournissons leurs profils hypothétiques d'expression génique pour une régulation génétique de l'HTA via la mADN. Bien qu'ils soient considérés comme peu fréquents, les eMethSNPs pourraient fournir une explication mécanistique pour les biomarqueurs génétiques prédisposant à l'HTA.

Mots clés : Hypertension artérielle, génétique, épigénétique, transcriptomique.

Contribution: *Nous avons participé à la conception de l'idée et effectué la recherche bibliographique. Nous avons également rédigé la première version du manuscrit.*

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Invited critical review

Genetic biomarkers of hypertension and future challenges integrating epigenomics

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ABSTRACT

Essential hypertension is a multifactorial disease, considered to be one of the world's greatest public health problems. Despite recent, major, technical advances aiming to elucidate its genetic component, the discovered biomarkers up to now were reported to have only small effects, explaining consequently a tiny fraction of its phenotypic variance and resulting in a large proportion of missing heritability. Likewise, little evidence is available with regard to the epigenetic regulation of essential hypertension, since no robust biomarkers have yet been reported.

In the current review, we discuss the main approaches used exclusively to study the genetics and epigenetics of essential hypertension, the biomarkers identified, their clinical utility and the difficulties to be overcome. Furthermore, we propose a new category of functional genetic–epigenetic biomarkers, eMethSNPs, and we provide their hypothetical gene expression profiles for a genetic functional regulation of hypertension via DNA methylation. Though believed to be infrequent, eMethSNPs could constitute a new category of mechanistically-based genetic biomarkers predisposing to essential hypertension.

Contents

1. Introduction

By 2025, one third of the world's adult population will be hypertensive (HT) [1]. Despite important advances in our understanding of the pathophysiology of hypertension (HTN), in the availability of prevention and treatment strategies, it remains one of the world's greatest public health problems [2] since it is a major risk factor for cardiovascular diseases (CVDs) [3]. Growing evidence gathered from population studies suggests a worldwide increase in the prevalence of HTN [3].

In contrast to rare familial forms [4], essential HTN is a polygenic trait [5]. Epidemiological studies that have investigated both HTN and blood pressure (BP) regulation have highlighted similar results for high heritability assessment [6]. Genetic and epigenetic factors may be involved.

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The genetics of HTN has been reviewed periodically for at least a decade, and a large number of interesting reviews have classically focused on its genetic regulation [7–10]. In the current paper, in addition to its genetic regulation, we discuss the epigenetic regulation of HTN. More interestingly, we propose a new category of functional biomarkers called eMethSNPs. Furthermore, we provide their hypothetical gene expression profiles for a genetic functional regulation of HTN via DNA methylation (DNAm).

2. Genetic biomarkers of essential hypertension

The genetic contribution to essential HTN comes in the form of multiple alleles (of unknown number and nature) in polygenes that can alter the function and/or the expression of the encoded protein(s), creating endo-phenotypes that complicate into HTN [5]. Family studies have shown that the heritability of essential HTN varies between 31% (single measurement of the systolic (SBP) and diastolic (DBP) blood pressure), 57% (long-term mean of the SBP and DBP phenotype), and 68% (SBP and DBP profiles over 24 h) [8].

Despite considerable recent technical advances aiming to elucidate the genetic components of HTN, discovered genetic biomarkers were reported to have small effects, consequently explaining only a tiny fraction of the phenotypic variance of BP and thus resulting in a considerable proportion of missing heritability.

2.1. Main approaches used to identify genetic biomarkers of hypertension

2.1.1. Candidate gene approach

Candidate genes are genes encoding proteins which could be involved in the regulation of HTN. The candidate gene approach concentrates on at least one gene and involves the use of classic epidemiological techniques, mainly case control studies for the HTN trait. The aim of this approach is to determine whether HTN is associated with given genetic markers, most frequently consisting of single nucleotide polymorphisms (SNPs). Based on our domain of expertise, we have focused the present review on SNPs even if other forms of genetic markers are also implicated [11]. In support of this information, a highly polymorphic variable number of tandem repeats localized 3′ on the human *apoB* gene were reported to be associated with HTN and to have different allelic frequencies in hypertensive individuals (HTs) when compared with normotensive individuals (NTs) [11]. The above-mentioned study, along with many others [12–14], points out the fact that HTN is not exclusively governed by SNPs.

Human candidate gene studies (Table 1) have identified more than 110 genes likely to be implicated in essential HTN. One of the most studied is the *angiotensin I converting enzyme* (*ACE*) gene. Unfortunately, the genetic variants reported were poorly replicated in the different candidate gene studies, and therefore cannot be considered as robust genetic biomarkers of HTN [15].

Despite the use of multiple drug therapy, it appears that 5% of HT patients are resistant to conventional antihypertensive therapy [16]. HTN may be termed resistant when a therapeutic plan that has included lifestyle measures has failed [17]. Subsequent studies have evidenced genetic predisposition acting through genetic biomarkers [18,19]. For example, in a prospective case control study conducted by Cruz-González et al. [17], the authors indicated that the rs2070744C allele in the *NOS3* gene increased the susceptibility to resistant HTN [17].

2.1.2. Genetic linkage studies

Due to the process of genetic recombination that occurs during meiosis, alleles having topographically close loci on the same chromosome are less often separated than alleles that are further apart. This implies that adjacent SNPs located on the same chromosome strand have high chances of forming a haplotype. Linkage analyses study the frequency with which SNP alleles are transmitted with the trait of interest to the next generation (parents to children), without there being any genetic recombination [20]. Since the 1990s, geneticists have concentrated on genetic linkage analyses in order to identify genes involved in the regulation of HTN. Linkage analyses are usually performed on the whole genome, hence the name genome-wide linkage analyses (GWLAs). Because GWLAs are not based on prior knowledge, they can lead to the identification of new loci, giving rise to new hypotheses concerning the pathogenesis of HTN. After finding susceptibility loci, a fine mapping step is needed to narrow the genomic region of interest in order to isolate causal genetic biomarkers [20].

At present, all human chromosomes have been reported to contain regions linked to BP and HTN [21]. Only the long arm of

Table 1
Candidate genes probably involved in the regulation of blood pressure.

Metabolic pathway	Gene symbol	Genomic localization	Nomenclature
Renin angiotensin aldosterone system	*ACE**	17q23	*Angiotensin converting enzyme*
	*AGT**	1q42-q43	*Angiotensinogen*
	*AGTR1**	3q21-q25	*Angiotensin II receptor, type 1*
	AGTR2	Xq22-q23	*Angiotensin II receptor, type 2*
	CYP11B1	8q22	*Cytochrome P450, family 11, subfamily B, polypeptide 1*
	CYP11B2	8q24.3	*Cytochrome P450, family 11, subfamily B, polypeptide 2; aldosterone synthase*
	*REN**	1q32	*Renin*
Sympathetic nervous system	*ADRB1*	10q24-q26	*Adrenergic, beta-1-, receptor*
	ADRB2	5q32-q34	*Adrenergic, beta-2-, receptor*
	ADRB3	8p12-p11.2	*Adrenergic, beta-3-, receptor*
	DRD1	5q35.1	*Dopamine receptor D1*
	DRD2	11q23	*Dopamine receptor D2*
	DRD3	3q13.3	*Dopamine receptor D3*
	DBH	9q34	*Dopamine beta-hydroxylase (dopamine beta-mono-oxygenase)*
	NPY	7p15.1	*Neuropeptide Y*
	NPY1R	4q31.3-q32	*Neuropeptide Y receptor 1*
	PNMT	17q21-q22	*Phenylethanolamine N-methyltransferase*
Sodium transporters	*SCNN1A**	12p13	*Sodium channel, nonvoltage-gated 1 alpha (alpha ENaC)*
	SCNN1B	16p13-p12	*Sodium channel, nonvoltage-gated 1 beta (beta ENaC)*
	SCNN1G	16p13-p12	*Sodium channel, nonvoltage-gated 1 gamma (gamma ENaC)*
	SLC12A3	16q13	*Solute carrier family 12 (sodium/chloride transporters), member 3*
	SLC12A1	15q15-q21.1	*Solute carrier family 12 (sodium/potassium/chloride transporters), member 1*
Steroids	*HSD11B2*	16q22	*Hydroxysteroid (11-beta) dehydrogenase 2*
	NR3C2/MLR	4q31.1	*Nuclear receptor subfamily 3, group C, member 2; mineralocorticoid receptor*
Natriuretic peptides	*NPPB*	1p36.2	*Natriuretic peptide precursor B*
	*NPPA**	1p36.21	*Natriuretic peptide precursor A*
	NPPC	2q24-qter	*Natriuretic peptide precursor C*
	NPR3	5p14-p13	*Natriuretic peptide receptor C/guanylatecyclase C*
Other pathways	*ABCB1**	7q21.1	*ATP-binding cassette, sub-family B (MDR/TAP), member 1*
	*ADD1**	4p16.3	*Adducin 1 (alpha)*
	*ADD2**	2p14-p13	*Adducin 2 (beta)*
	*ADD3**	10q24.2-q24.3	*Adducin 3 (gamma)*
	CYP3A5	7q22.1	*Cytochrome P450 family 3, subfamily A, polypeptide 5*
	*GNB3**	12p13	*Guanine nucleotide binding protein (G protein), beta polypeptide 3*
	EDN-1	6p24.1	*Endothelin-1*
	*LPL**	8p22	*Lipoprotein lipase*
	*NOS3**	7q36	*Nitric oxide synthase 3*
	*PPARG**	3p25	*Peroxisome proliferator-activated receptor gamma*

* Candidate genes studied in our laboratory.

chromosome 2 (Chr. 2q) has been repeatedly linked to BP and/or HTN in 9 different European populations [22–30] and in 5 non-European populations, namely African [31], Afro-American [32], Chinese [33,34] and Samoan [35].

2.1.3. Genome-wide association studies

Genome-wide association studies (GWASs) are based on the concept that common complex diseases are governed by several SNPs with a minor allele frequency greater than 5% in the study population [15]. GWASs have made it possible to take a big step forward compared with GWLAs, because they have been conducted on thousands of ethnically homogeneous individuals, thus investigating hundreds of thousands of SNPs at a time [8].

GWASs use dense maps of SNPs located throughout the human genome to investigate associations between the different genetic variants and the phenotype studied [15]. Unlike the candidate gene approach that targets a set of potential genes, GWASs investigate a large part of the genome without any preconception concerning the identity of the genes involved [15]. The advantage of this approach is that new information can be discovered concerning previously unidentified loci. In this regard, a GWAS published in May 2009 [36] reported that rs2681472 located in the *plasma membrane calcium-transporting ATPase 1* gene (*ATP2B1*) is associated with an increased risk of HTN [36] (Table 2). Padmanabhan et al. [37] found that, in contrast to rs2681472, the SNP rs13333226 in the promoter of the *uromodulin* (*UMOD*) gene was associated with a decreased risk of HTN [36]. The latter genetic biomarker is an expression SNP (eSNP) possibly reducing urinary uromodulin excretion [37].

More recently, in a larger scale meta-analysis (combining data on 200,000 individuals of European ancestry) to detect SNPs with smaller effect sizes, Ehret et al. [38] reported seven other genetic biomarkers that were associated with HTN to different degrees (Table 2). Of the SNPs reported, three were found to be intergenic (Table 2). In addition, only one genetic biomarker, rs1799945 in the *hemochromatosis* (*HFE*) gene that reached genome-wide significance level, was non-synonymous, resulting in an amino acid change with no possible damaging effect on HFE protein structure [39]. Three others were located in gene introns and investigations to detect cis-acting effects in diverse existing expression SNP data sets did not reveal any significant association. Generally speaking, geneticists connect an intergenic SNP to the closest nearby genes. However, eQTL (expression quantitative trait loci) studies have demonstrated that genetic biomarkers can regulate gene expression across the genome. Sex and body mass index interaction analyses were performed, but no further interactions were observed [40].

As expected, the large sample size increased the power to detect novel SNPs with small effect sizes. Accordingly, the magnitude of association for each novel SNP, though highly significant, was modest (0.046–0.17 unit difference per risk allele, Table 2).

3. Epigenetic biomarkers of essential hypertension

Over the last decade it has become ever more apparent that understanding the genome did not finish with unraveling the genetic code [41]. Regulatory information is needed to determine which parts of the genome are active or inactive, and to form a memory system that can be transmitted through cell divisions for proper cell function [41]. This information underpinning heritable gene regulation is encapsulated by the term epigenetics.

The best known epigenetic process is DNAm, characterized by the covalent addition of a methyl group to carbon 5 of a cytosine residue. mDNA is maintained during cell division only in mammals with CpG dinucleotides [41]. A second well-studied example of an epigenetic process is the modification of chromatin, or more precisely covalent modifications of the histones around which is wound the DNA double helix. In most cases, DNAm (especially in the proximal promoter region) is capable of repressing gene transcription [41]. By contrast, the relaxation of condensed histones is a key step for activating gene expression [41].

Although no epigenetic biomarker has yet been reported, three main observations support the participation of a DNAm component in the regulation of essential HTN. Smolarlek et al. [42], found that the overall level of DNAm is lower in the whole blood cells of HTs than in healthy individuals, and that it depends on the progression of the HTN [42]. In addition, Friso et al. [43] reported the proximal promoter of *HSD11B2* gene to be hypermethylated in peripheral blood mononuclear cells from HTs [43]. Another interesting clue towards the implication of epigenetic regulation is that mDNA profiles change (age dependently) during an individual's lifetime in ways specific to given cell types [42]. Therefore, age-related hyper/or hypomethylation could at least hypothetically modify normal gene responsiveness to HTN. To reveal epigenetic biomarkers implicated in HTN etiology, progression and prevention, NHLBI convened a

Table 2
Published genome-wide association studies showing single nucleotide polymorphisms associated with essential hypertension.

First author/date/ journal	Initial sample size	Replication sample size	Region	Reported gene(s)	Strongest SNP-risk allele	Context	RAF in controls	P	OR or beta coefficient and [95% CI]
Levy D May 10, 2009 *Nat Genet.*	29,136 individuals from European ancestry	34,433 individuals from European ancestry	12q21.33	*ATP2B1*	rs2681472-A	Intron	0.83	2×10^{-11}	0.17 increase in log(odds)
Padmanabhan S October 28, 2010 *PLoS Genet.*	1621 Swedish cases, 1699 Swedish controls	19,845 European ancestry cases, 16,541 European ancestry controls	16p12.3	*UMOD*	rs13333226-A	Promoter	0.81	4×10^{-11}	0.13 unit decrease
Ehret GB September 11, 2011 *Nature*	69,395 European ancestry individuals	Up to 133,361 European ancestry individuals	20q13.32	*GNAS, EDN3*	rs6015450-G	Intergenic	0.12	4×10^{-14}	0.11 increase in ln(odds)
			5p13.3	*NPR3, C5orf23*	rs1173771-G	Intergenic	0.60	3×10^{-10}	0.062 increase in ln(odds)
			10p12.33	*CACNB2*	rs4373814-G	Intergenic	0.55	9×10^{-8}	0.046 decrease in ln(odds)
			6p22.2	*HFE*	rs1799945-G	Non-synonymous	0.14	2×10^{-10}	0.095 increase in ln(odds)
			11q22.1	*FLJ32810, TMEM133*	rs633185-G	Intron	0.28	5×10^{-11}	0.07 in ln(odds) decrease
			10q23.33	*PLCE1*	rs932764-G	Intron	0.44	9×10^{-9}	0.055 increase in ln(odds)
			6p21.33	*BAT2, BAT5*	rs805303-G	Intron	0.61	1×10^{-10}	0.054 increase in ln(odds)

RAF: risk allele frequency, P: P-value, OR: odds ratio.
SNPs were extracted from the catalog of genome-wide association studies: http://www.genome.gov/gwastudies/ with a significance threshold of 5×10^{-8}.

working group of multidisciplinary experts in Bethesda, Maryland in June 2011 [44]. The working group has started to identify the main gaps in our current knowledge of HTN epigenetics and a manuscript in a peer-reviewed journal is planned for 2012 [44].

In addition to DNAm, histone modifications and microRNA regulation also seem to be involved in HTN regulation [45,46]. In murine models, Pojoga et al. [45] reported that when fed a high-salt diet, *lysine-specific demethylase-1* (*LSD1*) deficiency was associated with HTN [45]. LSD1 is known to induce histone 3 demethylation, one of the most actively and chemically modified histones [45]. In humans, using microarray-based miRNA expression profiling, Shuqiang et al. [46] compared miRNA expression in plasma samples from HTs and NTs [46]. Importantly, they found a novel link between human cytomegalovirus infection and HTN [46], thus revealing important insights into the pathogenesis of HTN.

3.1. Main approaches used for genome-wide DNA methylation scans

The introduction of powerful high-throughput genetic technologies, in particular SNP arrays, was one of the major steps forward that made large-scale GWASs possible. Epigenomic profiling technologies have only recently reached the stage at which genome-wide DNA methylation scans (GWDMS) are becoming feasible, a motivating step for searching across the genome for differentially methylated regions (DMRs) in HTN.

This section presents a brief description of two currently available approaches for performing a GWDMS. Each approach is subdivided into two main categories depending on the type of analysis (chemical conversion or affinity enrichment) and the type of detection system used (biochips or next-generation sequencing). The choice of a well-defined category depends on many variables including the number of samples examined, the amount of DNA available, the cost and availability of equipment, the detection of absolute or differential methylation, the need for quantitative regional results or other results with a deeper resolution.

3.1.1. Chemical conversion approach

The chemical conversion of cytosine by sodium bisulfite effectively turns cytosines into uracils [47]. When amplified by PCR, the uracils are considered as thymines, whereas methylated cytosine residues are amplified as cytosines [47]. The discovery and optimization of this conversion [47,48] have revolutionized the field of DNAm analysis, since this method shows single base pair resolution. Although microarrays can be coupled, bisulfite conversion technology is much better suited to sequencing-based approaches. Despite being promising, this approach has not yet been combined with microarrays and sequencing-based approaches to identify DMRs in HTN.

3.1.2. Affinity enrichment approach

Affinity enrichment of methylated regions using either specific antibodies for 5 methylated-cytosine residues or using methyl-binding domain-harboring proteins with affinity for methylated genomic DNA has been shown to be a powerful tool for the comprehensive profiling of DNAm [49]. This approach does not result in DNAm information at the single base pair level, but rather yields regional information (global quantification). Both, microarray and next-generation sequencing techniques can be further coupled to the affinity enrichment approach to analyze DNAm on a genomic scale.

In a recent epigenome-wide scan conducted on a supposed healthy aging population of 172 female twins, Bell et al. [50] reported 490 genomic regions that were differentially methylated with chronological age (age differentially methylated regions: aDMRs) [50]. Even with a large number of aDMRs, only four were age-related phenotype DMRs, thus associated with low density lipoproteins (*STAT5A*), lung function (*WT1*), and maternal longevity (*ARL4A*, *TBX20*) [50]. By contrast, none of the previously reported aDMRs were found to be associated with BP or HTN [50]. Therefore, the vast majority of age-related changes in DNAm were not associated with phenotypic measures of healthy aging.

4. Clinical limitations for genetic biomarkers of hypertension and future challenges

Investigating HTN regulation should not be only for cataloging, but also for understanding. We believe that the most important goal at present is to use the existing knowledge in order to develop adequate strategies to treat HTN and prevent its occurrence.

GWASs are powerful tools for identifying new loci associated with HTN [38], but the clinical usefulness of the SNPs identified is practically negligible at this stage. Indeed, many attempts have been made to develop genetic profiles based on the results of GWASs, but their value has been shown to be very limited for predicting the individual risk [36,38] and conventional risk factors and family history are still better predictive biomarkers [6].

With the emergence of novel sequencing techniques (exome sequencing) and next generation GWASs (exome chips), great efforts are being made to identify rare susceptibility variants with large size effects. Rare genetic variants are predicted to have larger effects than common ones [51]. In fact, the theory of evolution predicts that harmful alleles should be rare [51]. Since a disease is acknowledged to be the opposite of a healthy state, its causative genetic variants should be repressed and consequently not be common [51]. In addition, many rare familial diseases are caused by rare SNPs with large effects [51]. The most complete study is probably the one showing that a quarter of cases of intellectual deficiency linked to the X chromosome can be

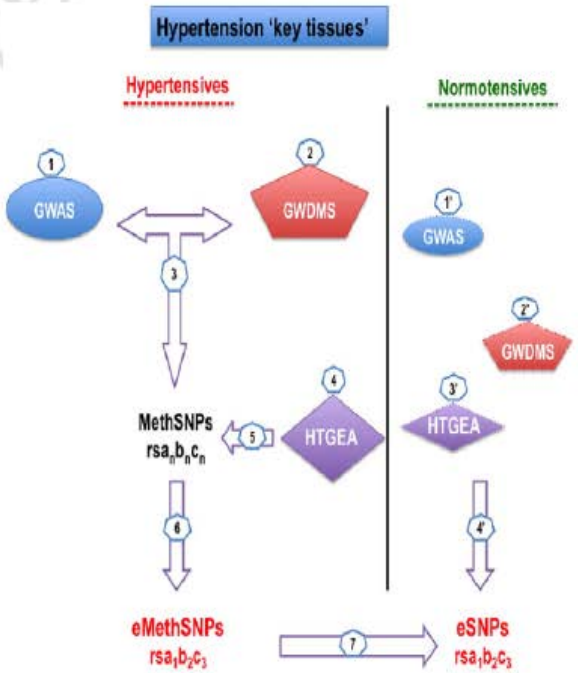

Fig. 1. Integrative approach for revealing eMethSNPs in hypertensive individuals. In the proposed approach, performing both a GWAS and GWDMS in 'key tissues' of hypertensive individuals leads to the identification of MethSNPs. When coupled with HTGEA, a new category of genetic biomarkers called eMethSNPs is generated. eMethSNPs ($rsa_nb_nc_n$) are then matched with their corresponding SNPs ($rsa_1b_2c_3$) in NTs. To identify a set of SNPs affecting gene expression through DNAm in HTs, their corresponding genetic biomarkers in NTs must be eSNPs exclusively. In NTs, GWDMS serve as a negative control for the methylation of eSNPs. The numbers show the successive steps of the proposed approach. GWAS: genome-wide association study, GWDMS: genome-wide DNA methylation scan, HTGEA: high-throughput gene expression analysis, HTs: hypertensive s, SNP: single nucleotide polymorphism, NTs: normotensives.

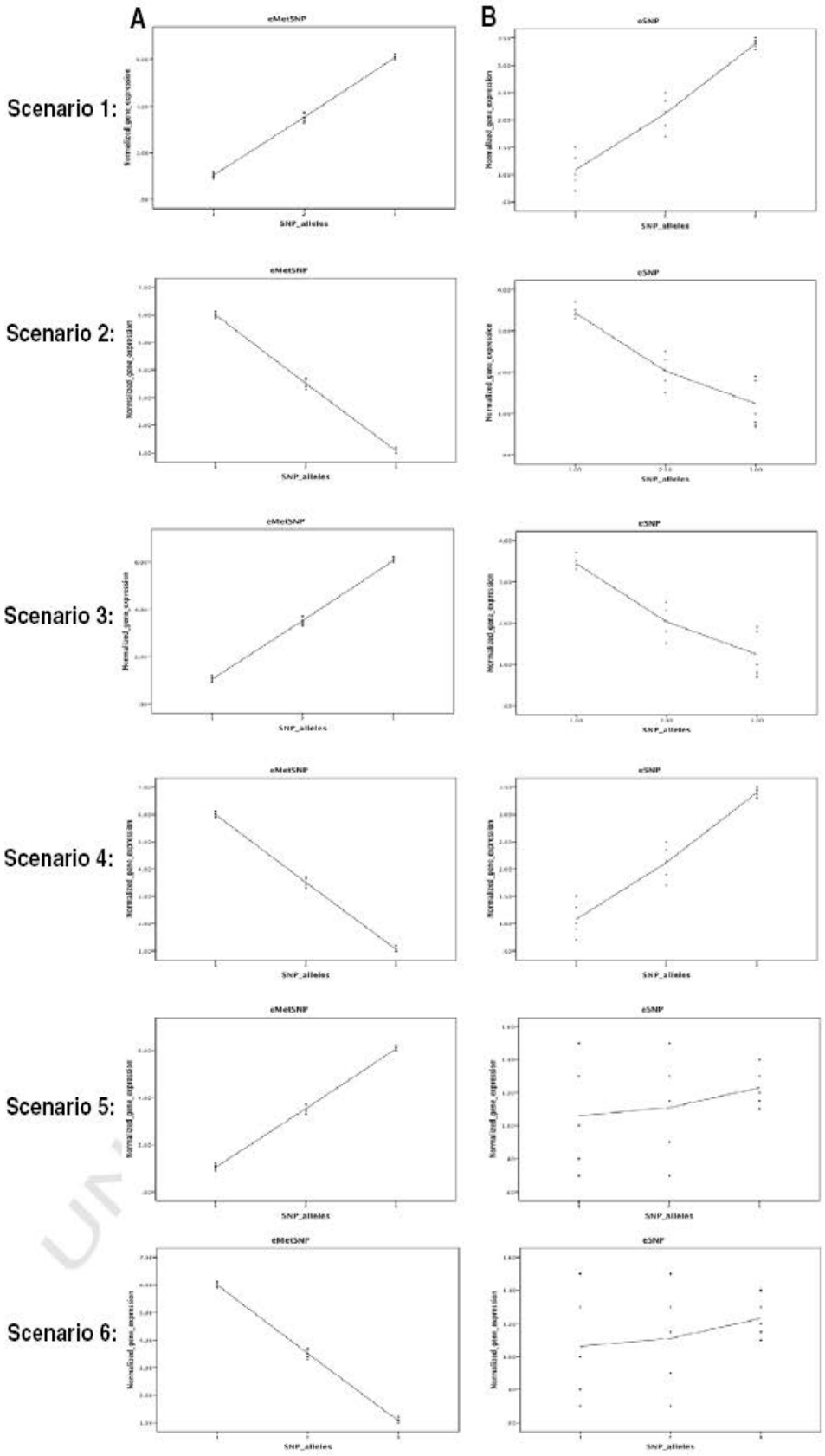

Fig. 2. Hypothetical gene expression profiles for eMethSNPs in hypertensives compared with eSNPs in normotensives. Normalized gene expression was plotted according to the SNP alleles (1: homozygous for the SNP major allele, 2: heterozygous, 3: homozygous for SNP minor allele). Gene expression profiles in hypertensives (black circles) and normotensives (gray circles) are shown under six scenarios.

attributed to rare coding SNPs, which were discovered by exome sequencing of the entire chromosome [52].

Furthermore, synthetic associations could explain the effects of common SNPs. In 2010, Dickson et al. [53] postulated that the associations detected for common SNPs identified in complex human diseases could, in principle, arise from the effect of nearby rare ones [53]. Goldstein et al. [53] explained that a frequent variant contributing to a disease or even a trait could be the synthetic association of a large number of rare SNPs in high LD with this SNP (D′ high, r^2 low) and with much stronger effects [53]. To the best of our knowledge, this hypothesis, which we consider relevant, has yet to be explored in regard to the regulation of HTN.

Studying pairwise epistasis may lead to the identification of new genetic biomarkers implicated in the regulation of HTN. Epistasis is defined as an interaction between two or more loci [54]. It is an important phenomenon because it can modify or mask the effect of a locus [54]. Scanning for epistasis may also identify genes that are not detected by the single locus consensus approach [55]. Investigating epistasis on a genome-wide scale is still a major challenge with some practical concerns. For example, studying pairwise epistasis in 500,000 SNPs would generate a huge number of comparisons (1.2×10^{11} comparisons) and, using simple Bonferroni adjustment, the significance level would be at 4×10^{-13}. At this level, more than 7000 individuals would be required to detect two SNPs jointly responsible for 1% of the phenotypic variance with 90% power [56]. While this is clearly not impossible, conducting such a large number of comparisons are not yet feasible on standard desktop computers. In the support of these concerns, Marchini et al. [56] reported that an exhaustive pair-wise search between 300,000 SNPs in 1000 cases/controls takes approximately 33 h on a ten node cluster [56]. Conducting such an analysis with 500,000 SNPs in 7000 individuals would therefore need at least one month of continuous analysis. Until these practical concerns are overcome, investigating pairwise epistases in a limited set of BP/HTN-associated SNPs in a two-stage design (discovery and replication populations) could generate relevant data and might constitute a first step towards explaining a small part of BP missing heritability [57].

Integrating gene-environment (GxE) interactions on a genome-wide scale could contribute to highlighting the effects of different loci depending on modifiable risk factors, particularly in HTN. However, this strategy calls for large consortia and methodological efforts. Successful GxE studies in a genome-wide context should take into account sample size, exposure assessment and heterogeneity, and have been extensively reviewed and discussed elsewhere [58]. Smith and Day [59] explained that the detection of an interaction required a sample size at least 4 times greater than that required for detecting a main effect of comparable magnitude [59]. Environmental factors may also act via epigenetic mechanisms to modify gene expression [58,60] in human complex diseases [61], although of importance they are yet to be studied in HTN.

Undergoing an integrative approach combining GWAS, GWDMS and high-throughput gene expression analyses in different HTN 'key tissues' (kidney, liver, heart and aorta) of HT individuals would lead to the identification of eMethSNPs (Fig. 1). Once identified in HTs, eMethSNPs ($rsa_1b_2c_3$) are matched with their corresponding genetic biomarkers (having identical SNP IDs) in NTs (Fig. 1). To identify a set of SNPs affecting gene expression through DNAm in HTs, their corresponding genetic biomarkers in NTs should be exclusively eSNPs (Fig. 1). Studying the transcriptome of different tissues implicated in the regulation of HTN is of high diagnostic and prognostic value as these primary models closely mimic the in vivo state, and generate physiologically relevant data [62]. This approach would reveal a new category of functional genetic biomarkers, called eMethSNPs, predisposing to essential HTN and acting via DNAm mechanisms.

In order to study the variation DNAm across the entire genome in a comprehensive and effective way (low false discovery rates), detailed methylome maps (at base pair resolution) are needed for the cells composing these tissues. Such DNAm maps are currently available for human embryonic stem cells and peripheral blood monocytes [63,64]. It is important to note that the eMethSNP approach would not be considered as unique for the discovery of HTN biomarkers since changes in the DNAm pattern have already been described in other chronic diseases such as type 1 diabetes and breast cancer [26,65]. For example, Harlid et al. [65] used a candidate CpG SNP approach to identify a novel possible risk SNP, rs7766585 in the *ESR1* gene, a locus that has been previously marked as a hotspot for breast cancer [65].

In Fig. 2 we provide hypothetical gene expression profiles for eMethSNPs and eSNPs in HTs and NTs respectively. In order to understand how DNAm acts on the genetic biomarkers associated with gene expression, eMethSNPs in HTs are compared with their corresponding eSNPs in NTs. The gene expression of eMethSNPs and eSNPs is illustrated and compared in order to generate six different scenarios for functional genetic regulation via DNAm (Fig. 2). eMethSNPs are associated with an increase or a decrease in gene expression (scenarios 1A-6A). This is also the case for eSNPs (scenarios 1B-4B) but with the additional possibility of having no effect (scenarios 5B and 6B). DNAm would therefore accentuate (scenarios 1 and 2), invert (scenarios 3 and 4) or even provide novel associations for eMethSNPs (scenarios 5 and 6) when compared with eSNPs. As expected, adjustment for methylation partially removes (scenarios 1 and 2) or completely removes (scenarios 3–6) the eMethSNP associations.

5. Concluding remarks

Various conclusions can be drawn from the existing reviews that discuss the genetics of HTN [10,15,66,67]. Some of these could be considered as common: (1) the genetic variants reported using candidate gene approaches were poorly replicated among studies and therefore cannot be considered as genetic biomarkers for HTN, (2) GWLAs showed that all human chromosomes contain regions linked to BP and HTN, (3) at present 9 robust genetic biomarkers for HTN have been identified by GWA studies, (4) the clinical usefulness of genetic biomarkers for HTN is almost negligible at this stage, (5) GxG, GxE and rare variants are expected to have large effects, (6) emerging evidence suggests that epigenetic (DNAm and histone modifications) and miRNA regulations are also implicated in HTN regulation, and (7) there is a real need to identify novel molecular pathways involved in HTN in order to define new preventive and/or treatment strategies.

The present review exclusively discusses and proposes the use of eMethSNPs. Although believed to be not very frequent in the genera population, eMethSNPs could provide relevant mechanistically-based genetic biomarkers of essential HTN.

Conflict of interest

None.

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Notre équipe d'accueil, l'EA-4373 'Génétique Cardiovasculaire', a une longue expérience dans l'étude de la génétique des MCV et a pu mettre en évidence de nombreux variants et phénotypes intermédiaires associés à la PA et l'HTA.

Jusqu'en 2008, l'approche gène-candidat était la principale méthodologie utilisée. Cependant, en 2009, notre équipe a initié une approche pangénomique dans le cadre du projet BioIntelligence soutenu par l'Agence d'Innovation Industrielle (A2I) et signé en avril 2010 avec l'INSERM transfert. Nous sommes tenus à la confidentialité en ce qui concerne les modalités de ce projet, néanmoins il est important de noter qu'il s'agit d'un projet collaboratif, financé par OSEO, et visant à proposer aux industriels des sciences de la vie, et en particulier aux industriels pharmaceutiques, des biomarqueurs pertinents en pharmacogénétique et pharmacogénomique. Notre thèse rentre dans ce contexte.

Les GWAS sont des outils performants pour identifier de nouveaux loci associés à des maladies et traits communs, fournissant ainsi des informations clés dans leurs physiopathologies. Cependant, l'utilité clinique des SNPs identifiés par GWAS est quasi négligeable à ce stade. En effet, de nombreuses tentatives ont eu lieu pour élaborer des profils génétiques en utilisant les résultats de GWAS, mais celles-ci avaient des valeurs très limitées lors de la prédiction du risque personnalisé, indiquant que les facteurs de risque conventionnels et les antécédents familiaux sont de meilleurs bio-marqueurs prédictifs [78]. Malgré que plusieurs entreprises fournissent des tests génétiques directs ou *direct-to-consumer genetic tests* qui évaluent « la prédisposition » d'un individu à certaines maladies complexes telles que le diabète type 2 et le cancer du sein, très peu de fournisseurs de soins de santé majeurs en Europe et aux Etats-Unis ont adopté ces tests pour la prédiction d'HTA. Afin d'organiser ce domaine émergent, la FDA a indiqué que les « *direct-to-consumer genetic tests »* devraient être considérés comme des dispositifs médicaux nécessitant leur approbation pour un usage commercial [78].

L'étude de la génétique d'HTA à l'échelle génomique a incontestablement révolutionné la manière d'appréhender son étiologie. Néanmoins, cette approche en est encore à ses balbutiements et de nombreux challenges méthodologiques restent à relever.

Comme évoqué précédemment, les facteurs environnementaux exercent un effet important sur la génétique de la PA et de l'HTA vie des interactions complexes. Ces interactions ne sont cependant que faiblement investiguées. Pourtant, elles sont en partie responsables des discordances observées (présentation des travaux , chapitre 3, publication N^{o}1). L'effet des SNPs varie avec le temps, comme le font leurs interactions avec des facteurs environnementaux (présentation des travaux , chapitre 3, publication N^{o}5).

Les généticiens définissent la proportion d'héritabilité expliquée (π) d'un trait pour être le ratio entre h^2 connue/h^2 totale. L'héritabilité connue constitue la proportion de la variation phénotypique expliquée par les effets additifs des SNPs connus [79]. Cependant, l'héritabilité totale, est la proportion de la variance phénotypique attribuable aux effets additifs de tous les variants génétiques, y compris ceux qui n'ont pas été découverts [79]. Le numérateur peut être calculé directement à partir des effets mesurés des SNPs, mais le dénominateur est déduit indirectement à partir des données démographiques.

L'opinion qui prévaut parmi les généticiens est que l'explication de *missing heritability* réside dans le numérateur, où plusieurs SNPs restent à découvrir [79]. Par contre, Eric Lander et ses collègues [79] expliquent qu'une partie importante de *missing heritability* peut ne pas être due à de nouveaux SNPs [79]. Selon eux, les études actuelles utilisent des estimations d'h^2 totale qui ne sont pas conformes, et qui surestiment sérieusement le dénominateur (h^2 totale) et sous-estiment donc la π [79]. En conséquence, même lorsque tous les SNPs affectant un trait sont découverts, la π peut être bien en deçà de 100%. Ils se réfèrent à cette 'lacune' par l'héritabilité fantôme ou *phantom heritability* [79].

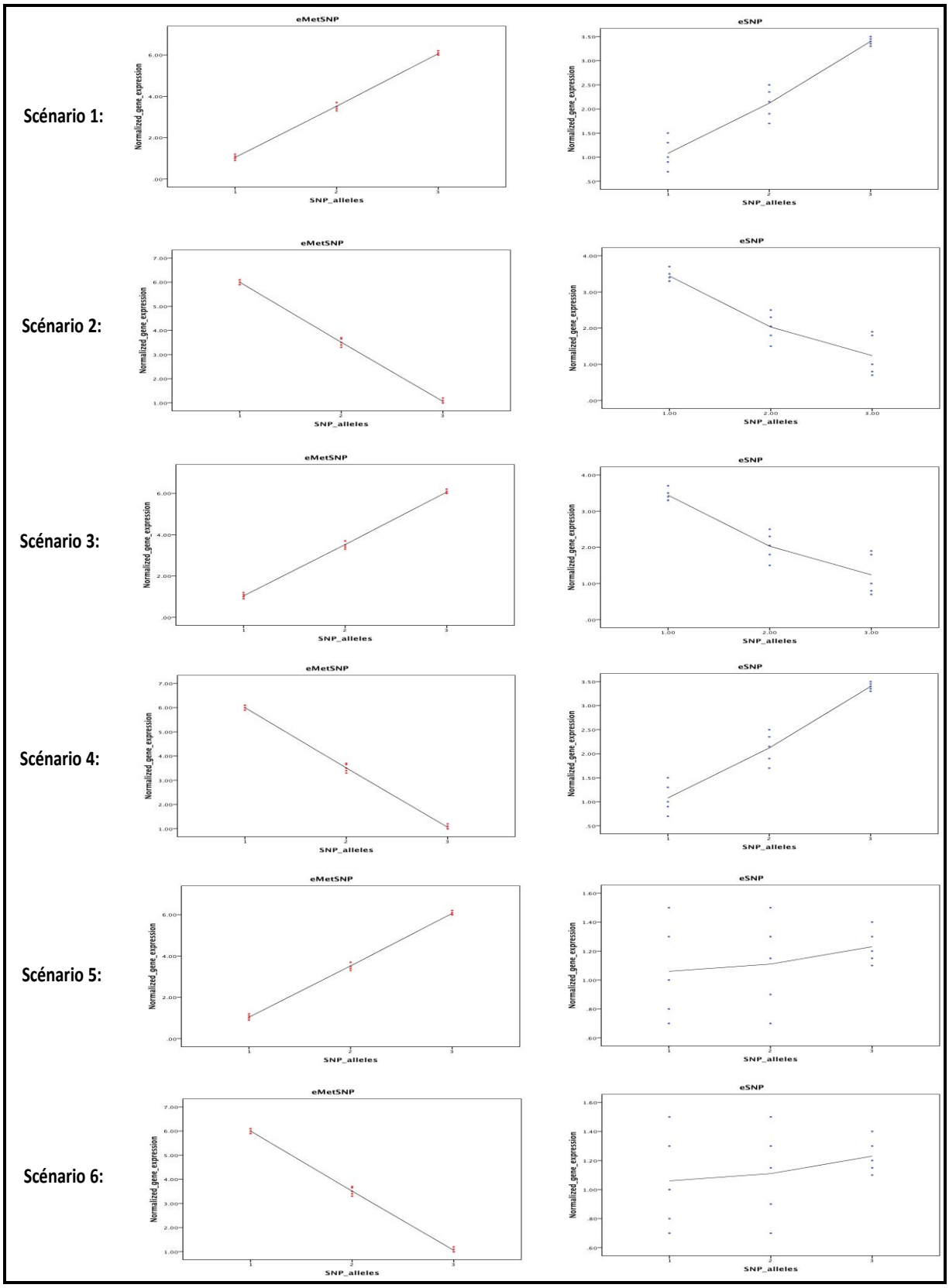
La réalisation d'une analyse intégrative combinant à la fois, les SNPs, l'expression génique et la mADN dans des tissus clés d'HTA pourrait identifier des SNPs affectants l'expression

génique via la mADN, fournissant ainsi des preuves concernant le partage des mécanismes génétiques et épigénétiques [80]. Cette approche pourrait identifier une nouvelle catégorie de SNPs fonctionnels, nommés eMethSNPs, prédisposant à l'HTA essentielle et exerçant leurs effets par les mécanismes de mADN.

Par comparaison aux analyses de mADN à l'échelle génomique, l'avantage le plus marquant des GWASs est la mise en place d'une carte détaillée de SNPs le long du génome humain qui a permis de choisir des 'SNPs étiquettes' assurant une bonne résolution de génotypage à un coût raisonnable (présentation des travaux , chapitre 3, publication N°9). Afin de mener ce même type de balayage épigénomique, de nouvelles cartes de méthylomes à haute résolution sont nécessaires pour décortiquer les profils de méthylation des cellules composant les tissus clés d'HTA (présentation des travaux , chapitre 3, publication N°9). De telles cartes (à résolution de pb unique) sont disponibles pour les cellules souches embryonnaires humaines, les fibroblastes fœtaux et les PBMCs humains [81].

Nous avons fourni dans la Figure 17 les profils d'expression génique hypothétiques pour les eMethSNPs chez des individus HT. Les profils d'expression génique des eMethSNPs chez les HT et des eSNPs chez les NT ont été illustrés et comparés afin de générer six scénarios différents (Figure 17). Comme le montre la figure précédente, la mADN pourrait accentuer (scénarios 1 et 2), inverser (scénarios 3 et 4) ou même créer de nouvelles associations des eMethSNPs (scénarios 5 et 6) chez les HT comparés aux eSNPs des NT. Comme prévu, l'ajustement à la méthylation supprimera partiellement (scénarios 1 et 2) ou complètement (scénarios 3-6) l'éffet des eMethSNPs (présentation des travaux , chapitre 3, publication N°9).

Figure 17: Profils hypothétiques d'expression génique des eMethSNPs chez les hypertendus comparés aux eSNPs chez les normotendus.



Les taux d'expression génique ont été normalisés et tracés selon les différents allèles du *single nucleotide polymorphism* (1: homozygotes pour l'allèle majeur, 2: hétérozygotes, 3: homozygotes pour l'allèle mineur). Les profils d'expression génique chez les hypertendus (cercles rouges) et normotendus (cercles bleus) sont présentés sous la forme de six scénarios.

Tout au long des trois années, notre travail a permis de mieux comprendre les déterminants génétiques de la PA avec des résultats précis et originaux dans le domaine des interactions gène-gène et gène-environnement. En effet, nous avons montré que la présence d'un état inflammatoire chronique de bas niveau pourrait anticiper le développement d'HTA. Cette constatation suggère que l'altération des taux des médiateurs inflammatoires observée chez les individus HT ne peut pas être uniquement attribuée aux lésions vasculaires induites par les niveaux élevés de PA. Lors de ce travail, un intéressant axe de recherche, notamment la transcriptomique, a été suivie pour mettre en évidence l'implication des médiateurs inflammatoires, lipidiques, immuns et autres.., dans la prédisposition à l'HTA.

Plus particulièrement, nous avons réussi à :

A- Identifier une nouvelle interaction épistasique fonctionnelle entre le rs5355C>T du gène *SELE* et le rs6046G>A du gène *F7* associée à la PAS.

De nombreuses études ont rapporté qu'un SNP pouvait réguler l'expression d'un gène localisé bien au-delà sur le chromosome voire sur un chromosome différent. Dans l'étude fonctionnelle de deux SNPs (rs6046G>A du gène *F7* et rs5035C>T du gène *SELE*) (présentation des travaux , chapitre 3, publication N^{o}2), nous avions réalisé une étude *eQTL* de ces deux SNPs et de l'expression de dix gènes impliqués dans les mécanismes d'inflammation. Il serait intéressant de réaliser si possible le profilage haut débit d'une centaine d'échantillons d'ARN provenant des tissus essentiellement impliqués dans la régulation de la PA tels que les reins, le cœur, le foie et les aortes. Nous pourrions ainsi révéler des voies moléculaires intéressantes en tant que cibles thérapeutiques. Ce type d'étude n'a jamais été réellement mis en place à cette échelle à cause des problèmes éthiques que cela implique.

Nous ne devrions pas négliger les SNPs candidats au sein des voies biologiques car ceux-ci pourront être d'une grande utilité. Tomaszewski et al [82] ont montré qu'un score de risque génétique, englobant les SNPs de la voie de signalisation des facteurs de croissance des fibroblastes, semble expliquer une plus grande proportion de la variation phénotypique d'HTA qu'un nombre similaire de top SNPs identifiés par GWAS [82]. Cela indique que les

connaissances biochimiques et moléculaires des modèles biologiques sous-jacents pourraient améliorer la variation expliquée.

Dans notre étude, nous avons trouvé que le SNP rs6046G>A du gène *F7* est fonctionnel suite à son association avec une augmentation des taux d'ARNm de *NAMPT* chez des individus supposés sains. La NAMPT a été au départ considérée comme une cytokine, car la recherche pour décrire ses rôles a été essentiellement orientée vers son implication dans le système immunitaire et l'inflammation. Elle a été suggérée comme un bio-marqueur pour l'inflammation pulmonaire aiguë [83]. La NAMPT semble avoir des propriétés pro-inflammatoires multiples et il est proposé qu'elle soit impliquée dans plusieurs mécanismes de la régulation du système immunitaire [84]. Par exemple, l'incubation des leucocytes humains avec de la NAMPT recombinante active ces cellules et induit la production des cytokines. Une revue récente récapitule les travaux de recherche effectués sur la NAMPT et ses rôles dans l'inflammation [84]. Ils soulignent que des taux élevés de NAMPT circulante sont observés dans le cas d'une multitude de maladies inflammatoires. L'expression de cette molécule dans les PBMCs inactivés (absence d'induction *in vitro*) et non exposés à des conditions dites d'inflammation ou de micro-inflammation, suggère une éventuelle implication de cette molécule dans l'inflammation de faible niveau. En effet, les sujets étudiés ne souffrent pas de maladies inflammatoires aigües ou chroniques et les taux circulants des marqueurs d'inflammation classiques, confirment l'absence d'aucune manifestation clinique d'inflammation.

Nous avons montré que les expressions des ARNm de *LL-37* et de *DEFA1-3* dans les PBMCs sont positivement associées avec la PAS et expliquent 4% de sa variabilité phénotypique (présentation des travaux , chapitre 3, publication N^{o}2). En parallèle avec nos observations, Bernard et al [85] ont mentionné que les dysfonctionnements chroniques des systèmes immunitaire et vasculaire sont fortement associés chez l'homme et chez l'animal HT [84]. Plus intéressant encore, des altérations du système immunitaire, associées aux lymphocytes T, ont été rapportées chez un modèle de rats HT [86;87]. Des études in vitro ont suggéré un lien entre le peptide *LL-37* et la fonction vasomotrice. En 2008, Berkestedt et al [88] ont montré que le peptide *LL-37* induit la relaxation endothélium-dépendante des segments de veines humaines ex vivo, un effet médié par le récepteur *FPRL1* endothélial [88].

Nos résultats sont aussi en accord avec ceux publiés par Saraheimo et al [89] qui ont montré que les taux circulants des DEFA1-3 sont associés à la PAS dans une population d'individus Européens diabétiques [89]. Sur la base des résultats de notre présente étude et les données de la littérature, l'expression des gènes *DEFA1-3* et *LL-37* pourrait être impliquée dans les processus immuno-inflammatoires contribuant au dysfonctionnement vasculaire et à l'HTA par la suite (présentation des travaux , chapitre 3, publication N°2).

B- Identifier deux interactions épistasiques associées aux niveaux de PAD.

Nous avons également observé deux interactions épistasiques pour tous les traits de PA : rs1041163T>C dans *VCAM1* interagit avec rs1367117G>A dans *APOB* et rs3741240G>A dans *SCGB1A1* interagit avec rs1800590T>G dans *LPL* (présentation des travaux , chapitre 3, publication N°3). Dans la population de réplication, ces 2 interactions étaient de nouveau trouvées significatives avec la PAD (présentation des travaux , chapitre 3, publication N°3).

L'étude de la bibliographie permis de mettre en évidence un mécanisme hypothétique concernant l'interaction entre le rs3741240G>A dans *SCGB1A1* et le rs1800590T>G dans *LPL*. Le SNP rs3741240 est un variant fonctionnel qui a été démontrer comme capable d'inhiber la phospholipase A2 et de diminuer les taux plasmatiques des HDL-C (*high-density lipoprotein-cholesterol*) [90]. L'ensemble de ces résultats suggère que la *LPL* et la *SCGB1A1* pourraient influencer la PA via les taux d'HDL-C [90;91].

Cette étude comme l'étude précédente a ainsi pu démontrer que les interconnexions pourraient exister entre différentes voies métaboliques et de ce fait d'éventuelles cibles thérapeutiques impliquées dans la physiopathologie de l'HTA pourront être isolées.

Il est important de noter que des 'difficultés pratiques' de modélisation, empêchent aussi la prise en compte des interactions épistasiques à l'échelle génomique. A titre d'exemple, la recherche d'épistasie par paires de loci entre 500,000 SNPs corresponds à mener $1{,}2x10^{11}$ analyses et portera donc le seuil de *genome-wide significance* à $P=4x10^{-13}$ (présentation des travaux , chapitre 3, publication N°9). A ce niveau, plus de 7,000 individus seront nécessaires pour détecter deux SNPs conjointement responsables de 1% de la variance phénotypique d'un trait donné ayant une puissance statistique de 90% (manuel de référence

épigénétique). La réalisation de ce grand nombre de comparaisons n'est pas encore possible sur les ordinateurs de bureau standard.

Dans cette même direction, Marchini et al (15793588) annoncent que l'étude des interactions épistasiques entre 300,000 SNPs chez 1,000 individus (cas/contrôles) nécessitera ≈33 heures d'analyses continues (15793588). Par conséquent, la réalisation de telles analyses entre 500,000 SNPs chez 7,000 individus faudrait au moins un mois de l'analyse en continu (présentation des travaux , chapitre 3, publication N°9). Dans ces conditions, force est de constater que les approches gène-candidat ne font pas encore partie du passé.

C- Identifier un nouveau SNP inter-génique fonctionnel (le rs7556897T>C *SLC19A3-CCL20*), capable d'interagir avec l'obésité pour modifier les niveaux de PA chez les enfants.

Ce résultat (présentation des travaux , chapitre 3, publication N°4) souligne la nécessité de placer les GWAS dans une démarche intégrée permettant de passer à une approche gène candidat lorsqu'un design plus complexe est nécessaire à une meilleure compréhension des associations observées.

Il est important aussi de signaler que la plupart des SNPs qui ont été identifiés par les GWAS sont localisés dans des régions inter-géniques non codantes. D'une façon générale, les généticiens se focalisent sur le gène le plus proche mais aucune preuve biochimique fonctionnelle n'indique que le SNP soit réellement associé à ce gène. Quand le gène le plus proche, normalement en position cis, code pour une protéine qui a des fonctions connues dans des tissus reliés de près ou de loin à la régulation de PA, les arguments en faveur du lien entre le SNP et ce gène sont plus forts et les désirs d'investigation moléculaire peuvent s'enclencher. Contrairement à d'autres traits, les relations SNP-ARNm en PA sont bien plus compliquées. Dans la plupart des cas, la fonction et/ou l'expression de la protéine codée par le gène le plus proche sont floues. Par comparaison aux récentes GWASs de PA, c'est le deuxième cas de figure qui a primé.

Enfin, notons que jusqu'à ce jour, aucune investigation fonctionnelle à haut débit n'a été menée sur des cellules primaires. CCL20 est une cytokine de 96 a.a faisant partie de la famille des chimiokines. Elle a été montrée fortement chimiotactique envers les lymphocytes

et attire faiblement les neutrophiles [67]. De nombreuses molécules peuvent induire l'expression de son gène, notamment le lipopolysaccharide (composant essentiel de la paroi des bactéries Gram négatif), le TNF-α et la NAMPT [92]. *CCL20* est exprimé dans de nombreux tissus, avec une grande abondance dans les lymphocytes du sang périphérique, d'où l'intérêt d'investiguer les PBMCs.

D- Acquérir des connaissances sur le rôle du rs5030878T>C du gène *FPR1,* de l'allèle KL-VS du gène *Klotho*, de l'haplotype CAT1/2 du gène *CAT* dans la régulation de PA.

Nous avons trouvé que le rs5030878T>C du gène *FPR1* et l'allèle KL-VS du gène *Klotho* interagissaient avec l'âge et le traitement antihypertenseur respectivement pour influencer les niveaux de PA (présentation des travaux , chapitre 3, publications N°5 et 6). Contrairement aux deux variants précédemment décrits, l'haplotype CAT1/2 du gène *CAT* n'était pas trouvé associé avec les niveaux de PA, cependant il interagissait avec la PAS pour influencer le vieillissement artériel.

Peu d'informations sont dévoilées sur le rôle mécanistique de *FPR1* dans la régulation de PA. Cependant, son ligand canonique, le tripeptide *N-formyl-methionine-leucine-phenylalanine* (fMLP) pourrait avoir un rôle [93;94]. Keitoku et al [91] ont montré que ce tripeptide hydrophobe produit des changements de tension passagers au niveau des artères coronaires humaines [91]. En effet, lors de sa liaison, fMLP déclenche la génération d'espèces réactives de l'oxygène en activant l'enzyme NADPH oxydase [95], un facteur important lors du dysfonctionnement endothélial aboutissant à l'augmentation des niveaux de PA [96]. Plusieurs domaines sont cruciaux pour une liaison à haute affinité fMLP-FPR1, avec un déterminant majeur situé dans la première boucle extracellulaire et ses domaines adjacents (8349692). Même si le SNP rs5030878T>C du gène *FPR1* est situé hors de la région du gène codante pour la première boucle extracellulaire, il en résulte une substitution intéressante d'acide aminé (Ile11Thr), car ceci ajoute des résidus hydrophobes à l'extrémité N-terminale extracellulaire.

D'une façon similaire, le mécanisme moléculaire par lequel l'allèle KL-VS pourrait moduler la PA n'est pas encore clair. Cependant, les études "*in vivo*" sont en faveur d'un rôle protecteur de ce gène contre le dysfonctionnement endothélial [97]. De même, les modèles de rats

OLETF ont montré que la surexpression de klotho pourrait améliorer la fonction endothéliale vasculaire et pourrait réduire la PA [97]. En se basant sur cette constatation, on pourrait suggérer un rôle fonctionnel pour l'allèle KL-VS par augmentation des niveaux de protéine Klotho. Du fait que l'allèle KL-VS est significativement associé à une baisse de la PAS et de la PP (présentation des travaux , chapitre 3, publication N°6), ceci suggère une implication dans la rigidité artérielle. Du fait que des niveaux bas de PAS et de PP reflètent dans la plupart des cas une rigidité artérielle plus faible, nous avons exploré une éventuelle relation avec cet allèle. Malheureusement, aucune association significative entre la PAD et le génotype KL-VS/KL-VS n'a été observée.

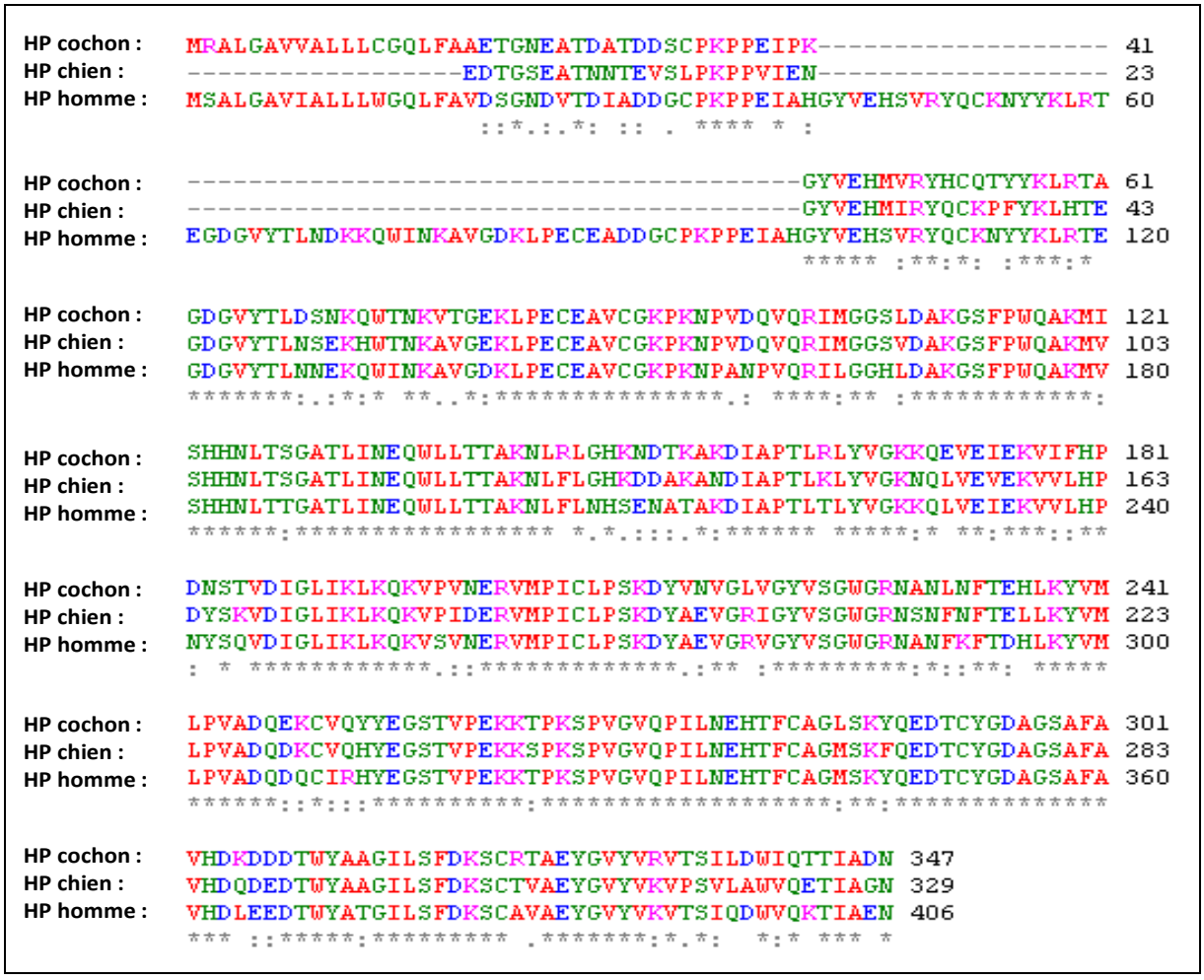
De plus, nous avons étudié dans quelle mesure l'haplotype CAT1/2 du gène *CAT* pourrait être impliqué dans le processus de vieillissement artériel (présentation des travaux , chapitre 3, publication N°7). Nous avons trouvé que l'haplotype CAT1/2 du gène *CAT* interagit avec la PAS et l'âge pour influencer le diamètre de l'artère carotide. Dans cette même direction, l'haplotype CAT1/2 était associé à un plus grand nombre de plaques d'athérome.

E- Identifier un SNP, le rs2000999G>A, expliquant quasiment la moitié de l'héritabilité génétique de l'haptoglobine.

Etant donné la position centrale de l'HP dans l'inflammation et l'homologie de 80% entre les séquences protéiques d'HP canine, porcine et humaine (Figure 18), la présente étude pourrait faire établir un lien indirect de l'inflammation avec l'HTA. En effet, en se basant sur les résultats moléculaires trouvés chez les modèles canins et porcins, un brevet vient d'être déposé au sein de l'US *Department of Health and Human Services, National institute of Health* ayant pour but de baisser les niveaux de PA élevés chez les patients ayant des taux élevés d'hémoglobine dans le sang (cas des anémies hémolytiques et malaria sévère). Cette invention serait efficace suite à l'injection des mimes peptidiques d'HP dans le sang [98].

En pratique clinique, le génotypage du rs2000999G>A que nous avons identifié (présentation des travaux , chapitre 3, publication N°8) pourrait donc identifier précocement des sujets à risque de développer l'HTA suite à la libération d'hémoglobine dans le sang des patients ayant une anémie hémolytique ou une malaria sévère.

Figure 18: Alignement multiple des séquences protéiques d'haptoglobine chez le cochon, le chien et l'homme.



L'alignement multiple a été obtenu avec le logiciel Clustal W. La comparaison de la séquence d'haptoglobine chez le cochon (347 a.a) avec celle du chien (329 a.a) a montré une homologie de 81%. La comparaison de la séquence d'haptoglobine chez le cochon (347 a.a) avec celle de l'homme (406 a.a) a montré une homologie de 79%. La comparaison de la séquence d'haptoglobine chez le chien (329 a.a) avec celle de l'homme (406 a.a) a montré une homologie de 79%. En moyenne nous avons remarqué une homologie de 80% entre les trois espèces.

* acide aminé conservé, : acide aminé moyennement conservé, . acide aminé peu conservé, a.a : acide aminé.

CONCLUSION :

Lors de ce travail, effectué sur des populations européennes (en majorité françaises), nous avons trouvé des variants génétiques qui semblent réguler la PA, et capables d'influencer ses niveaux via des interactions gène-gène et gène-environnement (Figure 19).

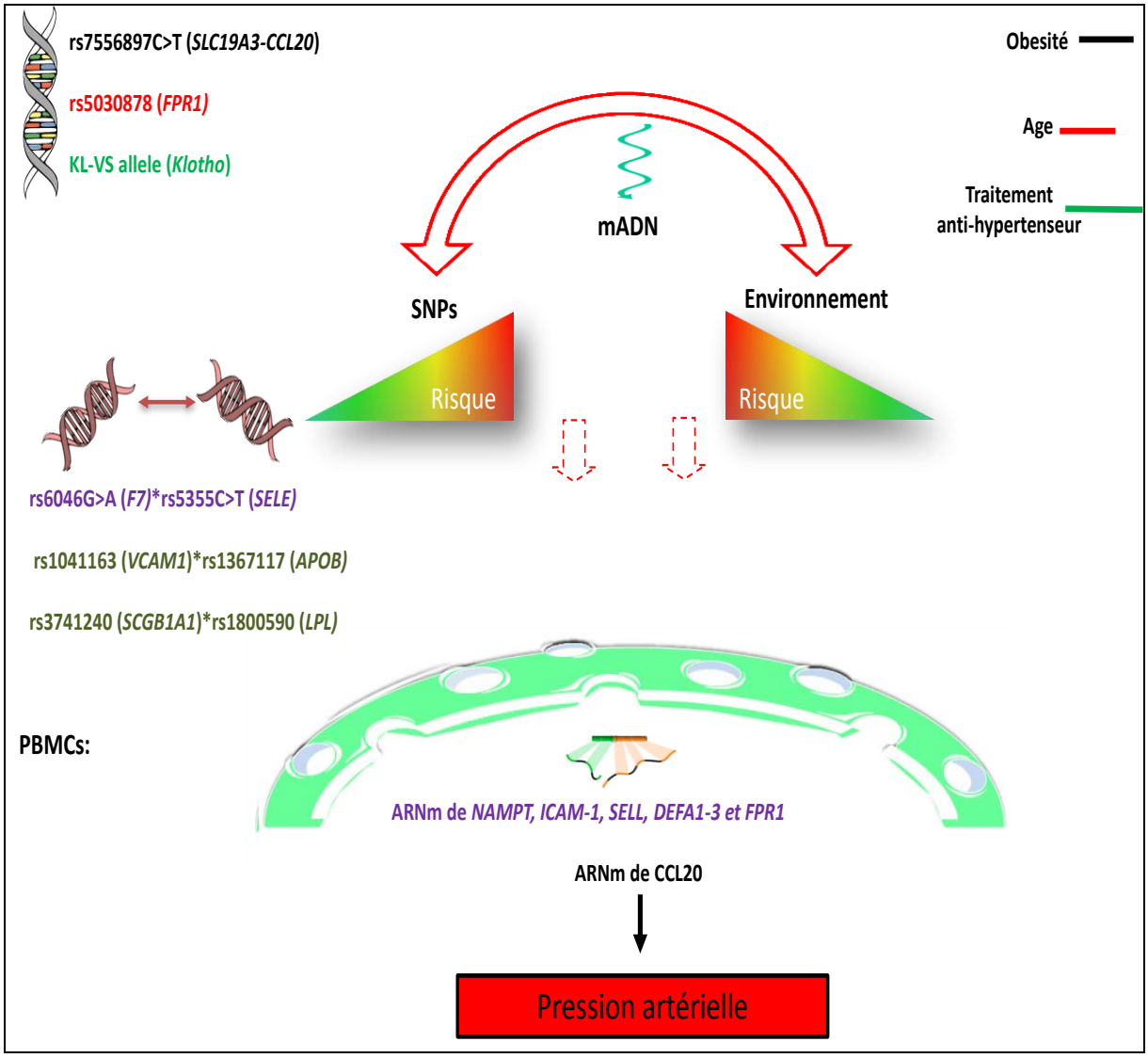
Les interactions identifiées dites 'fonctionnelles', au cours de notre thèse, montrent que l'inflammation de faible niveau est impliquée dans la régulation de la PA. L'ensemble de nos données implique une mosaïque d'interactions complexes entre l'inflammation, l'activation cellulaire au niveau des vaisseaux et les changements structurels dans les artères.

Sans aucun doute, l'intégration des interactions gène-gène et gène-environnement à l'échelle pangénomique, permettra de dévoiler une grande partie de la part d'ombre de l'héritabilité génétique. En attendant, l'investigation du rôle des SNPs rares dans l'étiologie de l'HTA révélera dans un futur prochain des voies inédites et isolera des biomarqueurs génétiques exerçant de plus grands effets.

Plus spécifiquement, un résultat majeur de notre thèse concerne la NAMPT qui a été identifiée comme étant une adipokine principalement sécrétée par le tissu adipeux viscéral à la fois chez l'humain et chez la souris [99]. Son expression était associée avec celle de TNF-α et l'IMC dans les PBMCs et le tissu adipocytaire des enfants supposés sains [99]. Dans cette même direction, nous avons démontré que l'expression des gènes *DEFA1-3*, *LL-37* et *CCL20* ont été liées à l'IMC. Jusqu'au jour où le rôle des SNPs identifiés dans la prédisposition à l'HTA et leur utilité clinique seront ultérieurement validés, il serait judicieux de lutter contre les facteurs de risque conventionnels d'HTA, surtout en maintenant un poids idéal (IMC<25Kg/m^2) en ayant une alimentation équilibrée et en pratiquant une activité physique régulière.

Au-delà de la génétique, l'épigénétique et plus spécifiquement la mADN, semble être impliquée dans la régulation de la PA et de l'HTA. Des investigations plus approfondies sont nécessaires.

Figure 19: Interactions gène-gène gène-environnement identifiées comme associées avec la pression artérielle.



Les variants génétiques sont schématisés en haut et à gauche de la figure. Les facteurs environnementaux sont schématisés en haut et à droite de la figure. Le code couleur a été utilisé pour designer une même interaction (gène-gène et gène-environnement). Parmi la panoplie des SNPs, rs6046G>A du gène *F7* et rs7556897C>T du locus *SLC19A3-CCL20* ont été trouvés fonctionnels via leurs associations avec l'expression génique de *NAMPT* et de *CCL20* dans les PBMCs des sujets supposés sains. L'ensemble de ces variants génétiques est rapporté pour être associer avec la PA dans des populations européennes (en majorité françaises).

mADN: méthylation d'ADN, PBMCs : *peripheral blood mononuclear cells,* SNPs : *single nucleotide polymorphisms*.

PERSPECTIVES :

A- Il est démontré que le niveau d'expression protéique d'un gène est biologiquement plus significatif que son niveau d'expression transcriptionnelle. Cela peut s'expliquer par des modifications post-transcriptionnelles qui peuvent affecter aussi bien la stabilité que le taux des ARNm disponibles pour être traduits en protéines correctement repliées, et par conséquent fonctionnelles. Dans notre laboratoire, il a été montré que le peptide LL-37 est exprimé dans les PBMCs et que cette expression est positivement corrélée avec son niveau d'expression transcriptionnelle dans ces mêmes cellules. D'autres études avaient déjà montré la présence de corrélation entre le taux d'ARNm et les peptides LL-37 dans d'autres tissus [100;101]. Ces résultats suggèrent que comme les ARNm, le taux peptidique de LL-37 dans les PBMCs pourrait être corrélé aux niveaux de PA. Toutefois, ces relations restent à vérifier en réalisant des études d'association entre l'expression protéique de *LL-37* et de *DEFA 1-3* dans les PBMCs et les niveaux de PA. En revanche, aucune corrélation n'est observée entre le taux circulant des peptides LL-37 et DEFA1-3 et leur expression dans les PBMCs (ARNm et protéines). Ceci implique que la source essentielle des niveaux plasmatiques de LL-37 et de DEFA1-3 n'est pas exclusivement les PBMCs, d'où l'intérêt de quantifier leur taux dans le sérum/plasma. De même, il est important de mener des études d'association entre le rs6040G>A du gène *F7* et les taux plasmatiques des DEFA1-3 et de LL-37 auprès de populations saines et HT, afin d'évaluer un éventuel rôle de ces peptides dans le risque de survenue d'HTA. D'une façon générale, il serait intéressant d'approfondir nos connaissances sur la génétique des endo-phénotypes d'HTA. L'identification des SNPs associés à l'augmentation de l'expression plasmatique de ces traits aidera à l'identification des individus prédisposés. Cette stratégie d'étude appliquée dans le domaine des maladies complexes, permettrait de combler en partie l'hétérogénéité des mesures de PA et d'HTA.

B- Certains auteurs affirment que seule une partie minime des SNPs associés à la PA et l'HTA a été identifiée [102] et que beaucoup de recherches sont encore nécessaires pour identifier des gènes plus spécifiquement associés à la régulation de PA chez les adultes et chez les enfants. Si ces SNPs seront communs à l'âge adulte et enfantin, ils pourraient fournir des indices importants concernant l'étiologie précoce de l'HTA [102].

Cet objectif représente une des nos perspectives majeures et pour se faire nous utiliserons le séquençage complet des exons du génome entier et les « «*exom chips »* qui certainement pourront améliorer la variation phénotypique de la PA et de l'HTA expliquée. En effet, avec l'émergence de ces nouvelles technologies, de grands efforts sont à mener afin de révéler des SNPs rares exerçant des plus larges effets. Plusieurs arguments sont en faveur d'un rôle important des SNPs rares dans la prédisposition aux maladies humaines complexes:

1- La théorie d'évolution prédit que les allèles délétères devraient être rares [103] et représente l'argument le plus fort. Il est admis que la maladie est le contraire de l'état sain et que par la suite ces variants génétiques doivent être 'réprimés' et ne devraient pas être fréquents [103].

2- Beaucoup de maladies familiales rares sont dues à des SNPs rares ayant des effets forts [103]**.** Cette déclaration ne s'applique pas uniquement aux maladies causées par les mutations mendéliennes rares, telle que la fibrose kystique [103] par exemple. Probablement, l'investigation la plus complète est celle qui a démontré que le quart des cas de déficiences intellectuelles liées au chromosome X peut être attribué à des SNPs rares codants, qui ont été découverts par le séquençage complet des exons du chromosome [104].

3- Les *Synthetic associations* pourraient expliquer les effets des SNPs fréquents [103]**.** C'est en 2010 que Goldstein et al (20126254) ont démontré par simulation, que les associations détectées pour des SNPs fréquents identifiés dans les maladies humaines complexes pourraient, en principe, provenir de l'effet des SNPs rares à proximité [105]. Ils expliquent qu'un variant fréquent contribuant à une maladie ou même à un trait serait l'image déformée ou '*synthetic association'* d'un nombre important de SNPs rares en fort LD avec ce SNP (D' élevé, r^2 faible) et à effets beaucoup plus forts [105]. La théorie de *Synthetic association* n'est pas prévue pour expliquer la plupart de l'héritabilité manquante, mais ce type d'effet doit être considéré comme une explication pour les effets apparents des SNPs fréquents. Dans l'état actuel de notre connaissance, cette hypothèse pertinente n'a pas encore été explorée dans le domaine de la PA et de l'HTA.

C- Il serait intéressant d'étudier l'impact des taux plasmatiques d'HP et du nouveau SNP que nous avons identifié sur les fonctions vasculaires et notamment l'HTA.

D- N'ayant pas eu le temps de tester le rôle des eMethSNPs dans la régulation de la PA, ce type de design serait appliqué dans le futur proche.

E- Une dernière étape serait l'expérimentation pharmaco-génomique/génétique de notre cheminement et de nos résultats dans l'espoir d'identifier des cibles préventives et thérapeutiques. Même si nous convenons qu'il est prématuré d'imaginer avoir des retombées thérapeutiques, nous sommes convaincus que de telles expérimentations doivent avoir lieu pour essayer d'établir une médecine personnalisée.

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AUTRES PUBLICATIONS

Contribution of vascular endothelial growth factor and related common genetic variants to the variation of blood lipids levels

Abbreviated title: VEGF related SNPs and plasma lipids levels

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Abbreviations: APO-E, apolipoprotein E; BMI, body mass index; BRC IGE-PCV, Biological Resources Bank "Interactions Gène-Environnement en Physiopathologie CardioVasculaire"; CVDs, cardiovascular diseases; GWAS, genome-wide association study; *MRPL14,* mitochondrial ribosomal protein L14 gene; SNPs, single nucleotide polymorphisms; TC, total cholesterol; VEGF, vascular endothelial growth factor

Abstract

The vascular endothelial growth factor (VEGF) is among the most significant stimulators of angiogenesis and its effect on cardiovascular diseases is considered important, although unclear yet. Recently, our team identified four polymorphisms (rs6921438, rs4416670, rs6993770, and rs10738760) explaining up to ~ 50% of the heritability of serum VEGF levels. In this study we tested the hypothesis whether these SNPs contribute to the variation of blood lipid levels in healthy subjects. The effect of these SNPs on apolipoprotein E (APO-E), triglycerides, total cholesterol, low and high-density lipoproteins (LDL and HDL) was evaluated using linear regression in a discovery and a replication sample (*n*=1,006 and 1,145 healthy unrelated adults from European origin respectively) followed by a meta-analysis. Their gene×gene and gene×environment interactions were also assessed. SNP rs6921438 was significantly associated with HDL ($P_{overall}$=1.2×10^{-7}) and LDL ($P_{overall}$=1.5×10^{-4}). A significant interaction between rs4416670 and hypertension for APO-E was also identified ($P_{overall}$=1.7×10^{-5}). Common genetic determinants were identified for VEGF and blood lipids, while epistatic and environmental interactions seem to play a role. These results offer new insights in the genetic regulation of cardiovascular diseases and investigation of associations between cardiovascular risk factors and these SNPs would be of interest.

Sypplementary key words: VEGF polymorphisms, lipid metabolism, cardiovascular diseases, HDL, LDL, epistatic interactions, gene×environment interactions

Introduction

The vascular endothelial growth factor (VEGF) family is one of the most important regulators of vascular biology. Especially, VEGF-A stimulates angiogenesis in a wide range of normal and pathological processes (1). Due to this significant position on blood vessel homeostasis, the role of VEGF on cardiovascular diseases (CVDs) and atherogenesis has been among the objectives of cardiovascular research during the last years (2). However, VEGF's effect on CVDs is still unclear.

The use of anti-VEGF agents in cancer therapy has shown significant cardiovascular side effects such as hypertension, cardiomyopathy and hemorrhagic events (3). Furthermore, VEGF protein or gene therapy has been tested in randomized clinical trials, particularly in patients with coronary artery disease, with results showing small clinical importance (2). Many studies have identified higher VEGF levels in patients with vascular diseases; nevertheless, it is not clear whether the up-regulation of VEGF is an adaptation to ischemia or if it is rather causal in the onset of these diseases (4-11). Concerning the involvement of VEGF in atherosclerosis, results seem to be conflicting. A possible neovascularization and proinflammatory effect of VEGF has been identified in animal models and *in vitro* studies, which leads to progression of atherosclerosis and plaque instability (12-17). In contrary, clinical trials using VEGF protein or gene therapy in humans (2) and in animal studies (18, 19) do not support a positive effect of VEGF on atherosclerosis progression. The complexity of CVDs combined with the pleiotropic effects of VEGF could partially explain the differences between studies.

An interesting point would be the assessment of the effect of VEGF on known cardiovascular risk factors in supposed healthy populations, such as lipids levels (20), as this would probably support possible implications of VEGF in the physiopathology of CVDs. Especially high-density and low-density lipoproteins (HDL, LDL respectively) are considered as independent risk factors for the development of CVD (20). Increased levels of circulating VEGF have been found in subjects with uncomplicated hyperlipidemia in a small sample-size study (21) and similar finding has been shown in a pilot study in patients with hypercholesterolemia (22). Significant associations were

found between HDL and VEGF levels in a supposed healthy population from Japan (23), while in a supposed healthy population of SAPHIR study, VEGF was negatively correlated with LDL, total cholesterol (TC) and apolipoprotein B only in women (24). Although the relations between lipids profile and VEGF levels are not yet clearly defined, these observations suggest an eventual implication of this molecule in lipids metabolism.

The genetic research can be used for the assessment of VEGF role in CVDs, through the identification of new possible molecular mechanisms. Via a recent genome-wide association study (GWAS), we identified four single nucleotide polymorphisms (SNPs) that explained up to ~ 50% of the heritability of serum VEGF levels (25). The investigation of the effect of these new genetic variants, as well as their interactions between them and with environmental factors, on blood lipids levels might give some insight in the relation between VEGF and blood lipids. Therefore, the aim of the present study is the assessment of genetic determinants of blood lipids levels using these four novel VEGF related SNPs, in supposed healthy discovery and replication populations.

Methods

Subjects

Discovery and replication population

Discovery (*n*=1,006) and replication (*n*=1,145) samples belong to two independent and non-overlapping populations extracted from the Biological Resources Bank (BRC) "Interactions Gène-Environnement en Physiopathologie CardioVasculaire" (IGE-PCV) in Nancy, East of France. They consist of supposed healthy unrelated adults of European origin (discovery population: Portugal, France; and replication population: Ireland, Greece). Individuals with chronic disorders (cardiovascular or cancer) or having a personal history of CVD have not been included. Subjects taking medication with blood lowering effect or having an effect on cardiovascular function were also excluded. The study protocols have been approved by the Local Ethics Committee of each recruitment centre and all subjects gave written informed consent for their participation in the study.

Data collection

For both populations, biological and clinical measurements, health and lifestyle information have been collected using appropriate, validated questionnaires and procedures as described previously (26, 27). Hypertension was defined as systolic blood pressure ≥140mmHg, diastolic blood pressure ≥90mmHg and smokers were identified based on current smoking status. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. Obesity was defined as BMI ≥30kg/m^2.

Apolipoprotein E (APO-E), triglycerides, TC, and HDL were measured in plasma (26, 27) and VEGF levels were measured also in plasma in a subsample of 403 individuals from the discovery population as previously described (25). LDL was calculated using the Friedewald formula (28).

Genotyping

DNA has been extracted from all participants and relative biobanks have been constructed in the BRC IGE-PCV. The SNPs rs6921438, rs4416670, rs6993770, and rs10738760 were genotyped by Genoscreen© (http://genoscreen.fr) using a Sequenom® iPLEX Gold assay – Medium Throughput Genotyping Technology (29) and in Kbioscience (http://www.kbioscience.co.uk) using the competitive allele specific PCR (KASP) chemistry coupled with a FRET-based genotyping system (http://www.kbioscience.co.uk/reagents /KASP/KASP.html) in the replication population.

Statistical analysis

Continuous variables are presented as mean value ± standard deviation and categorical variables are given in percentages. Hardy-Weinberg equilibrium was tested using the chi-square test. VEGF concentrations were natural log-transformed to normalize their distribution in a subsample of the discovery population. Correlations were evaluated by calculating the Pearson coefficient (r). Linear regression models adjusted for age, gender and BMI were used to test possible associations between VEGF plasma levels and the levels of the assessed lipid traits. Significance was assessed at a two-tailed P=0.05 level.

Genetic analyses were performed under the assumption of an additive model.

For the discovery population and the replication populations, linear regression models adjusted for age, gender and BMI were used for the assessment of the effect of each SNP (independent variable) in blood lipids concentrations (dependent variables). Further adjustments were performed in both populations for smoking and hypertension. Significance was assessed at a two-tailed P=0.0125 level (adjustment for multiple testing). In the case where more than one SNP are associated with one trait, a conditional analysis assessing the main effect of all significant SNPs in the same model of linear regression (adjusted for age, gender and BMI) was performed in order to clarify the independent determinants of the trait.

The environmental factors used for the gene×environment interactions assessment included BMI, smoking and hypertension. In the previous regression models the environmental factor and an additional interaction term (SNP×environmental factors) was added. Significant results were considered those with $P \leq 0.004$. For the significant SNPs implicated in gene×environment interactions, separate regression models using the environmental factor as the dependent variable were performed in order to control for a direct association between the SNPs and the factor.

The assessment of gene×gene interactions was tested using all possible pair-wise combinations between the four SNPs in both discovery and replication populations. In the regression models adjusted for age, gender and BMI, two SNPs and their interaction term were added. In order to adjust for multiple testing, significance was set at $P=0.008$.

All analyses were performed using PLINK 1.07 software (http://pngu.mgh.harvard.edu/purcell/plink) (30) and the SPSS statistical software version 16.0 (SPSS, Inc, Chicago, Illinois).

Meta-analysis for each quantitative trait was performed using a weighted inverse normal method via the function “metagen”, with a fixed effect, in the “META” R 2.15.1 package.

The significant results of this study were compared to previous findings in the literature (previous GWAS) by imputation analyses using Plink. SNPs with a correlation coefficient ≥ 80% were considered in linkage disequilibrium (LD). The GWAS investigator of HuGENavigator engine (31) and the NHGRI Catalog of published GWAS (http://www.genome.gov/gwasstudies) (32) were used in order to assess previous GWAS concerning blood lipid levels.

Results

Participants' data are presented in table 1. The characteristics of the genotyped SNPs are shown in table 2. All SNPs in both populations are in agreement with the Hardy-Weinberg equilibrium.

In the discovery population, significant associations were observed between rs6921438 and rs6993770, and the levels of HDL (β=-0.09mmol/l, P=1.2×10^{-4} and β=-0.01 mmol/l, P=8.3×10^{-3} respectively; table 3) and between rs6921438 with plasma LDL concentrations (β=0.14mmol/, P=6.7×10^{-3}, table 3). Specifically, the presence of the minor allele A of rs6921438 is associated with decreased HDL and increased LDL values, while the minor allele T of rs6993770 is associated with increased levels of HDL. These significant associations were only confirmed for SNP rs6921438 in the replication population (β=-0.07mmol/l, P=2.7×10^{-4} and β=0.12mmol/l, P=8.4×10^{-3} for HDL and LDL respectively; table 3). Conditional analysis including both rs6993770 and rs6921438 revealed that rs6921438 was the only SNP with significant direct effect on HDL levels (β=-0.09mmol/l, P=1.2×10^{-4}; β=-0.07mmol/l, P=2.7×10^{-4}). Of note, these results remained significant in both populations after adjustments for both smoking and hypertension. SNP rs6921438 explained 1% of the variability of HDL and 0.2% for LDL in both populations. Meta-analysis of the results of both populations gave highly significant associations between rs6921438 and both HDL and LDL levels (β=-0.08mmol/l, P=1.2×10^{-7} and β=0.13mmol/l, P=1.5×10^{-4} for HDL and LDL respectively; table 3). SNP rs6993770 was not significantly associated with HDL levels in joint analysis of the two populations. Furthermore, meta-analysis of the conditional analysis results including rs6993770 and rs6921438 verified that rs6921438 is the only independent determinant of HDL levels (β=-0.08mmol/l, P=1.2×10^{-7}).

Among nine previously published GWAS studies concerning lipids levels, rs6921438 was not reported in the lists of statistically significant SNPs (genotyped or imputed) (33-41). Therefore, it is a novel SNP associated with both HDL and LDL levels.

The gene×environment interactions assessment revealed that rs4416670 was interacting with hypertension for APO-E (P= 3.5×10^{-3}, 1.6×10^{-3}, and 1.7×10^{-5} respectively

in the discovery, replication populations and meta-analysis; table 4). The minor allele of the polymorphism was associated with lower levels of APO-E in hypertensive participants (β=-0.71mg/l, -0.75mg/l, and -0.73mg/l respectively; table 4). In order to test for a possible direct association of rs4416670 on hypertension, further analyses were performed in both populations. No significant associations were observed between the same SNP and hypertension.

Regarding epistatic interactions, in the discovery set, we found that the SNP rs6921438 interacted with rs6993770 for HDL levels (β=0.05mmol/l, P=2.4X×10^{-3}, table 4). Although this finding was not significant in the replication population (β=0.02mmol/l, P=0.035, table 4), it was significant in the meta-analysis of the results (β=0.03mmol/l, P =2.6×10^{-3}, table 4).

In a subsample of the discovery population (n=403) with VEGF plasma levels measurements, no correlation was found between VEGF plasma levels and any of the assessed lipid traits. Furthermore, VEGF levels were not associated with lipids levels in regression models.

Discussion

The present study assessed the effect of VEGF-related SNPs on blood lipid traits and found significant associations and gene×environment interactions for HDL, LDL and APO-E levels.

In the discovery population, rs6921438 and rs6993770 were associated with HDL and rs6921438 was associated with LDL plasma levels. The minor alleles of rs6921438 had a negative effect, while rs6993770 seems to be protective through increase of HDL levels. Furthermore, the association of rs6921438 with HDL and LDL was observed also in the replication sample, while similar results were observed when meta-analysing the results of both populations. Conditional analyses showed that only rs6921438 is significantly associated with HDL in the discovery and replication populations, as well as in the joint analysis. Thus, it seems that rs6921438 is the basic determinant of HDL levels among the four assessed SNPs.

The rs6921438 is an intergenic SNP in chromosome 6, close to *VEGFA* gene and located between the mitochondrial ribosomal protein L14 gene (*MRPL14*) and the *MCG45491* gene (C6orf223). We negatively associated this SNP with VEGF levels in a previous GWAS (25). Consequently, it appears that rs6921438 could have a negative effect in cardiovascular system through decrease of HDL, increase of LDL levels, while decreasing VEGF levels.

It should be mentioned, that to our knowledge, this is the first study that investigates the effects of VEGF-related SNPs with blood lipid traits. Also, rs6921438 has not been identified in any previous GWAS study concerning HDL and LDL levels (33-41). Furthermore, as the existing GWAS have managed to explain a small percentage of the blood lipid traits variance (e.g for HDL it ranges from 0.6 to 10%), other genetic variants remain to be found. Nevertheless, in the present study, rs6921438 explained 1% of HDL variability and 0.2% of LDL variability (in both populations). In this study, a candidate-gene approach was used based on SNPs identified from a GWAS concerning VEGF levels heritability. The use of GWAS-identified SNPs as candidate genes for other traits could help the elucidation of genetic relationships between phenotypes and new biological

mechanisms associated with pathologies. Hence, the present study, which suggests a common genetic regulation of blood lipid traits and VEGF, could support this methodology.

In order to assess the effect of this common regulation, we tested the association between VEGF and blood lipid levels, though the results were non significant. Therefore, plasmatic levels of VEGF have no direct implication in lipid metabolism, at least in supposed healthy individuals. However, Blann et al (21) demonstrated that subjects with hyperlipidemia have increased levels of VEGF compared with healthy controls even if authors found no correlation of VEGF with blood lipids levels. Additionally, similar results were observed in a small pilot study of hypercholesterolemia patients by Belgore et al (22). Both these studies have been performed in small sample sizes and they have included pathological populations in a case-control design. Thus, these results can not be directly compared with the present study, where healthy populations have been used. In the study of Kimura et al (23), serum VEGF levels were negatively correlated with HDL levels in healthy adults, however, this correlation was observed only in male population. Moreover, in this study measurements have been performed in serum samples, which demonstrate higher levels of VEGF compared with plasma levels that were measured in the present study (23, 25). Finally, Sandhofer et al (24) have shown that plasma VEGF levels were negatively associated with TC and LDL in a healthy female sample. Although the population of this study is larger than our discovery cohort, the female sample is significantly older compared to ours. As previously observed, VEGF levels increase with age, especially in women (42). In the abovementioned study, VEGF plasma levels in the female sample are higher than the levels of our study, and this could probably explain the different results between them. It should be mentioned that in our sample there were no significant differences between genders (data not shown). Taken together, it seems that in supposed healthy populations, VEGF is only marginally or not associated with blood lipid levels. Thus, although a common genetic background between VEGF and blood lipids, especially HDL and LDL, may exist, a clinical manifestation can not be detected in physiological situations. However, the functionality

of these SNPs and the clarification of molecular pathways that are implicated should be determined in other functional studies including transcriptomic analysis for the expression of key proteins.

Another interesting finding is the interactions of the SNPs between them and with environmental factors. It is currently widely accepted that gene×gene and gene×environmental interactions can explain a significant amount of genetic heritability (43-45). SNP rs4416670 was interacting with hypertension for decreasing APO-E levels. This SNP×hypertension interaction is not due to a direct relationship between the two factors. Hypertension is among the major risk factors for CVDs (46). Presence of altered blood lipid levels and high blood pressure are very common manifestations in subjects with CVDs. Thus, the identification and explanation of these types of interactions between risk factors and genes could be important for the understanding of the complex mechanisms that define the phenotypes in CVDs. Also, these interactions, if functionally validated, could improve the prevention and prognosis of the diseases.

In addition, an rs6921438×rs6993770 interaction for HDL was detected in the discovery cohort. This finding was not observed in the replication population, although, the epistasis was significant in the meta-analysis of the results from both samples. However, conditional analyses in both populations (separately and jointly analyzed) showed that when both SNPs were assessed in the same model only rs6921438 was significantly and independently associated with HDL levels in the discovery population. Therefore, this epistasis may reflect the effect of rs6921438 on HDL and not the presence of a true interaction between the two SNPs.

As the polymorphisms that were genotyped in this study have not been studied before in relation to blood lipid traits, there are no previous reports on possible gene×gene or gene×environment interactions.

This study was the first designed to assess common genetic regulation between VEGF and blood lipids. The selection of healthy populations is important for the understanding of these relationships as in pathological situations, like CVDs, the clinical profile of both blood lipids and VEGF is affected by many disease-related factors which

complicate the situation and do not allow the demonstration of comprehensive results. Another strong point of the study is the replication of the most significant results in an independent population, as well as the use of meta-analyses, that ensured the validity of findings. We acknowledge however the limited number of individuals with VEGF plasma levels measurements.

In conclusion, common genetic variants were identified for VEGF and HDL and LDL, through independent mechanisms. Also, APO-E was associated with interactions between one SNP and hypertension, which is known to be related with CVDs. The assessment of other CVDs risk factors associations with these polymorphisms could assist in the understanding of the normal regulation and the pathophysiological mechanisms that underlie these complex diseases.

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Tables

Table 1. Characteristics of the studies participants

Variable	**Discovery (*n*=1,006)**		**Subsample with VEGF plasma levels measurements (*n*=403)**		**Replication (*n*=1,145)**	
	Mean[a]	**SD**[b]	**Mean** [a]	**SD**[b]	**Mean** [a]	**SD**[b]
Age (years)	43.17	9.08	44.52	4.91	41.98	9.24
Gender (male %)	43.00		50.40		82.00	
Body mass index (kg/m^2)	25.19	4.15	24.92	3.94	26.70	3.91
Hypertension (%)	25.80		14.30		27.80	
Obesity (%)	10.12		7.93		15.90	
Smoking (%)	23.33		25.40		27.30	
High-density lipoprotein (mmol/l)	1.47	0.44	1.60	0.47	1.61	0.62

Low-density lipoprotein (mmol/l)	3.27	1.09	3.55	0.89	3.57	1.17
Total cholesterol (mmol/l)	5.51	1.09	5.73	1.03	5.92	1.18
Triglycerides (mmol/l)	1.24	1.36	1.30	1.91	1.63	1.30
Apolipoprotein E (mg/l)	42.68	15.14	41.84	17.63	47.39	18.33
Vascular endothelial growth factor (ng/l)			42.71	43.33		

[a] mean value for continuous variables and percentage for categorical variables

[b] ***SD***, standard deviation (only for continuous variables)

Table 2. Characteristics of the four studied genetic variants

Chromosome	**SNP**	**Discovery**		**Replication**		
		Minor allele	**MAF** [a]	**Minor allele**	**MAF**	
6	rs6921438	A	0.42	A	0.40	
6	rs4416670		C	0.47	C	0.45
8	rs6993770		T	0.30	T	0.31
9	rs10738760	A	0.48	G	0.45	

[a] ***MAF***, minor allele frequency

Table 3. Significant associations of SNPs with plasma lipids

SNP	Minor allele	Phenotype	Discovery		Replication		Meta-analyses	
			β [SE] [a] (mmol/l)	*P*	β [SE] [a] (mmol/l)	*P*	β [SE] [a] (mmol/l)	*P*
rs6921438	A	HDL [b]	-0.09 [0.02]	1.2×10^{-4}	-0.07 [0.02]	2.7×10^{-4}	-0.08 [0.01]	1.2×10^{-7}
rs6921438	A	LDL [c]	0.14 [0.05]	6.7×10^{-3}	0.12 [0.04]	8.4×10^{-3}	0.13 [0.03]	1.5×10^{-4}
rs6993770	T	HDL [b]	0.01 [0.02]	8.3×10^{-3}	-0.02 [0.02]	0.318	0.01 [0.01]	0.298

[a] ***β***, effect size; ***SE***, standard error

[b] ***HDL***, High-density lipoprotein

[c] ***LDL***, Low-density lipoprotein

Table 4. Significant gene×environment and gene×gene interactions with plasma lipids

SNP	Phenotype	Discovery		Replication		Meta-analyses	
		β [SE] [a]	*P*	β [SE] [a]	*P*	β [SE] [a]	*P*
rs4416670× hypertension	Apolipoprotein E	-0.71 [0.24]	3.5×10^{-3}	-0.75 [0.23]	1.6×10^{-3}	-0.73 [0.17]	1.7×10^{-5}
rs6921438× rs6993770	High-density lipoprotein	0.05 [0.01]	2.4×10^{-3}	0.02 [0.01]	0.035	0.03 [0.01]	2.6×10^{-3}

[a] ***β***, effect size; ***SE***, standard error; units for β coefficient are: mg/l for apolipoprotein E and mmol/l for high-density lipoprotein

Newly identified synergy between clopidogrel and calcium-channel blockers for blood pressure regulation possibly involves CYP2C19 rs4244285

Running title: Synergy between clopidogrel and calcium-channel blockers

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Abstract

Background: Interactions between clopidogrel and calcium-channel blockers (CCB) have been identified in regards to antiplatelet activity but not for blood pressure (BP) levels.

Objective: To determine the effect of joint use of clopidogrel and CCB on BP levels. Furthermore, the effect of the rs4244285 polymorphism of CYP2C19 gene on BP levels was tested.

Design: Cross-sectional observational study

Settings and Participants: Patients consecutively admitted to the intensive coronary care unit of the Coimbra University Hospital (Portugal) between May 2004 and July 2011, for acute myocardial infarction or unstable angina, were recruited. Patients for whom pre-admission medication data was not available were excluded. Final sample consisted of n=2,322. The genetic assessment was performed on a healthy European population (n=2,155).

Intervention: Data collected in both samples included age, gender, weight, height, BP and retrospective data for pharmacological therapy prior to admission was recorded, during the procedure of medical history assessment for the cardiovascular patients.

Measurements: Associations between clopidogrel and clopidogrel combined with CCB use and BP levels were assessed using linear regression models adjusted for relative co-factors. Similar models were applied for the assessment of the effect of rs4244285 on BP in the healthy sample.

Results: Clopidogrel use was significantly associated with lower diastolic blood pressure (DBP) (P= 0.034, beta= -1.47mmHg) in the total sample and in the CCB group (P= 0.030, beta= -3.07mmHg). The genetic analysis revealed an association between rs4244285 and both systolic and DBP (P=0.008, beta: -1.69mmHg and P=0.003, beta: -1.28mmHg respectively) in the healthy population.

Limitations: Causal relationships not assessed.

Conclusions: A synergy between clopidogrel and CCB for reduction of BP levels was observed and a novel genetic variant related to BP was identified.

Introduction

Hypertension is among the most significant causes of vascular events and the worldwide burden of high blood pressure is extremely high (1). The major complications of hypertension are related to thrombosis. Abnormalities of coagulation, platelets, and endothelial function support the existence of a prothrombotic or hypercoagulate state due to high blood pressure in patients with cardiovascular disease such as congestive heart failure (2).

Cardiovascular disease patients are usually managed with a range of medications including antiplatelet agents and antihypertensive treatment. Although antithrombotic medication offers significant benefit in such cardiovascular disease patients, they are associated with increase of haemorrhagic complications. This risk could be increased in hypertensive patients (2). For some antiplatelet agents, like acetylsalicylic acid (ASA), data support their safety and efficacy in hypertensive patients, while for others (eg clopidogrel) more research is needed (2). Furthermore, the effect of antiplatelet agents on blood pressure levels in patients with cardiovascular disease has not been assessed to date.

Clopidogrel is a thienopyridine commonly used as antiplatelet agent in cardiovascular disease patients. Apart from this action, clopidogrel has been shown to exert a protective effect on endothelial function and nitric oxide bioavailability by reducing oxidative stress and inflammation (3, 4). Therefore, clopidogrel is possible to exert other effects than its primary antiplatelet action. CYP2C19 is actively involved in the metabolism of clopidogrel as it mediates the production of its active metabolite, and polymorphisms of this gene have been associated with poor response to clopidogrel (5), which is however not always related to the clinical efficacy of the medication (6, 7). Another enzyme that is implicated in the metabolism of clopidogrel is CYP3A4 (4). A wide range of medications are known inhibitors of CYP450 enzymes and thus they are associated with interactions with clopidogrel metabolism and modification of its efficacy (8). Among these medications, calcium-channel blockers (CCB), which are commonly

used for hypertension treatment, are associated mostly with CYP3A4 inhibition (9). These drug-drug interactions could also be influenced by CYP2C19 genotype (8).

As both antiplatelet and antihypertensive agents can be co-prescribed in patients with cardiovascular disease, the possible interactions between them have been tested in previous studies. Some of them have identified a decreased platelet inhibition in patients treated with CCB (10, 11) which may decrease the clinical efficacy of clopidogrel (12), while others have concluded that this interaction may not have a significant clinical importance in different types of cardiovascular patients (13, 14). However, none of these studies has assessed the joint effect of these medications on blood pressure levels, when co-prescribed. Also, this aspect has not been previously assessed in relation to CYP450 isoforms polymorphisms.

The present cross-sectional observational study aimed to assess the effect of clopidogrel use on blood pressure levels in a large sample of cardiovascular disease patients and whether the co-treatment with CCB may modify this effect. Furthermore, to assess the possible relationship between clopidogrel-related metabolism and blood pressure levels, the effect of a common CYP2C19 polymorphism (rs4244285**)** was tested in relation to blood pressure levels. In order to obtain information on this CYP450 genetic variant's physiological role on blood pressure regulation, a supposed healthy European sample was selected for the genetic analysis.

Methods

Study populations

Two populations are assessed in a cross-sectional design, a sample of adult patients with cardiovascular diseases and a sample of supposed healthy unrelated adults. This investigation was conducted according to the principles outlined in the Declaration of Helsinki and was approved by the local ethics committee and institutional board. The genetic study protocol was approved by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine, Nancy, France. All individuals gave written informed consent.

Cardiovascular patients:

Patients of both sexes consecutively admitted to the intensive coronary care unit of the Coimbra University Hospital (Coimbra, Portugal) between May 2004 and July 2011, for acute myocardial infarction or unstable angina, were recruited. Acute myocardial infarction was defined according to the Universal Definition of myocardial infarction, as a positive cardiac biomarker (namely troponin I) with symptoms of ischemia or electrocardiogram changes indicative of new ischemia (ST and T wave and new bundle branch block) (15). Unstable angina was defined either by new onset angina (at least class III CCS), progressive angina, or angina at rest, with or without electrocardiogram ischemic changes, and a negative cardiac biomarker assay (16).

Patients for whom pre-admission medication data was not available were excluded. Based on complete availability of clinical data, a sample of 2,322 patients participated in the study.

Healthy individuals:

The assessment of the genetic effect of rs4244285 was performed in a sample of 2,155 unrelated adults of European origin, taken from populations that are available in the Biological Resources Bank (BRC) “Interactions Gène-Environnement en Physiopathologie CardioVasculaire” (IGE-PCV) in Nancy, East of France. They were selected on the basis of

the following criteria: (1) no antihypertensive drug therapy at recruitment and (2) complete clinical and genotypic data available.

Data collection

Data collected in both samples included age, gender, weight, height, and blood pressure (BP).

The body mass index was calculated as weight (kg) / square of height (m). Systolic (SBP) and diastolic (DBP) blood pressure were measured at rest under constant temperature (19°C-21°C) and standardized conditions (supine position) using a manual sphygmomanometer (Colonne à mercure, Mercurius) by expert nurses. The recorded values were the means of three readings on 20 min intervals. An adjustable BP cuff was used to correct errors due to variations in arm circumference.

In the sample of cardiovascular patients, retrospective data for pharmacological therapy prior to admission was also recorded, during the procedure of medical history assessment. Therapeutic data covered prescription of antihypertensive medications (CCB, angiotensin converting enzyme inhibitors, beta blockers, and diuretics), antiplatelet agents (ASA, clopidogrel), and statins.

All individuals of the healthy population underwent complete medical examination including anthropometric and biochemical measurements collected with standardized methods (17).

Genotyping assays

Genomic DNA was extracted from peripheral blood samples using the salting out method. The single nucleotide polymorphism (SNP) rs4244285 was genotyped in Kbioscience (http://www.kbioscience.co.uk) using the competitive allele specific PCR (KASP) chemistry coupled with a FRET-based genotyping system

(http://www.kbioscience.co.uk/reagents /KASP/KASP.html) in the healthy population. Call rate was >90%.

Statistical analysis

Analyses were performed using the SPSS® statistical software version 16.0 (SPSS, Inc, Chicago, Illinois) and Plink v01.7 (18).

Multiple linear regression models adjusted for age, gender, body mass index (BMI), ASA use, and treatment with any anti-hypertensive medication were applied to assess the effect of clopidogrel use on SBP and DBP in the sample of cardiovascular patients. Further adjustment was performed for statins use. In order to test the effect of clopidogrel on BP according to the CCB use, the sample of cardiovascular patients was split in two groups: CCB (n=535) and non-CCB (n=1,787) groups. Similar multiple regression models were applied separately in the two groups.

Analyses in the healthy sample were performed under the assumption of an additive model using the minor allele as reference. Hardy-Weinberg (HW) equilibrium was tested using the chi-square test. The assessment of the effect of rs4244285 on SBP and DBP was performed with multiple linear regression models adjusted for age, gender, and BMI.

Significance was assessed at a two-tailed P=0.05 level.

Role of the funding source

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Results

Both samples' characteristics are presented in table 1. Mean age is 68.6±11.6 years and 42.7±8.9 years respectively for cardiovascular patients and healthy individuals, while mean BMI is in the range of overweight.

Cardiovascular disease patients:

After adjustments for age, gender, BMI, use of ASA, and use of antihypertensive medication clopidogrel use was significantly associated with a lower DBP (P= 0.034, beta= -1.47mmHg), while its effect on SBP was not significant (P= 0.233). However, when adjusting also for statins use, this association was no longer statistically significant (P=0.216).

In the separate analysis in the CCB (n=535) and non-CCB (n=1,787) groups, clopidogrel use was significantly associated with low DBP only in the CCB group (analysis adjusted for age, gender, BMI, use of ASA, and use of other antihypertensive medication) (P= 0.030, beta= -3.07mmHg) and no association was detected in the non-CCB group (P=0.193). These results were not modified even after adjustment for statins use. Therefore, clopidogrel seems to improve the DBP lowering effect of CCB. The relative analysis for SBP did not give significant results.

Healthy individuals:

Genotyping was performed for the SNP rs4244285 [minor allele frequency, MAF= 0.14 (A allele)] of the CYP2C19 gene which was in agreement with HW equilibrium (P= 0.164).

In the regression models adjusted for age, gender, and BMI, only rs4244285 was significantly associated with low values of both SBP and DBP (SBP: P=0.008, beta: -1.69mmHg and DBP: P=0.003, beta: -1.28mmHg).

Discussion

Drug-drug interactions can play a significant role in the efficacy of medications and in the clinical outcome of patients. In the present study, an interaction between clopidogrel and CCB on blood pressure levels was identified in cardiovascular disease patients. A novel association between CYP2C19 rs4244285 genotype and blood pressure was also detected, possibly contributing to partially explain the interaction between clopidogrel and CCB.

The interaction of these commonly co-prescribed medications has been previously assessed in relation to the antiplatelet activity of clopidogrel. The common metabolic pathways of these agents through CYP450 enzymes triggered the hypothesis for this interaction. Most studies identify a reduction on the antiplatelet effect of clopidogrel due to CCB (10, 11). However, the joint effect of these medications on blood pressure has never been assessed to date.

In the present study, clopidogrel use was associated with lower diastolic blood pressure levels (DBP) as compared with its non-use. Clopidogrel acts as an antiplatelet agent through blocking the platelet adenosine diphosphate (ADP) receptors $P2Y_{12}$ (19). These receptors have been identified in vascular smooth muscle and they have been associated with human blood vessels contraction (20). However, a recent animal study indicated that the protective effect of clopidogrel in hypertension-related vascular functional changes was not directly mediated by the $P2Y_{12}$ blocking (21), while the contraction effect in the study of Wihlborg et al was not reduced in patients treated with clopidogrel (20). Therefore, clopidogrel's action on blood pressure levels could be mediated by its antioxidant and anti-inflammatory effects on endothelium (3, 20). However, in our study this significant effect was lost when adjustment for statins use was applied. Statins are medications that are also implicated in CYP450 metabolism and have been shown to interact with clopidogrel: competition between clopidogrel and statins for CYP3A4 can result in decreased conversion of clopidogrel prodrug to its active metabolite (22) leading to a decrease of its antiplatelet activity. Despite the fact that the impact of this interaction on clinical outcome is doubtful (23), co-treatment with statins could reduce the putative protective effect of clopidogrel on BP as well.

Moreover, a DBP lowering effect of clopidogrel was observed only in individuals treated with CCB even following adjustment for other antihypertensive medication and statin use. This finding might support the existence of a clinical synergy between the two medications. This

synergy seems to have a beneficial effect on the blood pressure of the cardiovascular patients included in this study. This could be clinically useful through the verification of efficacy and safety of the co-prescription, especially in hypertensive individuals, where the benefit/harm ratio (antithrombotic versus haemorrhagic complications) can be potentially reversed with antiplatelet treatments. As previously mentioned, $P2Y_{12}$ receptors are present in vascular smooth muscle and their *in vitro* activation leads to human blood vessels contraction (20), mediates proinflammatory and atherogenic actions in the vessel wall and stimulates vascular smooth muscle cell proliferation (24). These receptors belong to the G-protein coupled receptor family and their activation leads to Ca^{+2} entry through calcium-channels (25). Therefore, an additive effect of clopidogrel and CCB could be speculated. Taking into account that, in animal models, clopidogrel's effect on vasculature is not directly mediated by $P2Y_{12}$ (21), we could hypothesize that in cardiovascular patients, the effect of clopidogrel on blood pressure is modest if any, but, its combination with CCB could lead to an enhanced anti-hypertensive action of CCB, possibly through the $P2Y_{12}$ receptors. Furthermore, a variety of CCB are metabolized by CYP3A4 and their inhibitory effect has been associated with decreased bioactivation of clopidogrel (11). This metabolic competition could also result in decreased inactivation of CCB. Relative studies are focused on the effect of this competition for CYP3A4 in terms of clopidogrel activation only. However, a slower inactivation of CCB, which could result in lower blood pressure, could be hypothesized and could offer another explanation of the clopidogrel*CCB synergy identified in the present study.

Given the fact that the interaction between these drugs has been shown to decrease the antiplatelet efficacy of clopidogrel, the question whether this interaction might lead to an improved blood pressure profile should be addressed. It is important though to mention that the role of this interaction has been tested before in terms of cardiovascular clinical outcome and not platelet inhibition. Three relatively recent studies have not identified a clinically significant adverse effect of clopidogrel and CCB co-treatment (13, 14, 26). Therefore, as a metabolically negative interaction between two drugs implicated with CYP450 isoforms does not necessarily lead to clinical complications, the nature of this synergy on blood pressure could be real and needs to be further assessed in future studies where blood pressure levels should be the primary outcome.

Furthermore, the present study indicated a novel association of a CYP2C19 polymorphism with blood pressure. In particular, rs4244285 was associated with a decrease in both SBP and DBP in a large sample of healthy adults. The rs4244285 variant is a G/A mutation at nucleotide 681 in exon 5 that creates an aberrant splice site (27) and it has not been identified in any genome-wide association study concerning blood pressure levels or hypertension. Ma et al have indicated an association of rs10509676 of CYP2C19 gene with hypertension in a case-control study of individuals with Chinese Han origin (28). The SNP rs10509676 is in linkage disequilibrium (LD) with rs12767583, which is also in LD with rs4244285. Although, this study cannot be directly compared to ours due to differences in design, sample size, and individuals' ethnicity, it could offer some support on our findings concerning blood pressure regulation by CYP2C19 gene. Furthermore, linkage analysis indicate that rs4244285 is in LD with 14 other polymorphisms of CYP2C19 including the tag SNP rs12571421. Therefore, following the replication of our results, the use of rs12571421 could probably be proposed in diagnostic and clinical assays.

A multidimensional effect of CYP2C19 on blood pressure levels could be hypothesized. CYP2C19 has been shown related with the *in vitro* progesterone metabolism through 21-hydroxylation (29). Recently, CYP2C19 and CYP3A4 were shown to improve 21-hydroxylation of progesterone in 21-hydroxylase deficiency patients. This effect was modified by a gain of function allele of CYP2C19. This study concluded that both CYP2C19 and CYP3A4 could through this action modulate the levels of mineralocorticoid such as aldosterone and that CYP2C19 polymorphisms might play a role (30). We could therefore hypothesize that a loss-of function polymorphism like rs4244285 could have the opposite effect, decreasing aldosterone levels and thus exerting a BP lowering effect.

This polymorphism has been previously implicated in pharmacogenetic studies related to clopidogrel responsiveness. No data are available for its association with CCB metabolism. The clarification of the mechanism explaining the molecular basis of the BP lowering effect of concomitant clopidogrel and CCB use could help identify patients who are more likely to present this favorable effect based on their genetic profile.

The direction of the association between rs4244285 and BP levels is also interesting in the present investigation. The allele related with decreased SBP and DBP level is the minor allele, A which is associated with poor metabolism of various medications including clopidogrel (5),

while clopidogrel was shown to reduce DBP when combined with CCB. These findings are not controversial. First, we did not report a clear association of clopidogrel with blood pressure. Second, we identified a synergy between clopidogrel and CCB in a population of cardiovascular patients. The effect of an interaction between medications is not necessarily additive, especially when the molecular basis of this interaction is still undefined. Nevertheless, the effect of this polymorphism on clopidogrel's primer effect is doubtful as a recent large study observed no differences in the efficacy and safety of clopidogrel based on CYP2C19 polymorphisms compared to a placebo group (7). Therefore the effect of this variant on clinical outcome is not clear. As rs4244285 has not been tested before in hypertension traits and in relation to CCB metabolism, no conclusion can be made concerning its effect and mode of action. However, the identification of its association with blood pressure in healthy state can give new directions for further research.

This study included large samples of European individuals. Another strength of the study is the fact that analyses were adjusted for a significant number of factors associated with blood pressure and the medications that have been assessed. However, due to the cross-sectional design, some information concerning previous medical and drug history of the patients (eg duration of treatment, specific type of medications, and dosage) are missing. Another limitation of our study is that, being observational, although it does allow us to measure associations between clopidogrel use and BP, it cannot demonstrate strictly causal relationships. Finally, although our findings could have clinical significance for cardiovascular and especially hypertensive patients, the explanation of the implicated mechanisms can only be hypothesized.

In conclusion, we identified a (positive) synergic effect of clopidogrel and CCB on the reduction of blood pressure levels. A novel association between a CYP2C19 polymorphism and blood pressure levels was also observed. It would be interesting to evaluate, in the future, the relation between these two observations. As these two types of medications are commonly used in combination in cardiovascular patients, the understanding of the possible clinical effects and the underlying mechanisms is of utmost importance for the outcome and safety of patients. These results can offer new research directions for the understanding of pharmacokinetic and pharmacogenetics mechanisms that explain them as well as for the clinical assessment of these effects.

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Conflicts of interest:

Authors declare that there are no conflicts of interest.

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Table 1. Cardiovascular disease patients and healthy individuals' characteristics

	Cardiovascular disease patients (n=2,322)		Healthy individuals (n=2,155)	
	% Mean	Standard deviation	% Mean	Standard deviation
Age (years)	68.6	11.6	42.7	8.9
Body mass index (kg/m^2)	27.6	4.0	25.9	4.1
Systolic blood pressure (mmHg)	137.7	24.1	127.3	16.9
Diastolic blood pressure (mmHg)	73.5	13.8	79.24	11.2
Male gender	68.4		63.0	
Clopidogrel	23.5			
Acetylsalicylic	48.2			
Angiotensin-converting-enzyme inhibitor	48.0			
Beta blockers	35.0			
Calcium-channel blockers	23.0			
Diuretics	27.1			
Statins	48.4			

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Genome-wide meta-analysis points to CTC1 and ZNF676 as genes regulating telomere homeostasis in humans

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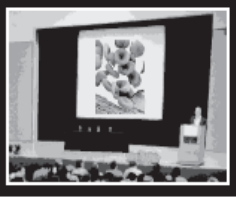
NEWS & VIEWS

Conference Scene

Functional genomics towards personalized healthcare and systems medicine



5th Biologie Prospective Santorini Conference, Santorini Island, Greece
30 September–2 October 2010

The 5th Biologie Prospective Santorini Conference explored the themes of systems biology, nutrigenomics and pharmacogenomics, all of which are related to personalized health, personalized therapy and personalized medicine. The conference started with a satellite meeting on genome-wide scan studies where the need for simplified models, the quality of the phenotypes and the input of epigenetics were dominant remarks. All of the omics approaches were then applied during the 3 days' sessions to multifactorial diseases (e.g., diabetes, atherosclerosis, cancer and inflammation) and often focused on gene–gene and gene–environment interactions. Afterwards, a fundamental session on drug metabolism, theranostics and pharmacogenetics and their practical aspects showed that the translation to clinical practice is finally happening although much slower than expected.

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The 5th Biologie Prospective Santorini Conference gathered 170 participants from 30 countries, originating from academic institutions and industries specialized in genetic-based research, particularly genome-wide association studies (GWAS), with application in chronic diseases (e.g., cardiovascular diseases [CVDs], cancer, inflammation and infection) and in pharmacogenomics and nutrigenomics.

The meeting was organized by the Institut National de la Santé et de la Recherche Médicale (INSERM) team 'Génétique Cardiovasculaire' and by Biologie Prospective with the collaboration of the American Association of Clinical Chemistry (AACC) divisions and the Chinese Society of Laboratory Medicine (CSLM), under the auspices of international organizations (International Federation of Clinical Chemistry and Laboratory Medicine [IFCC], International Atherosclerosis Society [IAS], European Atherosclerosis Society [EAS], International Society of Nutrigenetics/Nutrigenomics [ISNN], International Society of Pharmacogenomics [ISP], Personalized Medicine Coalition [PMC], Asian–Pacific Congress of Clinical Biochemistry [APCCB], European Personalised Medicine Diagnostics [EPEMED] and the European Network for Pharmacogenetics) and national societies (New Society of French Atherosclerosis [NSFA], French Society of Clinical Biology [SFBC], Greek Society of Clinical Chemistry – Clinical Biochemistry [EEKX-KB] and Hellenic Society of Biochemistry and Molecular Biology [HSBMB]).

We thank all our sponsors, in particular the Gold (Bühlmann Laboratories, Randox and Genomatix) and Silver sponsors (Nestlé, Roche, Beckman Coulter Genomics, BioM, Illumina, Future Diagnostics, AbD Serotec, Affymetrix, Bruker, Diasys) and also Protagen, GlaxoSmithKline, Chembio Diagnostic Systems, Servier and Daiichi-Sankyo.

The 3 days of the conference raised intensive discussions on the genetics and omics technologies' influences on personalized health:

- Day 1: systems biology and infectious diseases;
- Day 2: inflammation, CVDs, obesity and nutrigenomics;
- Day 3: pharmacogenomics and cancer. A round table on 'pharmacogenomics and personalized medicine in clinical practice' ended the conference.

We briefly present here the most important points made by the speakers during the symposium.

Satellite meeting: GWAS

In its 5th edition, the Santorini Conference – Functional Genomics towards Personalized Health Care – initiated its first satellite meeting devoted to GWAS under the auspices of Philippe Froguel (Unité Mixte de Recherche [UMR] 8199, Centre National de la Recherche Scientifique [CNRS] and Pasteur Institute, Lille, France and Genomic Medicine, Imperial College London, UK) and Sophie Visvikis-Siest (EA4373 'Génétique Cardiovasculaire',

future medicine part of fsg

Université Henri Poincaré, Nancy, France). To this occasion, leading experts in this area were invited: John PA Ioannidis (Stanford University, CA, USA), Heather J Cordell (Institute of Human Genetics, Newcastle University, UK), Allen Roses (Deane Drug Discovery Institute, NC, USA), Eleftheria Zeggini (Wellcome Trust Sanger Institute, Cambridge, UK) and Martin Seifert (Genomatix Software, Munich, Germany) to debate the contribution of GWAS in the understanding of common disorders and the future expectations.

The meeting started with a statement of the art by John Ioannidis, recounting the evolution, within a decade, of human genome epidemiology from a discipline of case series or case–control studies of a few dozen participants to the accrual of large-scale teams and consortia including thousands of participants. The scope of findings has varied across different fields: 95 novel loci identified for lipid levels, 40 for Type 2 diabetes (T2D), 45 for Crohn's disease and 80 for bone mineral density versus only two, four and one for pancreatic cancer, smoking behavior and bipolar disorder, respectively. Indeed, we have just scratched the surface and very little is known about the implication of nongenetic exposure and how it maps across the phenome.

Two common disorders with a multifactorial component, CVDs and T2D, have been extensively studied since the advent of GWAS. Visvikis-Siest stressed the tremendous findings observed since 2007 in the genetics underpinning CVDs and related quantitative traits. However, these results weighed against the fact that no genetic variant identified by GWAS has been successfully integrated in cardiovascular risk assessment scores to date and even if the insights of personalized medicine based on GWAS findings is challenging, it is thus also premature.

As stated by Philippe Froguel, "GWAS can't read the future". Thus, their integration into personalized medicine should be through their ability to "illuminate the molecular understanding and physiology" of common disorders, such as T2D.

We are definitely witnessing a revolution of genetic epidemiology and yet the previous methodologies should not be abandoned but integrated in 'network studies' including gene–gene–environment interactions assessment and functional validations. Indeed, raw GWAS results do not give clear functional implications relevant to the physiopathology of common disorders and the missing heritability issue remains. This dark matter was consistently discussed by the experts. Philippe Froguel addressed the interest of studying rare/uncommon variants which should have higher penetrance. This 'rare variants (minor allele frequency < 0.01)/frequent disease' hypothesis has been experimented in the Meta-Analyses of Glucose and Insulin-related Traits Consortium (MAGIC) and a novel susceptibility gene for T2D and glucose has been highlighted. However, John Ioannidis still thinks that extending investigations on uncommon variants will lead to small effects anyway. In his opinion and in the opinion of Eleftheria Zeggini, this dark matter is also explained by imperfect phenotype definition and ascertainment; lack of power; epistatic (gene–gene) interactions; gene–environment interactions; differential effects in different populations; imperfect genome coverage for common variants; parent-of-origin specific effects; mutations; pedigree-specific effects and structural variants (rare or common alleles). Tremendous methodological improvement in GWAS should be performed to address all these limitations.

Meanwhile, alternative strategies should be undertaken. Sophie Visvikis-Siest proposed, for example, prospects on pediatric populations in which environmental effects are minimized in order to determine early genetic factors. This is a hint that the EA4373 Cardiovascular Genetics team is currently following on various quantitative traits: lipids, blood pressure and inflammatory markers.

Eleftheria Zeggini also described low-frequency and rare variant association analysis methodology using an alternative multivariate method to combine information across multiple variant sites. Several locus-specific approaches have been proposed and she discussed collapsing and allele-matching methods. These studies could be empowered by focusing on isolated populations, in which rare variants may have increased in frequency and linkage disequilibrium tends to be extended. This is the aim of the UK10k project gathering population-based cohorts with rich phenotype data (4000 whole genomes and 6000 whole exomes).

The following talks focused on post-GWAS on common disorders and Heather Cordell introduced the concept of maternal genotype and/or imprinting effect studying toxoplasmosis and possible genetic predisposition to this infection acquired *in utero*.

Allen Roses also highlighted phylogenetic analyses defining the evolutionary relations combining the use of rare and common polymorphisms (and not only SNPs) to determine the evolutionary genetics of complex disorders such as Alzheimer's disease. After tenfold sequencing of the *APOE–TOMM40* linkage disequilibrium region, he identified a polymorphic poly-T variant, rs10524523, in the *TOMM40* gene that provides greatly increased precision in the estimation of late-onset Alzheimer's disease risk and disease onset for *APOE* ε3 carriers.

It is clear that the need to upgrade 'conventional' GWAS strategies will succeed through technological advances and, thus, Martin Seifert from Genomatix, Carsten Rosenow from Illumina and Sören Deininger from Bruker discussed their last-generation sequencing tools, the results obtained with the '1000 genome project' and the progress with mass spectrometry technologies. Martin Seifert considered these upgrades in the core of evidence-based medicine. "As a matter of fact, next-generation sequencing technology is rapidly changing the scientific landscape and we cannot lose sight of the patient's profit: the technology's increasing efficiency will soon allow epigenomes, genomes and transcriptomes to be sequenced on an individual basis, thus improving knowledge of a patient's predisposition to given diseases". Indeed, treatments based on highly differentiated genetic diagnostics are becoming more feasible. For scientists and patients, "now is the right time to make this vision a reality".

Session: from genome-wide scan to systems biology

■ From genome-wide survey of human memory to drug identification & personalized medicine: opportunities & pitfalls

After the GWAS satellite meeting, the first session really in line with the conference spirit was introduced with a very unusual and stimulating presentation by Andreas Papassotiropoulos (University of Basel, Switzerland) on genetic liaison with human memory. He started by giving some examples of emotional memory. Using new tools based on genomic platforms, he demonstrated that genetics can identify molecular and signaling pathways related to human memory. He stressed the importance of precise phenotypes, which could then correlate with neuronal involvement.

For improving memory, he related some experiences with hydroxyfasudil but he preferred testing the simpler model of *Caenorhabditis elegans* and his knockout variant, which allows the study of the gene–environment interactions. Here again, the phenotype is of primordial importance. This introductory lecture between systems biology and future therapeutic approaches was exactly the theme of the conference.

■ Optimizing cancer pharmacotherapeutics using mathematical modeling & a systems biology approach

The second speaker, Jean Clairambault (Institut National de Recherche en Informatique et Automatique [INRIA], Paris, France), explored an entirely different aspect of systems biology, trying to convince us of the interest of mathematical equations based on cellular models and pharmacokinetics (PK) data for correcting drug dosage in cancer patients in function of their biological rhythms. Such a systems approach is really very useful to avoid toxic effects on non-malignant cells. He and his coworkers were using ATP-binding-cassette (ABC) transporter-regulated efflux and UDP glucuronosyltransferase genetic variant information on cultured Caco-2 cells with irinotecan as the anticancerous drug. The data obtained could help to optimize the drug infusion time schedules in patients while minimizing toxicity.

■ Molecular networks & the mechanism of action of drugs

With the third speech of Giulio Superti-Furga (Research Center For Medicine, Austrian Academy of Sciences, Vienna, Austria), we moved on to proteomics, an obligatory element of the systems biology approach. Each protein has multiple

domains and functions and Giulio Superti-Furga proposed the 'proteostasis' concept using the allosteric and cooperative data of many proteins. He was applying this concept to Gleevec®-based intestinal tumor treatment, showing how myristilation is modifying different domains of the BCR–ABL protein complex. Here again, cellular models and pathway analysis are necessary to understand the data obtained by the sophisticated mass spectrometry method. Drug targets are generally not a simple protein but larger multiprotein complexes.

Integration of genomic & epigenomic analysis in T2D

Finally, Christopher Bell (University College London [UCL] Cancer Institute, UCL, UK) introduced epigenetics in the systems biology approach with methylation data on CpG islands obtained in patients suffering from T2D in comparison with the same number of non-diabetic controls. He demonstrated the importance of controlling each technical step. He showed how the epigenetic data are complementary to the genomics data obtained by GWAS. The *FTO* obesity gene is at the fore of these interesting epigenetic responses, with a clear regulatory function in the brain. Mouse and zebrafish models were used to demonstrate the environmental effects linked to age. The involvement of epigenetics in evolutionary adaptation could then be better studied. However, epigenomic DNA methylation data must also be introduced in chronic disease routes.

Session: infectious diseases, genetics & new biomarkers

Studies on the effects of genetics on infectious disease were poorly known until the last few years, but evidence and characterization begin to be proved thanks to the development of new technologies, including information on the human genome. There is now epidemiological evidence supporting a role of genetics in susceptibility to disease infection. Mutation may be responsible for increasing the severity of a given pathology, but it can also protect from an infection. These studies need to be made in different large populations to distinguish the effect of genetics from that of the environment.

Such an approach should have been presented by Erwin Schurr (McGill Center for Study of Host Resistance, McGill University, Montréal, Canada), who was not able to be present in Santorini. However, the corresponding paper is due to be published in *Clinical Chemistry and Laboratory Medicine* [1].

The other speakers gave presentations that were more specific.

Progress of HIV-1 infection molecular epidemiology research in China

The complex genetic structure of HIV-1 epidemics can be seen in Asia, where various HIV-1 strains are co-circulating within and among different risk populations. Hong Shang (The First Hospital of China Medical University, Shenyang, China) spoke mainly about the genetic and epidemiologic profile of HIV-1 in different affected Chinese provinces. Some mutations may be responsible for a drug resistance mutation inducing a rise in acute HIV-1 infection. The high genetic diversity of HIV-1 adds considerable complexity to the development of efficacious vaccines.

Novel biomarkers for tuberculosis

Mycobacterium tuberculosis is a pathology affecting more than 2 billion people. There is still no real marker for the detection of this disease as presented by Gerd Michel (Foundation for Innovative New Diagnostics, Geneva, Switzerland). Many of the existing tests are not specific or not sensitive enough. The *M. tuberculosis* proteome contains 500 different proteins. A multiplex Luminex rapid immunoassay, a tuberculosis proteome microarray technology, has been developed and should allow to fight with efficacy tuberculosis associated or not associated with HIV infection.

Multiplex PCR coupled to biochip array technology for detection of ten sexually transmitted infections

Martin Crockard (Randox Laboratories, Crumlin, UK) developed a biochip array for the simultaneous detection of ten sexually transmitted infections. The array uses dual priming oligonucleotide technology for the multiple amplification of

highly specific pathogen sequences, before hybridization on a biochip array.

Session: inflammation as a common denominator of chronic & infectious diseases

The inextricable link between atherosclerosis & inflammatory chronic disease

Claudia Monaco (Kennedy Institute of Rheumatology, Imperial College London, UK) explained some cellular and molecular similarities in atherosclerosis and rheumatoid arthritis and showed that atherosclerosis is the most important complication of rheumatoid arthritis.

She demonstrated that Toll-like receptors (TLRs) drive local inflammation while TLR2 and TLR4 are the best-known members of the TLR superfamily. Their roles have been studied by her scientific group as the 'inextricable' link between chronic inflammatory disorders and CVDs. In addition, cytokine-dependent activation of the NF-κB pathway is a major mechanism involved in both diseases and could represent effective drug targets.

Novel biomarkers for better diagnosis of sepsis

Wei Cui (Peking Union College Hospital, Beijing, China) presented clinical criteria for diagnosis of sepsis and compared some current diagnostic biomarkers including C-reactive protein, procalcitonin and lipopolysaccharide-binding protein for this serious inflammatory condition. She told us that high expression of CD64 has been found to be a better predictive marker for sepsis than procalcitonin and C-reactive protein. As the early diagnosis of sepsis is crucial for treatment and prevention of death, according to recent results, miRNA-150 could also be an early sepsis marker. miRNA-150 is significantly downregulated in sepsis patients compared with healthy controls.

Inflammation & targeted immunotherapy

Jean-Yves Bonnefoy (Transgene, Strasbourg, France) told us that Transgene is developing a viral-based therapeutic vaccine for treatment of cancer and infectious diseases. He presented the TG4010 as an immunotherapeutic agent in cancer therapy, which gives a better survival of patients with normal levels of inflammatory proteins. He ultimately concluded that a combination of anti-inflammatory drugs and therapeutic vaccines may be more beneficial for cancer treatment.

Bioactive lipid mediators & resolution of vascular inflammation

Aksam Merched (INSERM U828, Pessac, France and Baylor College of Medicine, Houston, TX, USA) in an interesting presentation developed the role of bioactive lipid mediators in control of inflammatory homeostasis. With three animal study models including normal, switched off and overexpressed lipoxygenase gene, he demonstrated that 12 of 15 lipoxygenases provide anti-inflammatory signals and protection during normal progression of atherogenesis mediated by downstream products. The local inflammatory response could be controlled by agonist actions of these mediators on macrophages and vascular endothelial cells. As the plasma cholesterol level increased during switching of mice from chow diet to Western diet in this study, he finally proposed a 3D approach including use of lipid-lowering drugs, lipoxygenases, proresolution mediators and polyunsaturated fatty acids for prevention of hyperlipidemia and its treatment.

Genetics of inflammation markers & relationships with lipid metabolism

Ndeye Coumba Ndiaye (EA4373 'Génétique Cardiovasculaire', Nancy, France) discussed the role of inflammatory molecules, particularly haptoglobin, in CVD given its large genetic heritability. She also mentioned an ongoing GWAS in large pediatric populations, including the Suivi Temporaire Annuel Non Invasif de la Santé des Lorrains Assurés Sociaux (STANISLAS) cohort that has demonstrated so far that a unique new common genetic variant on the *HP/HPR* cluster explained an important proportion of the haptoglobin level variability.

Potential role of heat shock proteins in CVD: evidence from *in vitro* & *in vivo* studies

Majid Ghayour-Mobarhan (Mashhad University of Medical Sciences, Iran) talked about heat shock proteins' (HSPs)

discovery, their classification, functions and their role in atherosclerosis. He presented some of his epidemiological studies, clinical and animal experiences on HSPs. Anti-HSP60 and 65 antibodies are raised in obese and hyperlipidemic patients. HSP27 antibody titer falls beyond 12 h of postcoronary event.

When considering some animal studies, the role of HSPs in atherogenesis may not be straightforward, with different HSPs potentially having pro- or anti-atherogenic roles.

In addition to a potential role in the initiation of atherosclerosis, they may also be involved in the later stages of disease by inducing a proinflammatory autoimmune response and the recruitment of a HSP-specific inflammatory lymphocyte population.

He finally suggested that we should measure HSP expression in plasma, including serum antigen or antibody concentrations that may be useful as markers of disease susceptibility.

Session: CVDs & gene–environment interactions

■ Low-density lipoprotein-cholesterol-raising gene variants & future lipid-lowering drug usage

Genetic and environmental factors are important determinants of lipid profiles (low-density lipoprotein-cholesterol [LDL-C], high-density lipoprotein-cholesterol [HDL-C] and triglyceride [TG]). Using a 50K-SNP chip in 5592 participants in the WHITEHALL II study, Steve Humphries (UCL Genetics Institute and British Heart Foundation Laboratories, London, UK) has identified 21 SNPs in *PCSK9*, *SORT1*, *APOB*, *HMGCR*, *APOA5*, *CETP*, *LDLR* and *APOE* genes to be associated with LDL-C, 12 SNPs in *CETP, LIPC, LPL, APOA4* and *BUD13* with HDL-C and 16 SNPs in *GCKR, GIMP, TRIB1, RBP4, BAZ1B* and *LPL* with TG. Common alleles in these genes explained 14.6% of LDL-C traits. In combination, these common LDL-C-modifying alleles have substantial effects on LDL-C levels that, combined with Framingham risk score, predicts the use of lipid-lowering (statin) medication.

■ Interaction of dietary fatty acids with 5-lipoxygenase, *ALOX5* activating protein with subclinical markers of coronary artery disease

Population studies report contradictory results in regard to 5 lipoxygenase (*ALOX5*), lipoxygenase-activating protein (*FLAP*) and coronary artery disease (CAD). On one hand, it was reported that both loci predispose individuals to CAD. On the other hand it was shown that *ALOX5* and *FLAP* interact with ω3 and 6 fatty acids. Therefore, Michael Y Tsai (Department of Laboratory Medicine and Pathology, University of Minnesota, MN, USA) has investigated the interaction of SNPs in *ALOX5* with ω3 and 6 fatty acids in regard to carotid intima–media wall thickness and coronary artery calcification among 2847 participants in the Multi Ethnic Study of Atherosclerosis (MESA). One SNP near the 3′-end of *ALOX5* interacted with phospholipid fatty acids in order to modify the association with internal carotid intimal medial wall thickness and the extent of coronary artery calcification. In conclusion, Tsai's study confirms that ω3 and 6 fatty acid intake may potentially modify the genetic predisposition of *ALOX5* genes to CAD.

■ Use of phage display screening for biomarker discovery

Proteins secreted by atherosclerotic plaque into the bloodstream consist of new cardiovascular biomarkers. Danielle Hof (Institute for Clinical Chemistry, University Hospital Zurich, Switzerland) proposed to generate antibodies capable of specifically binding to plasmatic targets and to further identify them by mass spectrometry. Using a phage display approach, recombinant antibodies were generated against proteins in the secretomes against post-translational modifications that occur during the inflammatory processes, both taking place in the atherosclerotic plaque. The identification of these proteins is the first step towards the development of an immunoassay usable in routine clinics.

■ Are triglycerides causally related to CVD?

Triglycerides are inherited complex traits. With LDL-C and HDL-C they are used clinically to evaluate the risk of future

CVD. Using a 50K-SNP chip in the WHITEHALL II study, Philippa Talmud (Centre of Cardiovascular Genetics, UCL, UK) found that rs651821 in the *APOA5* cluster showed the strongest association with TG and with increased risk of coronary heart disease (CHD). Subsequent analyses demonstrated that rs651821 is in complete linkage disequilibrium with two other SNPs; together the rare haplotype explains 46% of TG variance and leads to decreased APOA5 expression, therefore increasing very low density lipoprotein plasmatic concentrations via reducing its catabolism. In contrast to rs651821 in the *APOA5* cluster, TG had no effect on CHD. In conclusion, *APOA5* cluster variants explain almost half of TG variance. If the relationship between TG and CHD is further clarified in other populations, *APOA5* might be used as a genetic instrument in Mendelian randomization to confirm this relationship.

Plasma plant sterols: a biomarker to predict lipid treatment efficacy?

Michel Krempf (University of Nantes, France) started his presentation by showing that intestinal absorption of cholesterol is estimated for years by using sitisterol and campesterol, plasma plant sterol concentrations, as markers. The status of cholesterol absorption is stable for each individual and poor or high absorbers could be individualized. Sitisterol and campesterol are not synthesized by humans and are poorly absorbed. It is believed that the plasma concentrations are an index of cholesterol absorption and could be a useful tool to identify cholesterol absorption status. Many studies have used this approach and have demonstrated a balance between absorption and synthesis estimated with lathosterol, a precursor of endogenous cholesterol synthesis: lower absorber being higher synthesizer and higher absorber being lower synthesizer. This paradigm was used to explain the large differences observed with cholesterol absorption treatment, such as ezetimide. In addition, this hypothesis is supported by numerous small clinical studies and recent analysis of larger trials has invalidated it. The origin of this discrepancy is still unclear but might be related to the use of sitisterol and campesterol to estimate cholesterol absorption.

Session: nutrigenomic epigenetics

Nutrigenomics: about mechanism, efficacy, disposition & programming

Martin Kussmann (Nestlé Research Center, Lausanne, Switzerland) introduced nutrigenomics, a domain that was recently catalyzed by the revelation of the human genome, thereby showing the first extra large extension to human bacterial and plant genomes. Nutrigenomics is influencing immune, digestive, mental, skeletal, muscular and metabolic diseases. Cohorts are very important in this research domain with nutritional intervention. The mechanisms are often linked to epigenetic regulation and are based on genetic, transcriptomic, proteomic and metabolic approaches. Animal models are also interesting tools for studying the intestinal and gut flora.

Phenotyping approaches & 'omics' applications in nutrition research

Hannelore Daniel (Center for Diet and Disease, University of Munich, Germany) presented an exciting proteomic profiling approach in nutritional human clinical trials, often with healthy volunteers. New proteomic phenotypes could be measured on plasma, saliva, urine and blood cells, which are the only readily available material, but it is also necessary to apply these profiling techniques to dynamic studies, requiring many samples. Mass spectrometry and nuclear magnetic resonance-based profiling are interesting technologies used for intervention studies with soy isoflavone and flaxseed.

The most interesting part of Hannelore Daniel's presentation was the human metabolomic study performed in Munich between more than ten institutions on 15 healthy volunteers who stayed 4 days in a study center with two blood samples taken per day. After a fasting period they received different standard liquid diet and were submitted to different tests and stress. She presented results on plasma amino acid charges during fasting and after glucose

administration, resulting in opposite variations. The relationship with metabolic pathways are then absolutely necessary for the interpretation of the data and the variation observed. It is then possible to 'titrate' the capacity of adaptation in time and space and to identify deviation from normal.

Long-term influence of diet & environment on gene expression

The Barker hypothesis states that adverse influences early in development and particularly during intrauterine life can result in permanent changes in physiology and metabolism, which result in increased disease risk in adulthood. In this context, obesity and insulin resistance are major public health problems in the 21st century. A better understanding of the factors involved in their development is crucial, especially if one third of obese children will remain so into adulthood. Using an ω3 fatty acid supplementation for murine models, Edgar Delvin (CHU Sainte-Justine, University of Montreal, Canada) has shown that its effect is exercised on first mouse generation and even persists until the second one. Therefore, based on clinical and experimental evidences, Barker's hypothesis could be supported. Furthermore, their experimental results have indicated that epigenetic DNA and perhaps histone modifications are involved.

Nutrigenomic mechanisms of the deficiency in methyl donors: experimental-based evidence & population studies

Neurologic and aging disorders are linked to homocysteine and methyl donor deficiency, but their mechanisms remain to be clarified. By using cellular models in a first step, Jean-Louis Guéant (INSERM U724, Université Henri Poincaré and University Hospital of Nancy, France) has demonstrated that methyl donor deficiency in NIE-15 murine neuroblastoma cells reduced proliferation and increased cell differentiation via upregulation of protein phosphatase 2A, pro-nerve growth factor and TNF-α converting enzyme (TACE). In the same direction, folate deficiency in H19-7 rat neuronal progenitor cells affected their proliferation, differentiation and synaptic plasticity through altered cytoskeleton proteins. In a second step, *in vitro* experiments on rat models were also used to assess the impact of methyl donor deficiency during gestation. The pups displayed increased homocysteine concentrations in the hippocampus and cerebellum, and reduced thickness of the CA1 pyramidal layer that was restored to normal in older rats when applying a normal diet. Homocystein accumulation decreases cystationine-β-synthase in Purkinje cells. Combined with defects in locomotion coordination and in proteins responsible for synaptic migration, vitamin B deprivation in early life may produce selective and persisting brain defects consistent with neurodegeneration. These results could be applied in the Outcome in Ankylosing Spondylitis International Study (OASI) cohort, composed of eldery subjects.

Nutrigenetics in the GWAS era

Nabila Bouatia-Naji (CNRS and Pasteur Institute, Lille, France) presented a large overview on GWAS applications to nutrigenomic studies.

Session: nutrigenomics & gene interaction studies

Self-reported zinc & zinc intake–genetic loci interactions of health status & age-related diseases

Zinc (Zn) is an essential trace mineral for human health; its deficiency can lead to dermatitis and impaired immune function. Infants are particularly vulnerable because of the large amount of Zn required for growth during this period. By defining a Zn level score (significantly correlated to daily Zn intake), George Dedoussis (Harokopio University, Athens, Greece) has evaluated the impact of the -174G/C polymorphism and Zn diet score on plasmatic IL-6 levels. He found this SNP interacted with Zn diet score, as GG genotype was associated with higher plasma IL-6 levels compared with GC/CC genotypes, with increasing Zn diet score. In addition, a significant interaction was observed between another SNP and increased glucose levels; this interaction diminishes as total Zn intake increases

(per 1 mg/day). In conclusion, George Dedousis revealed that Zn intake interacts with gene variants to influence IL-6, associated with health status and diabetes

■ Validation of the use of peripheral blood mononuclear cells as surrogate markers for skeletal muscle tissue in nutrigenomic studies

Peripheral blood monocyte cells (PBMCs) are considered as a substitute of traditional tissue specimens that are not easily accessible. Iwona Rudkowska (Institute of Nutraceuticals and Functional Food, Quebec, Canada) has investigated the possiblity of using PBMCs for gene-expression analysis as an alternative to skeletal muscle tissue (SMT). By carrying out a transcriptomic analysis comparison of PBMCs versus SMT after 8 weeks supplementation with n-3 polyunsaturated fatty acid in 16 insulin-resistant subjects, Iwona Rudkowska found that 88% of transcripts (32341 transcripts) were common in PBMCs and SMT. In addition, a strong correlation ($r = 0.84$; $p < 0.001$) was present between transcript expression levels of PBMCs and SMT. In conclusion, PBMCs express the majority of SMT transcripts with comparable expression levels between both tissues.

■ Genetic variants near *IRS1* & their association with T2D, hyperinsulinemia & insulin resistance in UK population-based cohorts & T2D patients

Genome-wide association studies identified a novel SNP, rs2943641, near *IRS1* loci that is highly associated with T2D, this association not being replicated in other European populations. Therefore, Nikolaos Yiannakouris (Harokopio University, Athens, Greece) conducted his study to validate the rs2943641C>T association with T2D risk and diabetes-related quantitative traits using data from UK population-based cohorts and T2D patients, and to explore potential associations with the risk of T2D of SNPs within and flanking the *IRS1* gene. The rs2943641C was associated with a 14% increased relative risk of T2D ($p = 0.006$), with CC subjects having an odds ratio of 1.21 compared with T allele carriers. In addition, the C allele of rs2943641 near *IRS1* was associated with higher fasting, glucose-stimulated hyperinsulinemia and impaired insulin sensitivity. Their data also suggest that rs2943641 and an *IRS1* putative promoter variant (rs6725556) may independently influence T2D risk, although further studies with larger cohorts are needed to confirm the etiological SNPs and to analyze their interactions in different populations.

■ Adaptive genetic variation & risk of metabolic syndrome & CVDs

Modern human populations have experienced diverse adaptation to environmental changes in the past 100,000 years and this adaptation has been imprinted on the human genome as allele frequency changes in certain genetic variants. By contrast, dietary habits and lifestyles have changed faster than our genomes have adapted to the environment. Chao-Qiang Lai (Nutrition and Genomics Laboratory, JM-USDA Human Nutrition Research Center on Aging at Tufts University, MA, USA) explained that some genetic variants interact with dietary intake and lifestyles to influence the risk of metabolic syndrome. In addition, he explained that the recent rise of metabolic syndrome is partially due to rapid environmental changes that are poorly fitting for genomes adapted to traditional environments.

Session: fundamental pharmacogenomics

■ From drug metabolism to pharmacogenomics & personalized healthcare

Urs A Meyer (Biozentrum, University of Basel, Switzerland), in an interesting presentation, defined pharmacogenetics as "linking of variation of human genes to clinical responses to drugs", gave a history and described the effects of pharmacogenetics on the safety and efficacy of drug therapy, highlighting examples of genetic variations that affect the response to treatment. Prepresciption genotyping and the use of pharmacogenomic biomarkers, either as required or recommended, help physicians to predict the drug response, prevent adverse drug reactions or determine drug

dosing based on the patient's drug-metabolizing status. While genotyping for *HLA-B*5701* is required to prevent hypersensitivity in HIV patients taking abacavir and is recommended for SLCO1B1 to prevent statin-induced myopathy in patients with hypercholesterolemia, routine genotyping for *CYP2D6* in women with breast cancer who are on tamoxifen is not accepted and is controversial. Furthermore, in spite of existing data about the role of CYP2C9 and VKORC1 in the metabolism of warfarin and CYP2C19 in the metabolism of clopidogrel, the clinical utility of these biomarkers of drug response remains controversial. In addition, some side effects such as carbamazepine hypersensitivity are only observed in Chinese populations, which demonstrates the importance of ethnic differences.

Polymorphisms affecting gene transcription in pharmacogenetic candidate genes: detection through allelic expression imbalance

In an exciting lecture, Wolfgang Sadee (The Ohio State University, OH, USA) first showed the general contribution of different types of SNPs – including coding SNPs that alter the encoded amino acids sequence, regulatory SNPs (rSNPs) in untranslated regulatory region of the gene and structural RNA SNPs (srSNPs), which are polymorphisms in the RNA transcript – to changes in protein activity and gene transcription. Then, he elucidated in detail the pharmacogenetic effects of rSNPs and srSNPs on changes in gene expression of drug-metabolizing enzymes, with a focus on rSNPs. rSNPs, which are likely to be more prevalent than coding SNPs, affect gene transcription and are confirmed by allelic expression imbalance, defined as transcription of two allelic variations of the same gene in different amounts due to a polymorphism in a regulatory part of the gene. This is also important for gene–gene interaction (epistasis). These rSNPs considerably affect efficacy and adverse drug effects in drug therapy. In a recent study that has been published in 2010, Wang *et al.* revealed that rSNPs in *CYP3A4* are as frequent as 4–8% and are responsible for two- to five-fold increases in gene expression, which subsequently leads to a decrease in statin dose requirement. rSNPs and srSNPs are now considered as drivers of evolution [2].

Transcriptional regulation & pharmacogenomics

The presentation of George P Patrinos (School of Health Science, University of Patras, Greece) had two relatively separate parts. In the first section, he reported the preliminary results of an ongoing study regarding the role of copy number variants, as a pharmacogenetic biomarker, in response to lithium. The results revealed at least two different chromosomal positions where losses of genomic fragments have been indicative for responsiveness to lithium treatment. In the second part of his speech, based on the results of another study, George Patrinos talked about the correlation between *KLF1* (a hematopoietic-specific transcription factor that induces high-level expression of adult β-globin) gene mutation and high levels of fetal hemoglobin in erythroid cells of β-thalassemic patients. KLF1 also indirectly suppresses fetal hemoglobin genes by activating BCL11A, a known suppressor of fetal hemoglobin gene transcription. This genetic variation accounts for different drug therapy in patients with the same β-thalassemia-causing mutation and occasionally an identical chromosomal background.

Relevance of drug transporters for pharmacotherapy

The imperative role played by different transport systems in drug PK and pharmacodynamics (PD) was the subject which Peter Meier-Abt (University of Basel, Switzerland) described in his sophisticated presentation. He showed the importance of drug transporters by reporting acute rejection in two heart transplant patients due to a St John's wort-induced intestinal P-glycoprotein (P-gp) drug transporter upregulation which led to a decrease in cyclosporin plasma concentration. The major transport systems involved in the uptake of organic compounds belong to the organic anion transporters and organic cation transporters and the organic anion-transporting polypeptides of the SLCO superfamily of polyspecific drug transporters. The kinetics of this system depend, to a large extent, on the pH of the cell

and they are highly substrate specific. On the other hand, ABC (multiple drug resistance [MDR]) proteins are the other major transporter system which transports various molecules across extra- and intra-cellular membranes. Differences in drug transporter expression and activity contribute to variability of the PK and PD of drugs and statins are among the most extensively studied medications. The *SLCO1B1*1b* gene polymorphism was associated with increased transport activity and decreased systemic bioavailability of statins and the *SLCO1B1*5* variant has been considered a major factor in limiting the uptake of statins and increasing statin exposure and, consequently, increasing the risk of myopathy. On the other hand, in a recently published study, MDR3 (*ABCB4*) and MRP2 (*ABCC2*) polymorphisms have been associated with drug-induced liver injury and cholestasis in patients who were on risperidone and amoxicillin [3].

SNPs in genes encoding G proteins in pharmacogenetics

G proteins communicate signals from many neurotransmitters and drugs outside the cells, which cause changes inside the cells; therefore, SNPs in genes encoding G proteins are considered, in pharmacogenetic studies, as a "bottleneck in signal transduction pathways", as described impressively by Winfried Siffert (Institute of Pharmacogenetics, University Hospital Essen, Germany). G proteins consist of α, β and γ subunits. Due to significant effects on the activity level of G proteins, polymorphisms in subunits of this molecule are a matter of interest pharmacogenetically. The *GNβ3* C825T polymorphism in the gene of the G-protein β3-subunit is associated with the occurrence of a splice variant termed Gβ3s which is a biologically active Gβ-protein that may mediate the enhanced signal transduction observed in cells with the 825T allele. The vasoconstriction response to endothelin-1, angiotensin II and α2-agonists was significantly enhanced in patients with the C825T polymorphism. T allele carriers of the C825T polymorphism also showed an increased blood pressure reduction in response to clonidine, a central α2-adrenergic receptor stimulant, and showed a better response to selective serotonin reuptake inhibitor antidepressant treatment. On the other hand, the functional *G-1211A* SNP in the *GNAS* promoter causes the impairment of transcription factors' binding sites, significantly changes Gα protein expression and lipolysis levels in human fatty tissues; therefore, A allele carriers benefit from sibutramine antiobesity therapy, while G allele carriers can lose weight without the drug and are at risk for exaggerated heart rate when using the drug. Overall, functional SNPs in genes encoding G proteins, receptors or downstream effectors are excellent candidates to predict drug response.

Session: pharmacogenomics translational aspects

Translation of technologies into clinical practice: the example of drug metabolism enzymes & transporters

Richard Dean Hockett (Affymetrix, CA, USA) briefly and comprehensively reviewed the genes involved in the drug metabolism pathway. He believed that pharmacogenetic research has been restricted to the search for polymorphisms in one or several genes thought to be involved in a drug's disposition. However, now we know that drugs pass through complex metabolic pathways rather than experiencing a single enzymatic modification, it has become clear that pharmacogenetic research must expand beyond the candidate gene approach. Richard Hockett then introduced the well-validated drug metabolism enzymes and transporters (DMET) Plus chip developed by Affymetrix Inc., which can genotype large amounts of variant in numerous genes involved in drug disposition. Finally, he reported the results of their recently published, large-sample study in which the authors comprehensively assessed the allele frequencies of 165 variants comprising 27 DMET genes from 2188 participants across three major ethnic populations (Caucasians, Africans and East Asians) [4].

Systems characterization of drug combination therapies in transgenic mice: focus on hypercholesterolemia

In a nice presentation, Lars Verschuren (TNO Quality of Life, Zeist, The Netherlands), using the results of the

study in which he compared the genes affected by combination therapy with statin and ezetimibe versus single drug treatment in transgenic mice, described a 'systems biology approach'. In this approach, genes affected by individual drugs are characterized. Considering all data for each single drug therapy may help us to recognize the genes involved in combination therapy and consequently their clinical effects.

Gene-expression meta-analysis of response to infliximab in rheumatoid arthritis

In her short but informative lecture, Alessandra CL Cervino (TcLand expression, Nantes, France) presented a meta-analysis including seven studies (208 patients) on the pharmacogenetic response to infliximab, an anti-TNF agent used in the treatment of rheumatoid arthritis. Interestingly, each study reported completely different sorts of genes as being responsible for the variability observed in response to treatment. Alessandra Cervino explained how she and her colleagues used a six-step process (identification of public data, clinical homogenization, technical homogenization, quality control, probe to gene mapping and algorithm choice) to prepare a standardized meta-analysis. Finally, she presented a list of involved genes as the result of the meta-analysis.

Auto-antibody signatures for disease diagnosis in cancer & autoimmune disease

During their lifespan every person produces antibodies directed against human antigens (auto-antibodies; AAs), which reflect the immune response to a continuous remodeling of cells or tissues caused by a chronic disease process. This fact is the fundamental principle of the technology that UNIarray® (Protagen AG, Germany) – systematic identification of AA signatures – has been made based on and this was introduced in a nice presentation by Peter Schulz-Knappe (Protagen AG, Dortmund, Germany). UNIarray® provides a new *in vitro* diagnostic and companion diagnostic for patient stratification and therapy selection using AAs in the blood. There are numerous advantages of using AAs in serum/plasma compared with genomic based markers in diagnostic applications, including:

- AAs are stable in serum and plasma;
- AAs are present in serum or plasma in high concentrations;
- No enrichment or elaborated sample preparation is required;
- AAs are not subject to circadian rhythms or other short-term changes in physiological states.

The companion diagnostic capability of AA detection has particularly been shown in multiple sclerosis and prostate cancer.

Pharmacogenomics translational aspects: thienopyridine genetics

Clopidogrel, the most well-known member of the thienopyridine family and the second top-selling drug in the world, is currently the antiplatelet treatment of choice for preventing secondary cardiac events across the spectrum of acute coronary syndrome and in patients undergoing percutaneous coronary intervention. However, up to 30% of patients do not display adequate antiplatelet response and patients with coronary disease with lesser degrees of platelet inhibition in response to clopidogrel appear to be at increased risk for cardiovascular events. Such variability may arise from genetic alterations in genes involved in the PK and PD of clopidogrel. Using clopidogrel as an example, Sandra Close Kirkwood (freelance consultant, USA) explained, in an exciting lecture, how genetics can be translated into clinical practice. Clopidogrel is an inactive prodrug requiring two-step oxidation by hepatic cytochrome P450 (CYP) to generate its active metabolite, which irreversibly targets the adenosine diphosphate P2Y12 receptor that consequently blocks activation of the glycoprotein IIb/IIIa pathway and prevents platelet aggregation. SNPs in genes involved in the PK/PD of clopidogrel can potentially change the response to this drug. Among the hepatic enzymes involved in the metabolization process of clopidogrel (CYP2C19, 3A4/5, 1A2, 2B6 and 2C9), SNPs of CYP2C19 have been associated with significant changes in response to drug treatment and metabolizing status.

Several studies showed that *CYP2C19*2/3* and *CYP2C19*17* polymorphisms are associated with decreased and increased activity of CYP2C19, respectively. Accordingly, a US FDA Box Warning released in March 2010 warned about the diminished effectiveness of clopidogrel in poor metabolizers (*CYP2C19*2/3* SNP carriers).

■ mRNA flow fish: a novel approach in HIV tropism detection & a valuable tool for the decision over CCR5 blockers

As the scientific director of FlowCytoGen Laboratories Ltd, as well as product manager of Zafiropoulos Co., George Lallas (Zafiropoulos Company, Athens, Greece), in a stimulating and exciting talk, presented HIV Monitoring Assay (HIV Reservoirs Assay ViroTect®VR and HIV Tropism Assay ViroTect®Tropism), a fast, quantitative, accurate and cost-effective theragnostic method for personalizing and monitoring virus therapy. Using a combination of these two arrays provides the possibility of determining the efficacy of HIV treatment in individual patients. Following therapy, patients can be completely suppressed or can have persistent replication in T cells or monocytes; therefore, therapy can be tailored to a patient's own reservoirs (T cells or monocytes) to achieve better immunologic recovery and prolong the interval to first treatment failure. On the other hand, determining the tropism of HIV (i.e., the cell type that HIV infects) in individual patients may make them candidates for CCR5 inhibitors (CCR5 is used only with a specific strain of HIV-1).

Pharmacogenomics & personalized medicine in clinical practice: introduction to the round table discussions

■ Changing clinical practice based on evidence generated in the real world: a series of comparative effectiveness study examples

Pharmacogenomics in clinical practice: example from the real world

Bryan Dechairo (Medco Health Solutions Inc., MD, USA) introduced this session by discussing the problems arising when considering the events from scientific innovations to clinical applications. Going by classical examples (warfarin, tamoxifen) he showed that translation from bench to bedside currently takes approximately 14 years. If the patient's and private payer's acceptance of personalized medicine is greater than that of physicians and government payers, the pharmacogenomics implementation could be quicker. Understanding impact on patient outcomes and cost of test implementation are essential for reimbursement decisions. At least, physician education paired with patient empowerment will be key for future success, which can be summarized in decreasing the time from discovery to clinical practice and in changing clinical practice through evidence of clinical utility.

Impact of ABC transporters in epilepsy & stroke

Alberto Lazarowski (Buenos Aires University, Argentina) showed the importance of ABC transporters (particularly P-gp, the multidrug resistance gene product, but also multidrug-resistance-associated proteins), which are known to play a role in antiepileptic drug extrusion, but are not by themselves sufficient to fully explain the phenomenon of drug-resistant epilepsy. It has been shown that ABC transporters are overexpressed in refractory epilepsy. The study of the *MDR-1* gene's SNPs (15) could explain the differences in efficacy of antiepileptic drugs.

Application of pharmacogenetics in psychiatry: clinical & technical aspects

Adrian Llerena (CiCAB-Clinical Research Centre, Extremadura University Hospital, Badajoz, Spain) highlighted the necessity of developing clinical psychopharmacology (by independent clinical trials and pharmacovigilance) as knowledge of genetics increases. If personalized medicine (prediction for a subject) is not currently feasible in the field of psychiatry, stratified medicine (prediction for a group) is conceivable. As genetics and physiology are tightly connected, the use of pharmacogenetics in clinical psychiatry must take into account PK of drugs but also therapeutic

response, metabolism modulations and adverse reactions.

Cardiopharmacogenomics: application of pharmacogenomics in cardiology

Jerry Yeo (University of Chicago, IL, USA) noticed that, currently, in cardiology, two pharmacogenomic tests were relabeled by the FDA (CYP2C9 and VKORC1 for warfarin and CYP2C19 for clopidogrel. However the pharmacogenomics testing for predicting warfarin responsiveness does not seem to improve health outcomes and, in the Center for Medicaid and Medicare Services (CMS), it is covered only for candidates for anticoagulation therapy and in the context of a prospective, randomized controlled clinical study. Regarding clopidogrel, interindividual variability in response to this drug is multifactorial (lack of compliance, obesity, nature of coronary event, genetic factors in absorption and metabolism, and drug–drug interactions), but the role of *CYP2C19*2* and *CYP2C19*3* alleles in reduced metabolism of clopidogrel is well recognized even if other alleles could be implicated at a lower level. Another example is given by *KIF6*, which encodes a kinesin (family of dimeric motor proteins involved in the intracellular transport of organelles, protein complexes and mRNAs). The *KIF6* Trp719Arg carrier status (Trp719Arg replaces a nonpolar residue with a basic residue near the coiled-coil structure and affects cargo binding or regulation of the motor domain) affects response to statins and CHD event reduction.

Thus, pharmacogenetic markers will increasingly be used for personalizing drug therapy in cardiology. Their clinical utility is recognized, since they can be used for dose adjustments (warfarin), selection of alternative drugs or dose escalation for loss-of-function carriers (clopidogrel) or selection of patients for aggressive therapy (statins).

Clinical applications: pharmacogenetics of immunosuppressants

Ron Van Schaik (Pharmacogenetics Core Laboratory, Erasmus MC Rotterdam, The Netherlands) showed the interest of pharmacogenetics of immunosuppressants for clinical applications, with examples of tacrolimus, cyclosporin (two calcineurin inhibitors) and prodrug mycophenolate mofetil. Tacrolimus is metabolized by CYP3A4 and CYP3A5 and displays nephrotoxicity. It has been shown that *CYP3A5* genotyping can be useful for guiding tacrolimus dosing. Mycophenolate mofetil has also to be dosed facing the possibility of acute rejection and *UGT1A9* -275T>A carriers have a lower mycophenolic acid bioavailability and a higher risk of acute rejection. Finally, *ABCB1* genotype of the donor seems to be correlated with cyclosporin nephrotoxicity.

Development & use of theranostics biomarkers: needs of guidelines

Genotype-guided dosing of coumarin derivatives: the European Pharmacogenetics of Anticoagulant Therapy Consortium trial

Vangelis G Manopoulos (Democritus University of Thrace, Alexandroupolis and Academic General Hospital of Alexandroupolis, Greece) emphasized the necessity of monitoring the anticoagulant effect of coumarins, due to inter-/intra-patient variability in the response: clinical habits consist of testing the international normalized ratio (INR). Coumarin responses are affected by demographic, clinical and genetic factors, among which are the polymorphisms in two genes: *CYP2C9* and *VKORC1* (the coumarin target enzyme in the vitamin K cycle, vitamin K epoxide reductase complex subunit 1). *CYP2C9*2* and/or **3* allele carriers have increased risk of major bleedings and thus require lower daily doses of coumarins. For *VKORC1*, 28 SNPs have so far been identified. Patients carrying *VKORC1* haplotype A (-1639A/1373T) are more sensitive to coumarins whereas patients carrying the *VKORC1* haplotype B (-1639G/1373C) show coumarin resistance. Thus, polymorphisms in the *CYP2C9* and *VKORC1* genes together account for approximately 40–50% of the variability in coumarin maintenance dose requirements. However, as large prospective randomized clinical trials are missing, the European Pharmacogenetics

of Anticoagulant Therapy (EU-PACT) wants to assess, in a single-blinded and randomized controlled trial with a follow-up period of 3 months, the safety and clinical utility of genotype-guided dosing in daily practice for the three main coumarin derivatives used in Europe. The proposed study will be undertaken in seven different European countries at 13 different centers. The aims of the study are first to improve effectiveness of anticoagulation therapy and second to improve the safety of the treatment, the quality of life of the patient and to determine whether genotype-guided dosing is cost effective.

Thus, the EU-PACT trial will evaluate a pharmacogenetic approach to anticoagulation therapy with coumarin derivatives using pharmacogenetic-based dosing algorithms, which include relevant polymorphisms of *CYP2C9* and *VKORC1* genes.

Development & use of theranostic biomarkers: impact on the industry business model; needs of guidelines & market access issues, the European case

Alain Huriez (European Personalized Medicine Diagnostics [EPEMED] and TcLand expression, Nantes, France) pointed out the necessity of a rigorous strategy for developing personalized medicine without forgetting an important constraint consisting of cost minimization and effectiveness.

Companion diagnostics generate great hopes in the pharmaceutical industry for optimizing each step of the drug value chain, particularly when companion diagnostics will be proposed to current marketed blockbusters. However, a small number of companion diagnostics have reached the market. Europe is far behind the USA when considering practical applications of molecular diagnostics, companion diagnostics or personalized medicine and huge needs remain in terms of education of the various stakeholders in Europe for a better understanding of the recent advances and the needs to bring such innovations to the EU market.

Serving this purpose, EPEMED is a not-for-profit organization bringing together forces in personalized medicine, confronting industry, regulators, payers and insurers as well as governments. It will function as a platform for the harmonization of personalized medicine development and implementation across Europe, focusing on the crucial role of diagnostics.

Conclusion

This conference brought together scientists with backgrounds ranging from human genomics to epidemiology to improve our understanding of human genetic variability and its impact on the prediction, prevention, diagnosis and therapy of multifactorial disorders. In the spirit of this conference, new concepts should help the implementation of molecular biology in clinical practice. Through the different presentations, systems biology, animal and cellular models, metabolomics, proteomics and proteostasis concepts were developed. Blood cells, particularly white cells, are in many cases interesting tools, as are cohorts and biobanks, which are more and more important for studying genetic variations in large populations. For understanding health state evolution, we should remember that dietary habits and lifestyles have changed faster than human genome adaptation to our different environments. This is also true for drug responses. Drugs use pre-existing genes and enzymes for their biotransformation, genes which were devoted to xenobiotics from the surrounding trees or endobiotics from our normal metabolism (such as steroids). The gradual introduction of genetic and genomic biomarkers in personalized therapy over recent years was discussed in a round table [5], which led to the creation of the European Society of Pharmacogenomics and Theranostics (ESPT) [101].

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Website

101 European Society of Pharmacogenomics and Theranostics
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Résumé :

L'hypertension artérielle est un trait polygénique ayant une héritabilité estimée entre 31% et 68%. Les variants génétiques découverts n'avaient qu'un faible effet et n'expliquaient qu'une infime partie (1%) de sa variabilité phénotypique. Ce résultat était à l'origine du concept d'héritabilité manquante. Nous pensons que l'héritabilité manquante pourrait s'expliquer par des interactions gène-gène (épistasiques), gène-environnement et la méthylation d'ADN. Une fois complétées par des études transcriptomiques, ces approches pourraient révéler les voies moléculaires impliquées. C'est le postulat principal que nous avons poursuivi au cours de cette thèse.

Ainsi, dans des populations européennes (en majorité françaises), nous avons identifié une nouvelle interaction épistasique fonctionnelle entre le rs5355C>T du gène *SELE* et le rs6046G>A du gène *F7* associée à la pression artérielle systolique. Dans cette même direction, nous avons rapporté deux autres interactions (rs1041163T>C dans *VCAM1* interagit avec rs1367117G>A dans *APOB* et rs3741240G>A dans *SCGB1A1* interagit avec rs1800590T>G dans *LPL*) pour être associées avec la pression artérielle. De plus, nous avons trouvé que le SNP intergénique rs7556897C>T dans le locus *SLC19A3-CCL20* interagissait avec l'obésité pour influencer la pression artérielle diastolique. En parallèle aux études précédentes, nous avons montré que le rs5030878T>C du gène *FPR1* et l'allèle KL-VS du gène *Klotho* pourraient être impliqués dans la régulation de la pression artérielle via leurs interactions avec l'âge et le traitement antihypertenseur respectivement. Contrairement aux autres variants génétiques étudiés, aucune association entre l'haplotype CAT1/2 du gène *CAT* et les niveaux de PA n'a été observée. Nous avons aussi participé à l'identification d'un nouveau SNP, le rs2000999G>A, expliquant quasiment la moitié de l'héritabilité de l'haptoglobine dont le rôle antihypertenseur est en cours d'investigation. Finalement, nous avons proposé une nouvelle catégorie de SNPs les eMethSNPs.

En conclusion, nous rapportons une panoplie de variants génétiques qui pourraient réguler la pression artérielle via des interactions gène-gène et gène-environnement. Les interactions identifiées dites 'fonctionnelles' montrent que l'inflammation de faible niveau est impliquée dans la régulation de la pression artérielle.

Abstract :

Essential hypertension is a polygenic trait, its heritability varies between 31% and 68%. Discovered genetic variants were shown to have small effects, consequently explaining a tiny fraction (1%) of its heritability and resulting to a missing heritability. The missing heritability could be explained by gene-gene (epistatic), gene-environment interactions and DNA methylation. Once conducted, these approaches should be further complemented by transcriptomic analyses, and thereby to reveal the involved molecular pathways. This is the postulate followed during our thesis.

Therefore, in European populations (mainly French), we have shown that rs6046G>A in *F7* interacted with rs5355C>T in *SELE* in order to influence systolic blood pressure levels. In the same direction, we have reported two other interactions (rs1041163T>C in *VCAM1* interacts with rs1367117G>A in *APOB* and rs3741240G>A in *SCGB1A1* interacts with rs1800590T>G in *LPL)* to be associated with blood pressure. We have also found the intergenic SNP rs7556897T>C, located between *SLC19A3* and *CCL20* locus, interacting with obesity in order to influence diastolic blood pressure. In parallel to those studies, we have shown that rs5030878T>C in *FPR1* and KL-VS allele in *Klotho* may be implicated in BP regulation through their interaction with age and antihypertensive drugs respectively. In contrast with the above-investigated genetic variants, CAT1/2 haplotype in *CAT* was not associated with blood pressure levels. We have also participated to the identification of a novel SNP, the rs2000999G>A explaining up to the half of haptoglobin's heritablilty, a protein currently under investigation for its antihypertensive role. Finally, we proposed a new category of functional genetic-epigenetic biomarkers, the eMethSNPs.

In conclusion, we report a range of genetic variants that may regulate BP and influence its levels via gene-gene and gene-environment interactions. The identified 'functional' interactions show that low-level inflammation is involved in blood pressure regulation.