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The study of “ -omics ” determinants of VEGF in the personalised medicine of chronic diseases

Maria Stathopoulou

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Ecole Doctorale BioSE (Biologie-Santé-Environnement)

Mémoire

Présenté pour la candidature au diplôme

D'HABILITATION À DIRIGER DES RECHERCHES

par Maria STATHOPOULOU

**L'étude des déterminants « -omiques » du VEGF dans la
médecine personnalisée des maladies chroniques**

Le 17 Avril 2019

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Curriculum vitae

Identification

Last name : Stathopoulou
First name : Maria
Date of birth : 28/01/1981
Family situation : Married
Grade : Research Engineer
Institution : Inserm
Research Unit : Université de Lorraine, Inserm, IGE-PCV; U1122, Nancy

Education

- 2010** *PhD “The effect of vitamin D receptor gene (VDR) polymorphisms on osteoporosis risk of postmenopausal women with low calcium intake”*
Laboratory for the Research of the Musculoskeletal System (LRMS),
Medical School, National and Kapodistrian University of Athens
- 2010** *MSc “Bone Metabolic Diseases”*
Laboratory for the Research of the Musculoskeletal System (LRMS),
Medical School, National and Kapodistrian University of Athens
MSc dissertation project *“The effect of common polymorphisms of LRP5 gene on bone mineral density of postmenopausal women. Possible interactions with calcium intake”*.
- 2003** *BSc in Dietetics-Nutrition, Harokopio University of Athens, Greece*
BSc dissertation project *“Response in calcitonin therapy and association with CTR polymorphisms in osteoporotic postmenopausal women”*.

Research Experience

2015-today **Research Engineer, Inserm**

Université de Lorraine, Inserm, IGE-PCV « Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire» ; U1122.

2010-2014 **Post-doctorant Researcher**

Université de Lorraine, Inserm, IGE-PCV « Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire» ; U1122.

Fund by « BioIntelligence » project and a fellowship from “Région Lorraine”, France

2004-2010 **Doctoral Researcher**

Project “*The effect of vitamin D receptor gene (VDR) polymorphisms on osteoporosis risk of postmenopausal women with low calcium intake*”.

Laboratory for the Research of the Musculoskeletal System (LRMS), Medical School, National and Kapodistrian University of Athens.

Director : George P LYRITIS (Professor)

Fund by the Hellenic Society for Research of Metabolic Bone Diseases, Greece and a fellowship for doctoral studies from the State Scholarships Foundation, Greece

2004-2010 **Contractual Researcher**

✓ **2008-2009** Participation in the Project: “*Childhood obesity: assessment of resting energy expenditure, anthropometric indices, VO2max and dietary patterns in obese and non-obese Greek children*”. Harokopio University of Athens, Greece.

✓ **2007-2009** Researcher in RCT designed within the Project: “*Parenteral nutrition in preterm infants: assessment of different prescribing methods and types of nutrients*”. Harokopio University of Athens, “Mitera” and “IASO” maternity hospitals, Greece.

- ✓ **2007-2008** Researcher in RCT designed within the Project “*Development and assessment of the biological activity of a new functional food (jelly desert) enriched with Sideritis euboica extract and Fructooligosaccharides (FOS) consumed by healthy adults (25+)*”. Harokopio University of Athens, National and Kapodistrian University of Athens, Aristotelian University of Thessaloniki, Greece. Fund by the General Secretariat of Research and Technology and Yiotis S.A. Food Industry.
- ✓ **2007-2008** Participation in the Project: “*Development and implementation of a software tool for nutritional assessment, prescription and design of nutritional plan - effects on the quality of provided clinical nutritional therapy in hospitalized patients*”. Harokopio University of Athens, “Hygeia” hospital, Greece.
- ✓ **2006-2007** Researcher in RCT designed within the Project: “*Assessment of the biological activity of a new functional food (cake dessert) enriched with barley beta-glucan consumed by healthy adults (40+)*”. Harokopio University of Athens, National and Kapodistrian University of Athens, Aristotelian University of Thessaloniki, Greece. Fund by the General Secretariat of Research and Technology and Yiotis S.A. Food Industry.
- ✓ **2006-2007** Participation in the Project: “*Energy expenditure and dietary patterns of hemodialysis patients*”. Harokopio University of Athens, 401 military hospital, ‘Laikon’ hospital, Greece.
- ✓ **2005-2012** Researcher in the Project “*Wellness: Nutritional Assessment and Support of patients with severe mental health illness – Quality of life*”. Harokopio University of Athens, Greece. Fund by Pharmaserve Lilly S.A.C.I.
- ✓ **2005-2006** Researcher in RCT designed within the Project “*Identification of the properties of Chios Mastic which are beneficial for human health. An in vivo study*”, Harokopio University of Athens. Fund by the Chios Mastic Growers Association
- ✓ **2004** Researcher in the Project entitled “*Nutritional assessment for designing health promotion programs in toddlers 1-4y old in Greece*”, Harokopio University of Athens, Greece. Fund by FRIESLAND HELLAS S.A.

2002-2005 Assistant Researcher

Project “*Response in calcitonin therapy and association with CTR polymorphisms in osteoporotic postmenopausal women*”.

Medical School of Athens, National and Kapodistrian University of Athens, Harokopio University of Athens, Greece. Fund by Pharmaserve Lilly S.A.C.I.

Research contracts as Principal Investigator (PI)

2018 AAP-BMS interne "Projets incitatifs - Crédits CS-UL 2018", Project : “*Development of a system for the management and analysis of biological samples and “-omics” data related to VEGF*”, 7000 euros, 1 year duration

List of Publications

Original publications

1. Ligthart S, Vaez A, Vösa U, **Stathopoulou MG**, ... , Alizadeh BZ. Genome-wide association analyses of >200,000 individuals identify 58 genetic loci for chronic inflammation and highlights pathways that link inflammation and complex disorders. *Am J Hum Genet.* 2018 Nov 1;103(5):691-706.
2. Azimi-Nezhad M, Mirhafez SR, **Stathopoulou MG**, Murray H, Ndiaye NC, Bahrami A, Varasteh A, Avan A, Bonnefond A, Rancier M, Mehrad-Majd H, Herbeth B, Lamont J, Fitzgerald P, Ferns GA, Visvikis-Siest S, Ghayour-Mobarhan M. The Relationship Between Vascular Endothelial Growth Factor Cis- and Trans-Acting Genetic Variants and Metabolic Syndrome. *Am J Med Sci.* 2018 Jun;355(6):559-565.
3. Xie T*, Akbar S*, **Stathopoulou MG***, Oster T, Masson C, Yen FT, Visvikis-Siest S. Epistatic interaction of apolipoprotein E and lipolysis-stimulated lipoprotein receptor genetic variants is associated with Alzheimer's disease. *Neurobiol Aging.* 2018 May 3. pii: S0197-4580(18)30147-7. * equal first authors
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5. NCD Risk Factor Collaboration (NCD-RisC). (**Stathopoulou MG** in author list). Contributions of mean and shape of blood pressure distribution to worldwide trends and variations in raised blood pressure: a pooled analysis of 1018 population-based measurement studies with 88.6 million participants. *Int J Epidemiol*. 2018 Mar 19. doi: 10.1093/ije/dyy016.
6. Xie T*, **Stathopoulou MG***, Akbar S, Oster T, Siest G, Yen FT, Visvikis-Siest S. Effect of LSR polymorphism on blood lipid levels and age-specific epistatic interaction with the APOE common polymorphism. *Clin Genet*. 2018 Apr;93(4):846-852. * equal first authors
7. **Stathopoulou MG**, Xie T, Ruggiero D, Chatelin J, Rancier M, Weryha G, Kurth MJ, Aldasoro Arguinano AA, Gorenjak V, Petrelis AM, Dagher G, Dedoussis G, Deloukas P, Lamont J, Marc J, Simmaco M, Van Schaik RHN, Innocenti F, Merlin J-L, Schneider J, Alizadeh BZ, Ciullo M, Seshadri S, Visvikis-Siest S - The VEGF Consortium. A transnational collaborative network dedicated to the study and applications of the Vascular Endothelial Growth Factor–A in medical practice: The VEGF Consortium. *Clin Chem Lab Med*. 2018 Mar 28;56(4):83-86.
8. NCD Risk Factor Collaboration (NCD-RisC) (**Stathopoulou MG** in author list). Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *Lancet*. 2017 Dec 16;390(10113):2627-2642.
9. Arguinano AA, Naderi E, Ndiaye NC, **Stathopoulou M**, Dadé S, Alizadeh B, Visvikis-Siest S. IL6R haplotype rs4845625*T/rs4537545*C is a risk factor for simultaneously high CRP, LDL and ApoB levels. *Genes Immun*. 2017 Sep;18(3):163-169.
10. Aldasoro Arguinano AA, Dadé S, **Stathopoulou M**, Derive M, Coumba Ndiaye N, Xie T, Masson C, Gibot S, Visvikis-Siest S. TREM-1 SNP rs2234246 regulates TREM-1 protein and mRNA levels and is associated with plasma levels of L-selectin. *PLoS One*. 2017 Aug 3;12(8):e0182226.
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14. NCD Risk Factor Collaboration (NCD-RisC) (**Stathopoulou MG** in author list). A century of trends in adult human height. *Elife*. 2016 Jul 26;5. pii: e13410.
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21. Shahabi P, Cuisset T, **Stathopoulou MG**, Morange PE, Grosdidier C, Herbeth B, Siest G, Alessi MC, Visvikis-Siest S. Genetic determined low response to thienopyridines is associated with higher systemic inflammation in smokers. *Pharmacogenomics*. 2015;16(5):459-469

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25. **Stathopoulou MG**, Monteiro P*, Shahabi P*, Penas-Lledo E, El Shamieh S, Silva-Santos L, Thilly N, Siest G, Llerena A, Visvikis-Siest S. Newly identified synergy between clopidogrel and calcium-channel blockers for blood pressure regulation possibly involves CYP2C19 rs4244285. *Int J Cardiol* 2013;168(3):3057-3058. *equal second authors
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36. Skouroliahou M, Konstantinou D, Koutri K, **Stathopoulou M**, Kakavelaki C, Antoniadou M, Xemelidis N, Kona V, Markantonis S. Effect of ω -3 fatty acids on oxidative stress of preterm neonates feed through parenteral nutrition. *Eur J Clin Nutr*. 2010;64(9):940-947.

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38. Skouroliahou M, Kastanidou O, **Stathopoulou M**, Vourli G. Evaluation of the antioxidant effect of a new functional food enriched with *Sideritis euboica* in healthy subjects. *J Med Foods* 2009;12(5):1105-1110.

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41. Skouroliakou M, Kakavelaki C, Diamantopoulos K, **Stathopoulou M**, Vourvouhaki E, Souliotis K. The development and implementation of a software tool and its effect on the quality of provided clinical nutritional therapy in hospitalized patients. *J Am Med Inform Assoc* 2009;16(6):802-805.
42. Charopoulos I, Trovas G, **Stathopoulou M**, Kiriazopoulos P, Galanos A, Dedoussis G, Antonogiannakis E, Lyritis GP. Lack of association between vitamin D and calcitonin receptor gene polymorphisms and forearm bone values of young Greek males. *J Musculoskelet Neuronal Interact* 2008;8(2):196-203.
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Scientific reviews

1. Visvikis-Siest S, Gorenjak V, **Stathopoulou MG**. Personalised Medicine: The Odyssey from Hope to Practice. *J Pers Med*. 2018 Sep 21;8(4).
2. Gorenjak V, Akbar S, **Stathopoulou MG**, Visvikis-Siest S. The future of telomere length in personalized medicine. *Front Biosci (Landmark Ed)*. 2018 Mar 1;23:1628-1654.
3. Chatelin J*, **Stathopoulou MG***, Aldasoro Arguinano AA*, Xie T*, Visvikis-Siest S*. Pharmacogenomic challenges in cardiovascular diseases: examples of drugs and considerations for future integration in clinical practice. *Curr Pharm Biotechnol*. 2017;18(3):231-241. * equal first authors
4. Mazidi M, Rezaie P, Pascal Kengne A, **Stathopoulou MG**, Azimi-Nezhad M, Siest S. VEGF, the underlying factor for metabolic syndrome; fact or fiction? *Diabetes Metab Syndr*. 2017 Nov;11 Suppl 1:S61-S64.
5. Visvikis-Siest S, **Stathopoulou MG**. Beyond genome-wide association studies: identifying variants using -omics approaches. *Pers Med* 2015;12(6):529-531.
6. Visvikis-Siest S, **Stathopoulou MG**, Ndiaye NC. Common mutations and polymorphisms predicting adverse cardiovascular events: current view. *Pharmacogenomics* 2012;13(16):1875-1878.

7. **Stathopoulou MG**, Kanoni S, Papanikolaou G, Antonopoulou S, Nomikos T, Dedoussis G. Mineral intake. *Prog Mol Biol Transl Sci.* 2012;108:201-236.
8. **Stathopoulou MG**, Grigoriou E, Dedoussis GV. Calcium and vitamin D intake interactions with genetic variants on bone phenotypes. *Current Nutrition Reports* 2012;1(3):169-174.
9. Ndiaye NC*, Azimi Nehzad M*, El Shamieh S*, **Stathopoulou MG***, Visvikis-Siest S*. Cardiovascular diseases and genome-wide association studies. *Clin Chim Acta* 2011;412(19-20):1697-701. *equal first authors

Conference publications

1. Visvikis-Siest S, Aldasoro Arguinano AA, **Stathopoulou M**, Xie T, Petrelis A, Weryha G, Froguel P, Meier-Abt P, Meyer UA, Mlakar V, Ansari M, Papassotiropoulos A, Dedoussis G, Pan B, Bühlmann RP, Noyer-Weidner M, Dietrich PY, Van Schaik R, Innocenti F, März W, Bekris LM, Deloukas P. 8th Santorini Conference: Systems medicine and personalized health and therapy, Santorini, Greece, 3-5 October 2016. *Drug Metab Pers Ther.* 2017 May 24;32(2):119-127.
2. Siest G, Medeiros R, Melichar B, **Stathopoulou M**, Van Schaik RH, Cacabelos R, Abt PM, Monteiro C, Gurwitz D, Queiroz J, Mota-Filipe H, Ndiaye NC, Visvikis-Siest S. Conference scene: pharmacogenomics: from cell to clinic (part 2). *Pharmacogenomics.* 2014 Apr;15(6):739-44
3. Siest G, Medeiros R, Melichar B, **Stathopoulou M**, Van Schaik RH, Cacabelos R, Abt PM, Monteiro C, Gurwitz D, Queiroz J, Mota-Filipe H, Ndiaye NC, Visvikis-Siest S. Conference scene: pharmacogenomics: from cell to clinic (part 2). *Pharmacogenomics.* 2014 Apr;15(6):739-44
4. Siest G, Medeiros R, Melichar B, **Stathopoulou M**, Van Schaik RH, Cacabelos R, Abt PM, Monteiro C, Gurwitz D, Queiroz J, Mota-Filipe H, Ndiaye NC, Visvikis-Siest S. Conference scene: pharmacogenomics: from cell to clinic (part 1). *Pharmacogenomics.* 2014 Apr;15(5):593-99
5. Grigoriou E, Trovas G, Dontas I, Papaioannou N, **Stathopoulou M**, Dedoussis G. Serum 25-hydroxyvitamin D levels of healthy adult women in Greece. *Bone Abstracts* 2014; 3:PP272

6. Grigoriou E, Trovas G, Dontas I, Papaioannou N, **Stathopoulou M**, Dedoussis G. Serum 25-hydroxyvitamin D levels of healthy adult women in Greece. *Osteoporos Int*. 2014;25(2):S333-S334 – P475
7. Siest G, Ndiaye NC, Shamieh SE, Shahabi P, **Stathopoulou M**, Saleh AS, Godjo T, Albertini L, Visvikis-Siest S. Conference Scene: Systems biology and personalized health science and translation. *Pharmacogenomics*. 2013;14(16):1953-1964
8. Froguel P*, Ndiaye NC*, Bonnefond A, Bouatia-Naji N, Dechaume A, Siest G, Herbeth B, Falchi M, Bottolo L, Guéant-Rodriguez RM, Lecoœur C, Langlois MR, Labrune Y, El Shamieh S, **Stathopoulou MG**, Morandi A, Maffeis C, Meyre D, Delanghe JR, Jacobson P, Sjöström L, Carlsson L, Walley A, Elliott P, Jarvelin MR, Dedoussis GV, Visvikis-Siest S. A genome-wide association study identifies rs2000999 as a strong genetic determinant of circulating Haptoglobin levels. *Drug Metabol Drug Interact*. 2012;27(3):A40.
*equal first authors
9. Lyritis GP, **Stathopoulou MG**, Dedoussis GVZ, Trovas G, Theodoraki EV, Katsalira K, Georgoulas T, Hammond N, Deloukas P. The Effect of Vitamin D Receptor Gene Polymorphisms in the Bone Mineral Density of Postmenopausal Women Stratified by Calcium Intake. *Osteoporos Int* 2010;21:[Suppl1]S114. World Congress on Osteoporosis, International Osteoporosis Foundation and European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis, Florence, Italy, 2010 (Invited presentation).
10. **Stathopoulou MG**, Dedoussis GVZ, Trovas G, Katsalira K, Hammond N, Deloukas P, Lyritis GP. Association of Low-Density Lipoprotein Receptor-Related Protein 5 (LRP5) Gene Variants with Bone Mineral Density in Postmenopausal Women. *J Bone Miner Res* 2009;24 (Suppl 1).
Available at <http://www.asbmr.org/Meetings/AnnualMeeting/AbstractDetail.aspx?aid=babdcd6c-377c-4a42-8b3e-dd1dbf12544c>. American Society for Bone and Mineral Research 31st Annual Meeting, Colorado, USA, 2009
11. **Stathopoulou MG**, Dedoussis GVZ, Trovas G, Theodoraki EV, Katsalira K, Hammond N, Deloukas P, Lyritis GP. The effect of Vitamin D Receptor Gene polymorphisms in the bone mineral density of postmenopausal women stratified by calcium intake. *J Bone Miner Res* 2009;24 (Suppl 1).
Available at <http://www.asbmr.org/Meetings/AnnualMeeting/AbstractDetail.aspx?aid=d22367ad-8087->

4f58-84ee-454aca7683ea. American Society for Bone and Mineral Research 31st Annual Meeting, Colorado, USA, 2009.

12. Dedoussis G, Katsalira E, **Stathopoulou M**, Louizou E, Galanos A, Lyritis GP. The response to nasal calcitonin and alphacalcidol combined therapy is not related to VDR polymorphism in women with postmenopausal osteoporosis. *J Musculoskel Neuron Interact* 2004;4(2):222. 4th International Workshop on Musculoskeletal & Neuronal Interactions, 2004, Chalkidiki, Greece, 2004.

13. Katsalira E, Dedoussis G, **Stathopoulou M**, Giannikou P, Galanos A, Lyritis GP. VDR polymorphism is related to increased risk of vertebral fractures in postmenopausal osteoporotic women. *J Musculoskel Neuron Interact* 2004;4(2):221. 4th International Workshop on Musculoskeletal & Neuronal Interactions, 2004, Chalkidiki, Greece, 2004.

Lectures-conferences-symposia

1. **Stathopoulou MG**, Xie T, Akbar S, Oster T, Yen FT, Visvikis-Siest S. LSR and APOE interactions and their role in personalised medicine of type III hyperlipidemia. 9th Santorini Conference, Santorini, Greece, 2018 (poster presentation)

2. Gorenjak V, **Stathopoulou MG**, Petrelis AM, Visvikis-Siest S. Telomere length in personalised medicine. 9th Santorini Conference, Santorini, Greece, 2018 (poster presentation)

3. Aldasoro Arguinano AA, Dadé S, **Stathopoulou MG**, Derive M, Ndiaye NC, Xie T, Masson C, Gibot S, Visvikis-Siest S. TREM-1 variants associations with TREM-1 protein and mRNA levels and adhesion molecules. 9th Santorini Conference, Santorini, Greece, 2018 (poster presentation)

4. Gorenjak V, Aldasoro Arguinano AA, Dadé S, **Stathopoulou MG**, Vance D, Masson C, Visvikis-Siest S. Associations of polymorphisms in the region of the TREM2 gene with TNF- α levels and IMT-F. 9th Santorini Conference, Santorini, Greece, 2018 (poster presentation)

5. **Stathopoulou MG**, Xie T, Ruggiero D, Chatelin J, Rancier M, Weryha G, Kurth MJ, Aldasoro Arguinano AA, Gorenjak V, Petrelis AM, Dagher G, Dedoussis G, Deloukas P, Lamont J, Marc J, Simmaco M, Van Schaik RHN, Innocenti F, Merlin JL, Schneider J, Alizadeh BZ, Ciullo M, Seshadri S, Visvikis-Siest S; VEGF Consortium. The VEGF Consortium. 9th Santorini Conference, Santorini, Greece, 2018 (poster presentation)

6. Gorenjak V, Aldasoro Arguinano AA, Dadé S, **Stathopoulou MG**, Vance D, Masson C, Visvikis-Siest S. The polymorphism rs6918289 located in the downstream region of the

TREM2 gene is associated with TNF- α levels and IMT-F. Personalized and Precision Medicine International Conference – PEMED 2018, Paris, France, 2018 (poster presentation)

7. **Stathopoulou MG**, Alizadeh B, Casas J, Dagher G, Deloukas P, Innocenti F, Lamont J, Marc J, Merlin J-L, Schneider J, Simmaco M, Van Schaik RHN, Ciullo M, Seshadri S, Visvikis-Siest S. The VEGF Consortium. 8th Santorini Conference, Santorini, Greece, 2016 (poster presentation)

8. Xie T, **Stathopoulou MG**, Akbar S, Oster T, Yen FT, Visvikis-Siest S. Epistatic interactions between VEGF, LSR and APOE on blood lipid traits. 8th Santorini Conference, Santorini, Greece, 2016 (poster presentation)

9. **Stathopoulou MG***, Shahabi P*, Chatelin J, Morange PE, Siest G, Alessi MC**, Visvikis-Siest S**. Interaction between CYP2C19 polymorphisms and smoking modifies the CRP level of post- percutaneous coronary intervention patients treated with thienopyridine. *equal first authors, ** equal second authors. Third ESPT conference Integration of Pharmacogenomics in clinical decision support, Budapest, Hungary, 2015 (poster presentation)

10. **Stathopoulou MG**, Petrelis AM, Buxton JL, Froguel P, Blakemore AIF, Visvikis-Siest S. The case of genetic determination of leukocyte telomere length in childhood. Third ESPT conference Integration of Pharmacogenomics in clinical decision support, Budapest, Hungary, 2015 (poster presentation)

11. NC Ndiaye, S EL Shamieh, **MG Stathopoulou**, G Siest, MY Tsai, A Benetos, HA Murray, JV Lamont, P Fitzgerald, S Visvikis-Siest. Epistasis in Blood Pressure Regulation. 7th Santorini Conference Biologie Prospective, Santorini, Greece, 2014 (poster presentation)

12. A Saleh, **MG Stathopoulou**, M Azimi-Nezhad, NC Ndiaye, H Murray, C Masson, J Lamont, P Fitzgerald, S Visvikis-Siest. Vascular Endothelial Growth Factor linking angiogenesis, inflammation and cardiovascular diseases. 7th Santorini Conference Biologie Prospective, Santorini, Greece, 2014 (poster presentation)

13. **MG Stathopoulou**, AM Petrelis, JL Buxton, P Froguel, AIF Blakemore, S Visvikis-Siest. Genetic determination of leukocyte telomere length in children differs from adults. 7th Santorini Conference Biologie Prospective, Santorini, Greece, 2014 (poster presentation)

14. P Shahabi, T Cuisset, **MG Stathopoulou**, PE Morange, C Grosdidier, B Herbeth, G Siest, MC Alessi, S Visvikis-Siest. Genetic-determined low response to thienopyridines is associated with higher systemic inflammation in smokers. 7th Santorini Conference Biologie Prospective, Santorini, Greece, 2014 (poster presentation)

15. Choi SH*, Ruggiero D*, Sorice R*, Song C, Nutile T, Vernon Smith A, Pina Concas M, Traglia M, Barbieri C, Ndiaye NC, **Stathopoulou MG**, Lagou V, Battista Maestrale G, Sala C, Debette SA, Kovacs P, Lind L, Lamont J, Fitzgerald P, Tonjes A, Gudnason V, Ingelsson E, Toniolo D, Pirastu M, Bellenguez C, Vasani RS, Leutenegger AL, Destefano AL, Visvikis-Siest S, Seshadri S**, Ciullo M**. Six novel loci associated with circulating VEGF levels identified by a meta-analysis of genome-wide association studies. *PLoS Genet.* 2016 Feb 24;12(2):e1005874. * equal first authors, ** equal last authors. 64th American Society of Human Genetics Annual Meeting in San Diego, CA, USA, 2014 (poster presentation)
16. Grigoriou E, Trovas G, Dontas I, Papaioannou N, **Stathopoulou M**, Dedoussis G. Serum 25-hydroxyvitamin D levels of healthy adult women in Greece. European Calcified Tissue Society Congress, Prague, Czech Republic, 2014 (poster presentation)
17. Grigoriou E, Trovas G, Dontas I, Papaioannou N, **Stathopoulou M**, Dedoussis G. Serum 25-hydroxyvitamin D levels of healthy adult women in Greece. World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases, Seville, Spain, 2014 (poster presentation)
18. **Stathopoulou MG**, Monteiro P, Shahabi P, Penas-Lledo E, El Shamieh S, Silva Santos L, Thilly N, Siest G, Llerena A, Visvikis-Siest S. Effects of synergy between clopidogrel and calcium-channel blockers in blood pressure levels. Involvement of CYP2C19 polymorphisms. Second ESPT Conference Pharmacogenomics: From Cell to Clinic, Lisbon, Portugal, 2013 (oral presentation)
19. **Stathopoulou MG**, Monteiro P, Shahabi P, Penas-Lledo E, El Shamieh S, Silva Santos L, Thilly N, Siest G, Llerena A, Visvikis-Siest S. Effects of synergy between clopidogrel and calcium-channel blockers in blood pressure levels. Involvement of CYP2C19 polymorphisms. 1st public LURIC meeting, Mannheim, Germany, 2013 (poster presentation)
20. **Stathopoulou MG**, Monteiro P, Shahabi P, Penas-Lledo E, El Shamieh S, Silva Santos L, Thilly N, Siest G, Llerena A, Visvikis-Siest S. Effects of synergy between clopidogrel and calcium-channel blockers in blood pressure levels. Involvement of CYP2C19 polymorphisms. 3rd Congress of the EUSPM, Riolo Terme, Italy, 2013 (oral presentation)
21. Froguel P, Ndiaye NC, Bonnefond A, Bouatia-Naji N, Dechaume A, Siest G, Herbeth B, Falchi M, Bottolo L, Guéant-Rodriguez RM, Lecoeur C2, Langlois MR, Labrune Y, El Shamieh S, **Stathopoulou MG**, Morandi A, Maffei C, Meyre D, Delanghe JR, Jacobson P, Sjöström L, Carlsson L, Walley A, Elliott P, Jarvelin MR, Dedoussis GV, Visvikis-Siest S. A genome-wide association study identifies rs2000999 as a strong genetic determinant of

circulating Haptoglobin levels. 6th Santorini Conference Biologie Prospective, Santorini, Greece, 2012 (poster presentation)

22. Kaliora AC, Kountouri AM, **Stathopoulou MG**, Andrikopoulos NK. Antioxidant and anti-inflammatory properties of mastic terpenes. A natural product of the Mediterranean. International PSE Symposium On Terpenes. Istanbul, Turkey, 2010 (poster presentation)

23. Charopoulos I, **Stathopoulou M**, Trovas G., Tourni S, Lyritis GP, Papaioannou N. The correlation of polymorphisms of the vitamin D receptor genomic region with the top bone density of Greek men. 18th Congress of the Hellenic Society for the Study of Bone Metabolism, Athens, Greece, 2010 (oral presentation)

24. Lyritis GP, **Stathopoulou MG**, Dedoussis GVZ, Trovas G, Theodoraki EV, Katsalira K, Georgoulas T, Hammond N, Deloukas P. The Effect of Vitamin D Receptor Gene Polymorphisms in the Bone Mineral Density of Postmenopausal Women Stratified by Calcium Intake. World Congress on Osteoporosis, International Osteoporosis Foundation and European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis, Florence, Italy, 2010 (Invited poster presentation)

25. **Stathopoulou MG**, Dedoussis GVZ, Trovas G, Theodoraki EV, Katsalira K, Hammond N, Deloukas P, Lyritis GP. The effect of Vitamin D Receptor Gene polymorphisms in the bone mineral density of postmenopausal women stratified by calcium intake. American Society for Bone and Mineral Research 31st Annual Meeting, Colorado, USA, 2009 (poster presentation)

26. **Stathopoulou MG**, Dedoussis GVZ, Trovas G, Katsalira K, Hammond N, Deloukas P, Lyritis GP. Association of Low-Density Lipoprotein Receptor-Related Protein 5 (LRP5) Gene Variants with Bone Mineral Density in Postmenopausal Women. American Society for Bone and Mineral Research 31st Annual Meeting, Colorado, USA, 2009 (poster presentation)

27. **Stathopoulou MG**, Dedoussis GVZ, Trovas G, Katsalira K, Hammond N, Deloukas P, Lyritis GP. VDR gene polymorphisms affect the bone density of postmenopausal women with low calcium intake. 17th Congress of the Hellenic Society for Research of Metabolic Bone Diseases, Crete, Greece, 2009 (oral presentation)

28. **Stathopoulou MG**, Dedoussis GVZ, Trovas G, Katsalira K, Hammond N, Deloukas P, Lyritis GP. Effect of LRP5 gene polymorphisms on bone density of female postmenopausal women. 17th Congress of the Hellenic Society for Research of Metabolic Bone Diseases, Crete, Greece, 2009 (oral presentation)

29. Skouroliakou M, Koutri K, **Stathopoulou M**, Vourvouhaki E, Giannopoulou I, Gounaris A. Comparison of two types of total parenteral nutrition prescription methods in

preterm neonates. 3rd ACCP-ESCP International Congress on Clinical Pharmacy, Orlando-Florida, USA, 2009 (poster presentation)

30. Stathopoulou CG, **Stathopoulou MG**. Nutritional support of patients with Chronic Obstructive Pulmonary Disease. 3rd Panhellenic Congress of Caregivers for Persons with Disabilities. Larissa, Greece, November 2008 (oral presentation)

31. Charopoulos I, Trovas G, **Stathopoulou M**, Kyriazopoulos I, Galanos A, Dedoussis G, Antonogiannakis E, Lyritis GP. The interaction of hygienic habits with the genomic polymorphisms of vitamin D and calcitonin receptors in bone density of the forearm of young Greek men. 15th Congress of the Hellenic Society for Research of Metabolic Bone Diseases, Paphos, Cyprus, 2007 (oral presentation)

32. Katsalira E, Dedoussis G, **Stathopoulou M**, Giannikou P, Galanos A, Lyritis GP. VDR polymorphism is related to increased risk of vertebral fractures in postmenopausal osteoporotic women. 4th International Workshop on Musculoskeletal and Neuronal Interactions, Chalkidiki, Greece, 2004 (poster presentation)

33. Dedoussis G, Katsalira E, **Stathopoulou M**, Louizou E, Galanos A, Lyritis GP. The response to nasal calcitonin and alfacalcidol combined therapy is not related to VDR polymorphism in women with postmenopausal osteoporosis. 4th International Workshop on Musculoskeletal and Neuronal Interactions, Chalkidiki, Greece, 2004 (poster presentation)

34. Katsalira E, Dedoussis G, **Stathopoulou M**, Giannikou P, Galanos A, Lyritis GP. VDR polymorphism is related to increased risk of vertebral fractures in postmenopausal osteoporotic women. 12th Congress of the Hellenic Society for Research of Metabolic Bone Diseases, Limnos, Greece, 2004 (oral presentation)

Teaching experience

1. Lecture on « *Genetic and skeletal health and development* ». Post-graduate course « Nutrition and development » of the Faculty of Dietetics-Nutrition of the Harokopio University of Athens 2010-2011, Athens, October 2010.

2. Lecture on « *Genetic and skeletal health* ». Seminar of Basic Education in Metabolic Diseases of Bone, Athens, January 2005.

3. Lecture on « *The BMI in the diagnosis of severe undernourishment* ». Educational Program of the Laboratory for the Research of the Musculoskeletal System (LRMS) of the Medical School of the University of Athens 2004-2005, Athens, November 2004.

Supervision of students and participation in evaluation committees

Co-direction (50%) of doctoral students with official autorisation (Autorisation à Codiriger une Thèse, ACT)

1. Vesna Gorenjak: « Outils biostatistiques /bioinformatiques pour une recherche de médecine personnalisée : télomères, facteur de croissance de l'endothélium vasculaire (VEGF) et molécules associées »

Codirected with Dr Sofia Siest

Date of ACT: 11/12/2017

Doctoral School BioSE, University of Lorraine

Co-financed by the « Région Lorraine », the project « FEDER-FSE Lorraine et Massif des Vosges 2014-2020 » and the project “Agence Nationale de la Recherche, programme d’Investissements d’avenir”, grant number ANR-15RHU-0004

Thesis defense: End of 2019

I will participate in the evaluation committee of this thesis.

2. Jérôme Chatelin: « Interactions gène × gène, gène × environnement et Médecine Prédictive, Préventive, Personnalisée et Participative (4P) dans l'inflammation »

Codirected with Dr Sofia Siest

Date of ACT: 16/09/2015

Doctoral School BioSE, University of Lorraine

Thesis defense: February 2019

I will participate in the evaluation committee of this thesis.

3. Ting Xie: « Interactions épistatiques pour le développement des algorithmes de prédiction des maladies cardiovasculaires: De l'approche gène-candidat au séquençage de prochaine génération »

Codirected with Dr Sofia Siest

Date of ACT: 16/09/2015

Doctoral School BioSE, University of Lorraine

Co-financed by the « Région Lorraine », the project « FEDER-FSE Lorraine et Massif des Vosges 2014-2020 » and the project “Agence Nationale de la Recherche, programme d’Investissements d’avenir”, grant number ANR-15RHU-0004

Thesis defense: December 2017

I have participated in the evaluation committee of this thesis.

Co-direction (50%) of doctoral students without official autorisation

4. Marc Rancier : « Intérêt de la génomique du VEGF en endocrinologie clinique »

Codirected with Dr Sofia Siest

Doctoral School BioSE, University of Lorraine

Thesis defense: March 2015

I have participated in the evaluation committee of this thesis.

5. Abdelsalam Saleh : « VEGF: Un biomarqueur potentiel dans la physiopathologie cardiovasculaire »

Codirected with Dr Sofia Siest

Doctoral School BioSE, University of Lorraine

Financed by Libyan funding

Thesis defense: March 2015

I have participated in the evaluation committee of this thesis.

6. Mohsen Azimi Nezhad : « VEGF-A et phénotypes intermédiaires des maladies cardiovasculaires : une approche de génomique fonctionnelle »

Codirected with Dr Sofia Siest

Doctoral School BioSE, University of Lorraine

Financed by Iranian funding

Thesis defense: September 2012

Co-direction (50%) of master students

1. Alexandros Petrelis : « Investigations biostatistiques/bioinformatiques des interactions gène × gène impliquées dans le risque cardiovasculaire - la longueur des télomères leucocytaires chez les enfants »

Codirected with Dr Sofia Siest

Master II Ingénierie Mathématique et Outils Informatiques, IMOI, Faculty of Sciences and Technologies, University of Lorraine

Period : 01/04/2014 - 30/09/2014

I have participated in the evaluation committee of this thesis.

Trainees (stagiaires)

1. Responsible for training of 4 secondees participating in the project « Mastiha treatment for obese with NAFLD diagnosis -MAST4HEALTH », within the framework of the HORIZON 2020 Marie Skłodowska-Curie actions - Research and Innovation Staff Exchange (MSCA RISE):

- ✓ Dr Dwaine Vance, Randox Laboratories, period: 02/10/2017 – 02/04/2018
- ✓ Lauren Doherty, Randox Laboratories, period: 02/10/2017 – 02/01/2018
- ✓ Eirini Chroni, Chios Mastiha Growers Association, period: 01/04/2018 – 31/08/2018
- ✓ Georgia Maurikou, Chios Mastiha Growers Association, period: 01/04/2018 – 31/08/2018

2. Professional tutor (50% with Christine Masson) of Chloé Hatriisse : « Certification d'un Centre de Ressources Biologiques »

License Professionnel « Biotechnologies appliquées à la santé », University of Reims Champagne-Ardenne)

Period : 30 January 2017 – 28 July 2017

Other activities linked to research

1. Member of the Steering Committee of the Biological Resources Centre (BRC) IGE-PCV (BB-0033-00051) of the Unit IGE-PCV; U1122, Manager and Coordinator of Collaborations, Responsible for the transfert of data and biological material, and Quality advisor
2. Administrator of the “Omics_BioBook” Biostatistics / Bioinformatics platform

Administrative activities

Member of the laboratory council of the unit IGE-PCV; U1122

Other activities

Member of the emergency standby personnel for the security of biological material stored in the freezers of the BRC IGE-PCV.

Awards and Fellowships

1. **2012** Fellowship for post-doctoral research entitled: “*Les Interactions Gène-Environnement dans l’era des analyses pangénomiques (genome-wide scan, GWS)*”, Région Lorraine, France.
2. **2010** Award for Best Research Study: “*Association of VDR polymorphisms with peak bone mass in Greek men*”, Hellenic Society for Research of Metabolic Bone Diseases, Greece.
3. **2010** Funding for the research program entitled: “*Vitamin D levels, bone markers and genetic factors in Greek sample of men and women*”, Hellenic Society for Research of Metabolic Bone Diseases, Greece.
4. **2009** Award for Best Research Study “*The effect of common polymorphisms of LRP5 gene on bone mineral density of Greek postmenopausal women*”, Hellenic Society for Research of Metabolic Bone Diseases, Greece
5. **2007** Award for Best Research Study: “*Interaction of nutrition and environmental factors with genetic polymorphisms of the VDR and CTR gene on peak bone mass in Greek men*”, Hellenic Society for Research of Metabolic Bone Diseases, Greece.
6. **2007** Funding for the research program entitled: “*Association study of genetic polymorphisms with bone mineral density and risk factors for osteoporosis*”, Hellenic Society for Research of Metabolic Bone Diseases, Greece.
7. **2005** Fellowship for doctoral studies from the State Scholarships Foundation, Greece
8. **2004** Award for Best Research Study: “*VDR polymorphism is related to an increased risk of vertebral fractures in postmenopausal osteoporotic women*”, Hellenic Society for Research of Metabolic Bone Diseases, Greece.
9. **2004** Funding for the research program entitled: “*Interaction of nutritional and environmental factors with genetic polymorphisms of the VDR and CTR gene on peak bone mass in Greek men*”, Hellenic Society for Research of Metabolic Bone Diseases, Greece.

Organisation of conferences

- 1) The 9th Santorini Conference, Santorini, Greece, 2018.
- 2) 8th Santorini Conference, Santorini, Greece, 2016.
- 3) Third ESPT Conference: Integration of pharmacogenomics in clinical decision support, Budapest, Hungary, 2015.
- 4) 7th Santorini Conference Biologie Prospective, Santorini, Greece, 2014.
- 5) Second ESPT Conference Pharmacogenomics: From Cell to Clinic, Lisbon, Portugal, 2013.
- 6) 6th Santorini Conference Biologie Prospective, Santorini, Greece, 2012.

Member of Scientific Societies

Hellenic Osteoporosis Foundation

European Society of Pharmagenomics and Personalised Therapy

European Society of Predictive Medicine

Résumé des travaux de recherche, d'enseignement et d'administration en français

Pendant mes études (Maîtrise, bac+4) en nutrition clinique et en diététique à l'Université Harokopio d'Athènes, en Grèce, j'ai été attiré par le domaine de la génétique et des interactions avec la nutrition. Mon projet de thèse a été réalisé au laboratoire de recherche sur le système musculo-squelettique (LRMS) de la Faculté de Médecine de l'Université Nationale et Kapodistrienne d'Athènes (Grèce) et s'intitulait «Réponse au traitement par la calcitonine et association au polymorphisme de la CTR chez la femme ménopausée ostéoporotique».

Après mes études et en parallèle à mon doctorat, j'ai été recrutée comme chercheur contractuel à l'Université Harokopio d'Athènes, en Grèce, et j'ai travaillé dans 9 projets de recherche concernant les effets de la nutrition sur la santé et les maladies chroniques. Ces projets comprenaient 4 essais cliniques randomisés, 1 étude d'intervention et 4 études observationnelles. Ils ont abouti à 8 articles originaux (publications 36, 37, 38, 39, 40, 41, 43, 44) et ils ont été présentés dans 3 conférences (conférences 22, 29, 30).

Mon introduction dans le domaine de la recherche en épidémiologie génétique des maladies chroniques a été efficace pendant mon doctorat grâce à mes projets de recherche centrés sur l'ostéoporose au Laboratoire LRMS de la Faculté de Médecine de l'Université Nationale et Kapodistrienne d'Athènes en Grèce. Les phénotypes liés à l'ostéoporose sont hautement héréditaires (par exemple, l'héritabilité de la densité minérale osseuse (DMO) est estimée à 50-80%) et, comme la plupart des maladies chroniques, elle est caractérisée par une régulation polygénique. Les études pangénomiques (GWAS) et gènes-candidats ont réussi à identifier des variants génétiques associés à la DMO et de nouvelles voies biologiques menant à de nouveaux agents thérapeutiques.

L'objectif de ma recherche de master II et de doctorat était d'évaluer les variants génétiques associées à la DMO, en tenant compte les interactions possibles avec apport alimentaire en calcium. Trois gènes-candidats ont été sélectionnés à cause de leur implication dans des voies pathophysiologiques classiques et nouvelles d'ostéoporose, le récepteur de la vitamine D (VDR), le récepteur de la calcitonine (CTR) et la protéine 5 liée aux récepteurs de lipoprotéines de basse densité (LRP5).

J'ai démontré que les polymorphismes de gènes CTR, VDR et LRP5 ne déterminent pas la masse osseuse chez les hommes jeunes (publication originale 42) et qu'ils ont un effet important sur la DMO uniquement lorsque la consommation de calcium est faible chez les femmes ménopausées. Par contre la consommation élevée de calcium a un effet protecteur sur la DMO contre la détermination génétique. Nous avons également souligné l'importance de

prendre en compte l'apport nutritionnel et les interactions de la nutrition avec les polymorphismes génétiques lors de l'évaluation du risque de maladies multifactorielles chroniques telles que l'ostéoporose. Les résultats correspondants ont été publiés dans deux publications originales (publications originales 34 et 35).

Pendant la période de post-doctorant à l'UMR INSERM; IGE-PCV «Interactions Gène Environnement-Physio-Cardio-Vasculaire» j'ai travaillé dans 5 thématiques de recherche:

- Projet 1: Etude du facteur de croissance de l'endothélium vasculaire (VEGF) et de son implication dans les maladies cardiovasculaires (MCV)
- Projet 2: Évaluation des interactions gène × gène et gène × environnement pour la régulation de la pression artérielle
- Projet 3: Etude d'association pangénomique sur les déterminants génétiques des taux d'haptoglobine
- Projet 4: Déterminants génétiques de la longueur des télomères chez l'adulte et l'enfant
- Projet 5: Projets pharmagénomiques du clopidogrel

J'ai participé à de nombreux contrats de recherche de l'Unité:

1. «Les interactions génétiques dans l'analyse des études pangénomiques» en bénéficiant d'une bourse par la Région Lorraine pour ma recherche post-doctorale
2. Projet «BioIntelligence». Il s'agissait d'un projet national coordonné par Dassault-Systèmes et regroupant de nombreux partenaires de recherche et industriels: EPI Orpailleur, Sobios, Aureus Pharma, Ipsen, Pierre Fabre, Sanofi-Aventis, Servier, Bayer CropScience, INSERM, Génopole Evry. Il visait à concevoir, développer et mettre en œuvre des logiciels utiles sur le plan clinique pour les sciences de la vie et les maladies multifactorielles. Les projets de recherche sur le VEGF et les interactions gène × gène et gène × environnement ont été utilisés dans le cadre de ce projet pour faciliter le développement de logiciels de voies métaboliques
3. Projet «Interactions gènes-environnement en physiopathologie cardio-vasculaire» financé par l'infrastructure BIOBANQUES Inserm dans le cadre de l'appel «Réseaux thématiques de biobanques». Ce projet visait à développer une plate-forme informatique appelée «Omics_BioBook», capable de gérer, stocker et transférer avec la «sécurité» les données provenant du BRC IGE-PCV via le réseau collaborateur de l'unité IGE-PCV; U1122
4. Projet «Omics» de STANISLAS Family Study. Ce projet reposait sur un contrat entre l'unité IGE-PCV; U1122 et le Wellcome Trust Sanger Institute au Royaume-Uni

5. Projet «TELARTA (Téломères et vieillissement artériel)». Il s'agissait d'un programme de recherche financé par l'ANR, où l'unité IGE-PCV; U1122 est responsable d'un package de travail

J'ai co-dirigé la thèse de Mohsen Azimi Nezhad: «VEGF-A et phénotypes intermédiaires des maladies cardiovasculaires: une approche de la génomique fonctionnelle». La soutenance de la thèse a eu lieu en septembre 2012 et a donné lieu à 6 publications.

J'ai co-dirigé la thèse de Marc Rancier : «Intérêt de la génomique du VEGF en endocrinologie clinique» et Abdelsalam Saleh: «VEGF: Un biomarqueur potentiel dans la physiopathologie cardiovasculaire» qui ont été présentées en mars 2015 et qui ont donné lieu respectivement à 4 et 3 publications. J'ai participé au comité d'évaluation de ces deux thèses.

J'ai co-supervisé le master II d'Alexandros Petrelis: «Investigations biostatistiques / bioinformatiques des interactions entre gènes impliquées dans le risque cardiovasculaire - longueur des téломères leucocytaires chez les enfants». La défense était en septembre 2014 et a abouti à une publication originale.

J'ai participé à l'organisation des conférences suivantes en tant que membres du comité d'organisation:

- 7ème Conférence Biologie Prospective de Santorin, Santorin, Grèce, 2014.
- 2ème Conférence ESPT Pharmacogénomique: De la cellule à la clinique, Lisbonne, Portugal, 2013.
- 6ème Conférence Biologie Prospective de Santorin, Santorin, Grèce, 2012.

Depuis 2015, j'ai été recrutée comme ingénieur de recherche à l'INSERM au sein de l'UMR U1122.

Jusqu'à aujourd'hui, je participe à 5 projets:

- Projet 1: L'étude sur le VEGF
- Projet 2: Effets des polymorphismes du LSR sur la lipidémie et la maladie d'Alzheimer
- Projet 3: Récepteurs déclencheurs exprimés sur les cellules myéloïdes (TREM) et l'inflammation
- Projet 4: La longueur des téломères en tant que biomarqueur pour la médecine personnalisée
- Projet 5: Nouveaux déterminants génétiques des molécules d'inflammation

Je participe également à 3 consortiums internationaux:

1. Consortium VEGF: Je suis le chef de projet du consortium et membre du comité de pilotage.
2. Consortium CHARGE: Je suis l'analyste principal et le responsable de contact pour l'IGE-PCV; U1122. J'ai participé aux 4 projets.
3. Groupe mondial collaborant sur les facteurs de risque liés aux maladies chroniques (NCD-RisC): Je suis l'analyste principal et le responsable de contact pour l'IGE-PCV; U1122. J'ai participé à 6 publications originales.

Depuis 2015, je participe aux contrats suivants de l'IGE-PCV; U1122:

1. «Traitement avec mastic des obèses en bonne santé avec NAFLD - MAST4HEALTH», financé par la Communauté européenne dans le cadre du projet H2020-MSCA-RISE-2015: Marie Sklodowska actions de recherche et échange de personnel.
2. «Combattre l'insuffisance cardiaque, FIGHT-HF», financé par l'ANR dans le cadre des projets «Recherche hospitalo-universitaire en santé», ANR-15RHU-0004 (2015-2020).).
3. Projet «TELARTA» financé par l'ANR dans le cadre du projet «ANR Programme de Recherche Translationnelle en Santé».
4. «Projet GEENAGE (génomique fonctionnelle, épigénomique et environnement pour la gestion du vieillissement saine et pathologique)», financé dans le cadre du projet LUE (Université d'excellence de Lorraine).
5. «Développement d'un système de gestion et d'analyse des échantillons biologiques et des données «-omiques» liées au VEGF», financé par le Pôle BMS «Biologie, Médecine, Santé» dans le cadre du projet AAP-BMS interne «Projets incitatifs - Crédits CS-UL 2018 ». Pour ce projet, je suis l'investigateur principal.

Depuis ma nomination en tant qu'ingénieur de recherche à l'Inserm en 2015, j'ai co-dirigé 3 doctorants en obtenant l'Autorisation à Codiriger une Thèse (ACT) de l'école doctorale BioSE:

1. Ting Xie: «Interactions épistatiques pour le développement des algorithmes de prédiction des maladies cardiovasculaires: De l'approche gène-candidat au séquençage de prochaine génération». La thèse a abouti à 5 publications. La soutenance a eu lieu en décembre 2017 et j'ai participé au comité d'évaluation.
2. Jérôme Chatelin: «Interactions gène x gène, gène x environnement et Médecine Prédictive, Préventive, Personnalisée et Participative (4P) dans l'inflammation». La thèse a abouti à 3

publications à ce jour. La soutenance aura lieu en 2019 et je participerais au comité d'évaluation.

3. Vesna Gorenjak: «Outils biostatistiques /bioinformatiques pour une recherche de médecine personnalisée : télomères, facteur de croissance de l'endothélium vasculaire (VEGF) et molécules associées». La soutenance est prévue pour fin 2019 et je participerais au comité d'évaluation. Jusqu'à présent, 4 publications ont été produites par cette thèse.

J'étais le tuteur professionnel du stagiaire de la Licence Professionnel Chloé Hatrisse: «Certification d'un Centre de Ressources Biologiques». Dans le cadre du projet H2020 MSCA RISE 2015, j'étais responsable pour la formation de 4 personnes dans le cadre du projet «MAST4HEALTH» pour les domaines de l'épidémiologie génétique et des analyses statistiques: Dwaine Vance, Laboratoires Randox, 6 mois, Lauren Doherty, Laboratoires Randox, 3 mois, Eirini Chroni, Association des producteurs de Chios Mastiha, 5 mois et Georgia Maurikou, Association des producteurs de Chios Mastiha, 5 mois.

J'ai participé à l'organisation des conférences suivantes en tant que membre du comité d'organisation:

- 9ème Conférence de Santorin, Santorin, Grèce, 2018.
- 8ème Conférence de Santorin, Santorin, Grèce, 2016.
- Troisième conférence ESPT: Intégration de la pharmacogénomique dans l'aide à la décision clinique, Budapest, Hongrie, 2015.

Lors de la 8ème Conférence de Santorin, j'étais également membre du comité des posters, chargé de l'évaluation des posters et de la sélection des lauréats pour les prix. J'étais président de ce comité lors de la 9ème Conférence de Santorin.

Je suis également membre du comité de direction du Centre de Ressources Biologiques (CRB) IGE-PCV (BB-0033-00051) situé dans l'Unité IGE-PCV; U1122. Je suis responsable et coordinatrice des collaborations et responsable du transfert des données et du matériel biologique. Je suis également référent qualité et je participe à la mise en œuvre de la démarche qualité de la certification selon la norme NF S 96-900.

Je suis également l'administrateur de la plate-forme «Omics_BioBook». Cette plateforme est une plateforme biostatistique / bioinformatique, développée en collaboration avec l'Inserm Grand Est, l'Université de Lorraine et l'infrastructure BIOBANQUES. Il s'agit d'une plateforme de haute performance dotée d'une capacité de stockage élevée (60 To pour le stockage

et 80 To pour le serveur de réplication automatique), conçue pour répondre aux avancées méthodologiques et technologiques du stockage et de l'analyse de données «-omiques», afin de garantir la sécurité des données du CRB IGE-PCV, et d'assurer la rapidité et la performance des analyses à grande échelle des consortiums internationaux dans lesquels l'IGE-PCV; U1122 participe.

Je suis membre du conseil de laboratoire de l'Unité IGE-PCV; U1122. Je participe aux réunions annuelles qui ont pour objectif de planifier les décisions stratégiques pour l'avenir de l'unité.

Résumé du projet de recherche en français

L'étude des déterminants « -omiques » du VEGF dans la médecine personnalisée des maladies chroniques

Objectifs

L'objectif général du projet est de développer des algorithmes de prédiction du risque pour la prédiction personnalisée du risque des MCV et d'autres maladies chroniques sur la base des déterminants «-omiques» du VEGF. Des données volumineuses provenant de déterminants «-omiques» (génétique, transcriptomique, protéomique et épigénétique) du VEGF et des molécules associées seront combinées avec les facteurs environnementaux et avec des interactions épistatiques avec d'autres molécules impliquées dans les processus physiopathologiques communs aux maladies chroniques (angiogenèse, inflammation, métabolisme des lipides). Les objectifs spécifiques du projet sont:

1. L'identification des déterminants «-omiques» du VEGF et de leurs interactions avec les facteurs environnementaux en santé par le biais de méthodes multidisciplinaires «-omiques» robustes et valables (y compris la génétique, l'épigénétique et la transcriptomique) dans de grandes populations longitudinales et familiales saines d'ascendance européenne.
2. Identification des déterminants «-omiques» du VEGF qui affectent les facteurs de risque de MCV, de la maladie d'Alzheimer (AD) et de l'ostéoporose, ainsi que leurs interactions avec les facteurs environnementaux chez des populations saines.

3. L'identification des déterminants «-omiques» du VEGF qui affectent le risque de MCV, de AD et d'ostéoporose dans les populations cas-témoins et les études prospectives. Cette approche permettra de tester la spécificité de différents déterminants «-omiques» sur différentes maladies.

4. Le développement de modèles et d'algorithmes de prédiction du risque basés sur les VEGF «-omiques». Leur valeur prédictive sera évaluée dans le cadre d'études prospectives visant à identifier les groupes à risque parmi les individus tout au long de leur vie. Leur utilité et l'efficacité de la stratification seront comparées aux algorithmes conventionnels de prévision du risque, en tenant compte à la fois de l'âge et du sexe.

Le projet utilisera les ressources disponibles dans l'IGE-PCV; U1122 et le consortium VEGF coordonné par l'Unité. Les ressources en population incluent des cohortes reconnues (SFS, Framingham Heart Study (FHS); étude Cilento, LLs, ORIGIN), des populations cas-témoins, des populations issues des biobanques (BRC IGE-PCV, BBMRI-ERIC, une infrastructure paneuropéenne, et UK Biobank) et des collaborations avec d'autres consortiums tels que CHARGE, METASTROKE et MEGASTROKE pour lesquels les données «-omiques» souhaitées sont disponibles. La plate-forme «Omics_BioBook» sera utilisée pour le stockage et l'analyse des données.

Mise en oeuvre du projet

Le projet sera basé sur 4 axes, un pour chaque objectif spécifique.

Axe 1: Identification des déterminants «-omiques» du VEGF en santé

L'objectif principal de l'axe 1 est d'identifier les déterminants génétiques, transcriptomiques et épigénétiques de la biologie du VEGF en santé en utilisant de méthodes «-omiques» robustes et validés dans de grandes cohortes familiales et longitudinales d'individus en bonne santé.

Déterminants génétiques des taux de VEGF

Nouvelles méta-analyses pangénomiques (GWAS) seront utilisées avec l'ajout de nouvelles populations disponibles via le consortium CHARGE et l'étude ORIGIN. L'objectif est de rassembler plus de 20000 personnes, augmentant ainsi la puissance de l'étude et le potentiel d'identification de nouveaux variants qui augmenteront le pourcentage de variabilité des niveaux de VEGF expliqués. Une liste de polymorphismes indépendants significatifs sera développée, tandis que des polymorphismes atteignant un niveau de signification de 0,05 par correction FDR (critère moins strict comparé à la correction classique de Bonferroni

appliquée dans GWAS) peuvent également être ajoutés à la liste des gènes candidats déterminants du VEGF. De nouvelles découvertes seront également prévues par les études d'interaction gène × environnement. Des répliques seront réalisées au UK Biobank. Un score génétique de tous les marqueurs indépendants sera développé.

Déterminants transcriptomiques des taux de VEGF

Le séquençage de l'ARN par TrueSeq (Illumina) sur des instruments HiSeq2500 (75 pb paired end reads) a été effectué pour 220 personnes appartenant à 70 familles de la cohorte SFS. Ces données et d'autres données transcriptomiques par la méthode de séquençage d'ARN ou de qPCR disponibles dans les populations saines participant au consortium VEGF seront combinées afin de quantifier l'expression des isoformes pro- et anti-angiogéniques du gène du VEGF dans les cellules sanguines mononucléaires périphériques. Après leur quantification, une analyse d'association et des méta-analyses seront effectués afin d'identifier les isoformes qui affectent davantage les niveaux de VEGF.

Déterminants épigénétiques des taux de VEGF

Le profil de méthylation de l'ADN à l'aide de la puce Infinium Methylation450K (Illumina) capable d'interroger 450000 sites CpG et de quantifier les niveaux de méthylation de l'ADN est disponible pour 220 personnes de la cohorte SFS et des autres populations en bonne santé participant au consortium VEGF. Des méta-analyses seront effectuées pour identifier les sondes de méthylation associées de manière significative aux niveaux de VEGF.

Axe 2: Identification des déterminants «-omiques» du VEGF associés aux facteurs de risque de maladies chroniques

L'objectif principal de l'axe 2 est d'établir et de quantifier l'effet des déterminants «-omiques» du VEGF sur les facteurs de risque des maladies chroniques et leurs interactions avec les facteurs environnementaux. Ces facteurs de risque comprennent les lipides sanguins, la pression artérielle, le métabolisme du glucose, l'obésité et les traits d'inflammation : la pression artérielle systolique, la pression artérielle diastolique, la pression artérielle moyenne, la protéine C réactive, l'haptoglobine, l'interleukine 6, le facteur de nécrose tumorale α , la glycémie à jeun, les taux d'insuline, l'indice de masse corporelle, la tour de taille, le rapport taille / hanche, les lipoprotéines de haute et bas densité, l'apolipoprotéine E, les triglycérides, indice de produit d'accumulation de lipides (LAP) et indice d'adiposité viscérale (VAI). Le

risque calculé par le système SCORE sur dix ans d'un premier événement MCV fatal, telle qu'une crise cardiaque, un accident vasculaire cérébral, un anévrisme de l'aorte ou un autre événement sera également utilisé comme phénotype d'intérêt. Ces phénotypes sont disponibles dans les cohortes du Consortium VEGF, CHARGE et la UK Biobank, réunissant un échantillon puissant. Les interactions directes, gène × gène et gène × environnement seront testées. Cet axe identifiera des déterminants spécifiques «-omiques» du VEGF qui affectent des voies spécifiques et des facteurs de risque de maladies.

Axe 3: Identification des déterminants moléculaires du VEGF associés au risque de maladies chroniques

L'objectif principal de l'axe 3 est d'identifier les déterminants «-omiques» du VEGF qui pourraient être utilisés pour prédire le risque de maladie. On utilisera des populations cas-témoins atteintes de MCV, d'AD et d'ostéoporose. Ces populations sont disponibles dans le CRB IGE-PCV, les consortia VEGF, CHARGE, METASTROKE et MEGASTROKE, et les biobanques BBMRI-ERIC et UK Biobank. Dans ces populations, les déterminants «-omiques» du VEGF identifiés dans les axes 1 et 2 seront testés pour déterminer les associations avec un risque accru de maladies évaluées. Nous nous attendons à identifier des déterminants «-omiques» spécifiques pour chaque maladie et à conduire à la sélection des biomarqueurs les plus puissants à ajouter aux modèles de prévision du risque du prochain axe.

Axe 4: Développement et évaluation de la valeur prédictive d'algorithmes personnalisés de prédiction du risque basés sur les déterminants «-omiques» du VEGF

L'objectif principal de l'axe 4 est de développer et de démontrer la valeur prédictive indépendante et additive de déterminants du VEGF spécifiques aux MCV, à l'AD et à l'ostéoporose, identifiés dans les axes précédents pour l'évaluation du risque de maladie par des algorithmes de prédiction computationnels. La valeur prédictive sera évaluée dans de grandes cohortes prospectives dans le but d'identifier les groupes à risque parmi les individus tout au long de leur vie. L'utilité prédictive incrémentielle et l'efficacité de stratification des nouveaux algorithmes seront comparées aux algorithmes classiques de prédiction du risque tels que le système SCORE, le risque cardiovasculaire de Framingham (FGCVDRP) pour les MCV et l'outil d'évaluation du risque de fracture FRAX WHO pour l'ostéoporose.

Résultats attendus

Les principaux résultats attendus sont:

- Déterminants «-omiques» du VEGF à utiliser pour la détermination moléculaire des MCV, de l'AD et de l'ostéoporose.
- Nouveaux modèles de prédiction du risque basés sur les «-omiques» du VEGF - algorithmes pour les MCV, l'AD et l'ostéoporose.

Ces résultats auront un impact important sur la connaissance de la biologie du VEGF et de son implication dans les maladies évaluées, mais démontreront également son utilité en tant que biomarqueur de la médecine personnalisée grâce à la prédiction précoce du risque.

Les connaissances générées par le projet seront mises à la disposition de la communauté scientifique et du public à la suite de plusieurs publications dans des revues internationales à facteur d'impact élevé dans les domaines médical, génétique et épidémiologique et lors de conférences scientifiques.

Résumé des activités de valorisation et de transfert et visibilité scientifique en français

Nombre total de publications (revues à comité de lecture): 66

- ✓ Nombre de publications originales (revues avec comité de lecture): 44
- ✓ Nombre de revues scientifiques (revues à comité de lecture): 9
- ✓ Nombre de publications des conférences (revues à comité de lecture): 13

Conférences-colloques: 34

Sur les 53 publications originales et revues scientifiques, 48 ont été trouvées dans la base de données Web Of Science en novembre 2018. Les analyses de ces 48 publications ont révélé ce qui suit:

Somme des citations: 3528

Somme des citations excluant les auto-citations: 3482

Moyenne de citations par article: 73.5

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Research, teaching and administrative activities

Assistant Researcher (2002-2005) in the project: Response in calcitonin therapy and association with CTR polymorphisms in osteoporotic postmenopausal women.

Medical School of Athens, National and Kapodistrian University of Athens, Harokopio University of Athens, Greece

During my bachelor studies in clinical nutrition and dietetics (4 years university studies) in Harokopio University, Athens, Greece, I first came in contact and was attracted by the field of genetics and interactions with nutrition. My bachelor dissertation project was performed in the Laboratory for the Research of the Musculoskeletal System (LRMS), Medical School, National and Kapodistrian University of Athens, Greece and was entitled “Response in calcitonin therapy and association with CTR polymorphisms in osteoporotic postmenopausal women”.

The study was performed in osteoporotic postmenopausal women treated with nasal calcitonin and alfacalcidol combined therapy (n=160). Selected candidate genes implicated in classical and novel osteoporosis pathways were tested including the vitamin D receptor (*VDR*) (rs1544410) and the calcitonin receptor (*CTR*) (rs1801197).

The study indicated the *VDR* polymorphism was associated with an increased risk of vertebral fracture. Furthermore, none of the polymorphisms affected the response to calcitonin therapy. These results were presented in 3 conferences (*conferences 32, 33 and 34*) and received one award for Best Research Study in 2004 from the Hellenic Society for Research of Metabolic Bone Diseases (*award 8*).

This study was funded by Pharmaserve Lilly S.A.C.I.

My participation included the DNA isolation and genotyping and databases preparation. This work introduced me to genetic epidemiology, clinical research and human chronic diseases fields.

Contractual researcher (2004-2010)

Harokopio University of Athens, Greece

Following my bachelor studies and in parallel with my doctorate, I was recruited as a contractual researcher in Harokopio University of Athens, Greece, in 9 research projects concerning the effect of nutrition in health and disease. These projects included 4 randomized clinical trials, 1 intervention study and 4 observational studies. They have resulted in 8 original papers (*publications 36, 37, 38, 39, 40, 41, 43, 44*)¹⁻⁸ and they have been presented in 3 conferences (*conferences 22, 29, 30*).

My contribution in these studies included patients' recruitment, data collection, analysis and interpretation of data and drafting of the manuscripts. As these projects are not directly linked with my research interests on genetic epidemiology are not presented in details in this report. However, their input in my research experience was significant as they enhanced my experience in multidisciplinary fields, including different study design, statistical analysis and data interpretation and they increased my skills in clinical research.

Doctoral researcher (2004-2010): Assessment of genetic variants associated with bone mineral density in Greek populations and gene × environment interactions

Laboratory for the Research of the Musculoskeletal System (LRMS), Medical School, National and Kapodistrian University of Athens, Greece

My introduction in the field of genetics epidemiology research was effective through my master and doctoral research projects focused on osteoporosis. Osteoporosis is a chronic disease characterized by deterioration of bone mass and increased susceptibility to fragility fractures⁹. It causes an extreme health and economic burden. It is responsible for more than 8.9 million fractures annually and an osteoporotic fracture happens every 3 seconds. Osteoporotic fractures are linked with decrease mobility, functionality and quality of life and with increased mortality and morbidity. Also, given that by 2050, the worldwide incidence of hip fracture is projected to increase by 310% in men and 240% in women, it is clear that it represents a major public health issue problem¹⁰ (<http://www.iofbonehealth.org/facts-statistics>). Osteoporosis related traits are highly heritable (e.g. bone mineral density (BMD) heritability estimated to be 50-80%)¹¹ and, as most chronic diseases, it is characterized by polygenic regulation¹². Both candidate genes and genome-wide associations studies

(GWASs) have been successful in the identification of genetic variants associated with BMD and they have also provided novel biological pathways which led to new therapeutic agents¹³. The aim of my master and doctoral research was the assessment of genetic variants associated with lumbar spine and femoral neck BMD, taking into account possible interactions with nutrients known to affect the traits, especially calcium¹⁴. Selected candidate genes implicated in classical and novel osteoporosis pathways were tested including the vitamin D receptor (*VDR*), the calcitonin receptor (*CTR*) and the low-density lipoprotein receptor-related protein 5 (*LRP5*).

The first study was a cross-sectional study conducted on a sample of 301 healthy young men aiming to assess the genetic effects on peak bone mass. The population aged 18-25 years was selected during graduation examinations for the Greek Armed forces. Distal bone mineral content (dBMC), distal BMD (dBMD) and ultradistal BMD (UdBMD) at the forearm were measured by single X-ray absorptiometry (Osteometer DTX-100, Denmark). Additionally, there were assessments of the corresponding geometrical areas, such as radial area (ArRad), ulnar area (ArUlna) and ultradistal area (ArUd). The polymorphisms rs1801197 of the *CTR* gene and the rs1544410 of the *VDR* gene were genotyped. Direct and epistatic interactions between the two polymorphisms were assessed. We found no significant direct association or any epistatic effect between forearm bone mass variables and geometrical areas and the two polymorphisms. This study indicated that these two polymorphisms of known candidate genes for osteoporosis are not determining the peak bone mass in Greek males (*original publication 42*)¹⁵. In this work, I was involved in the study design, DNA samples preparation and genotyping, databases preparation and statistical analysis and manuscript revision. I have presented these results as oral presentations (*conferences 23 and 31*) which received the award for Best Research Study in 2007 and 2010 from the Hellenic Society for Research of Metabolic Bone Diseases (*awards 5 and 2*).

The second study was a cross-sectional study on 600 postmenopausal women aiming to assess the genetic effects on bone deterioration during menopause. The subjects were unselected postmenopausal Caucasian women of Greek origin who were asked to voluntarily participate in the study through advertisements in four randomly selected Centers of Open Protection for the Elderly in the Athens region (community centers that aimed for primary health prevention and social support of elderly persons). The BMD of the lumbar spine (L2–L4) and hip was measured using dual-energy X-ray absorptiometry with the Lunar DPX-MD device. The genotyping was performed for the polymorphisms rs1784235, rs491347, rs4988321, and rs4988330 of the *LRP5* gene and the rs1544410 (*Bsm-I*), rs11568820 (*Cdx-2*), and rs731236

(Taq-I) polymorphisms of the *VDR* gene. For the polymorphisms of the *LRP5* gene, we found that rs4988321 was associated with lumbar spine BMD (P-value=0.002). We also showed a significant interaction of the rs4988321 polymorphism with calcium intake (P-value=0.016), where the carriers of the A allele had lower lumbar spine BMD compared to GG homozygotes (P-value=0.001) only in the lowest calcium intake group (<680mg/day), while in the highest calcium intake group no differences were found in BMD between genotypes (figure 1). For the polymorphisms of *VDR* gene, we have identified significant associations between Taq-I and Bsm-I with the presence of osteoporosis [odds ratio (OR)=2.141, 95% confidence interval (95% CI)=1.098–4.174, P=.025, and OR=2.216, 95% CI=1.141–4.306, P=.019, respectively] and between Cdx-2 polymorphism and lumbar spine BMD (P=.025) (figure 2) only in the group of low calcium intake (<680 mg/day). In the higher calcium intake group, the polymorphisms of *VDR* gene were not associated with the assessed traits.

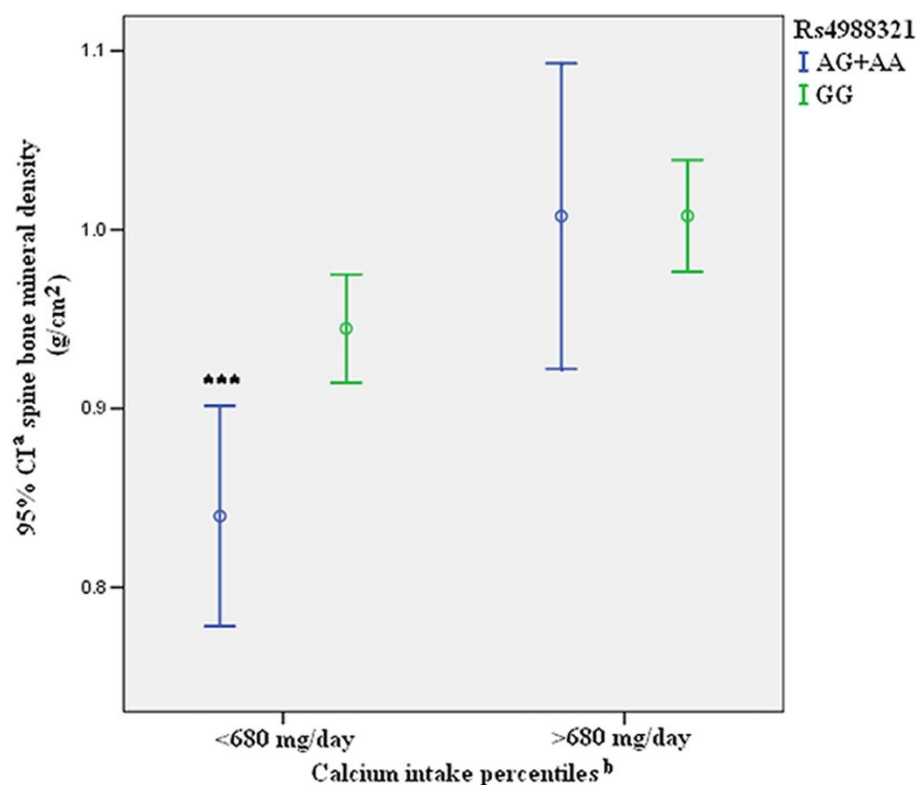


Figure 1. Adjusted spine bone mineral density (mean±95% confidence interval) by rs4988321 genotype and calcium intake groups.

^a Confidence interval (CI).

^b Percentiles derived using the median value of calcium intake (680 mg/day) as a cutoff point.

(Calcium intake was assessed using two 24-hour recalls and values were averaged).

*** P-value=0.001 for the comparison of mean adjusted spine bone mineral density between rs4988321 genotypes in the <680 mg/day calcium intake percentile (univariate analysis of variance using age, weight, height as covariates).

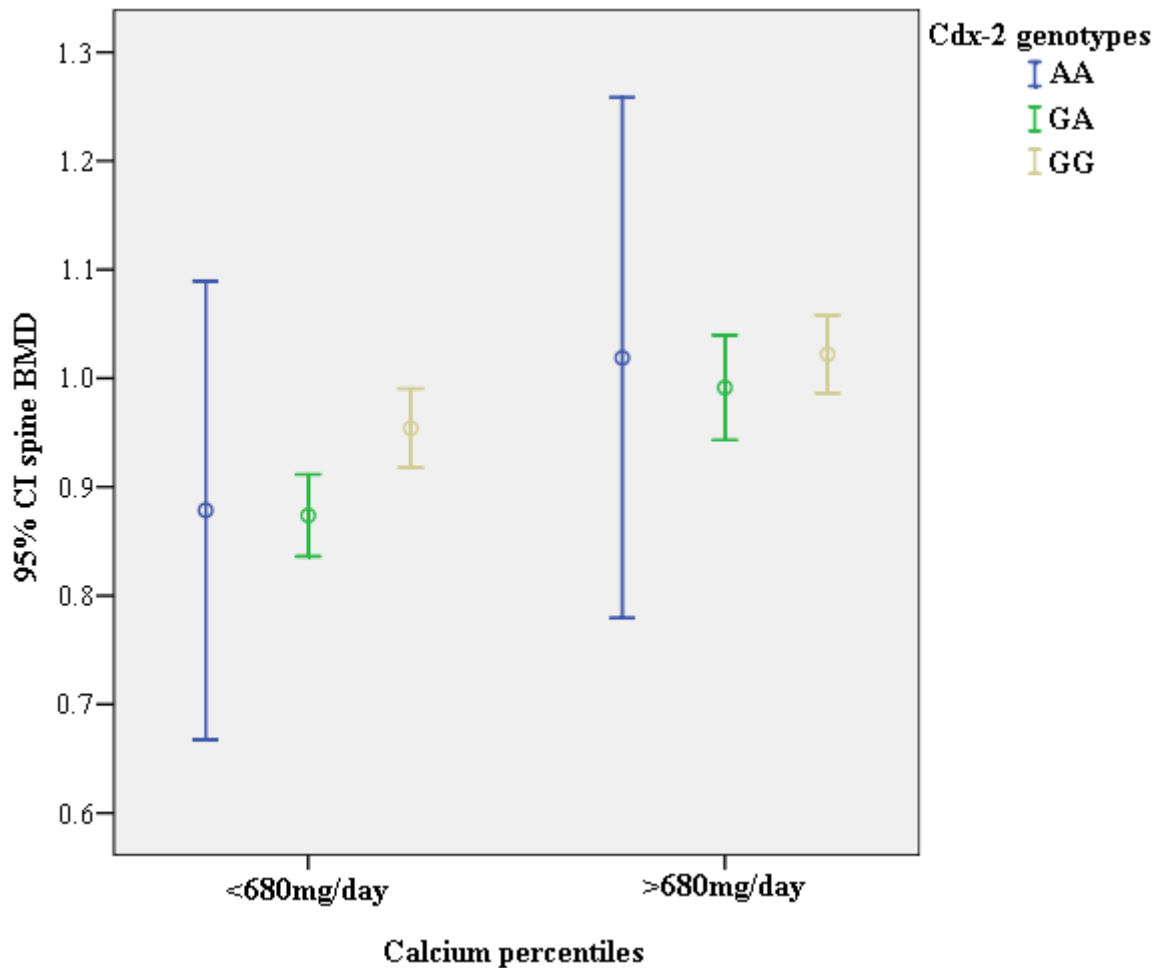


Figure 2. The effect of Cdx-2 genotypes on spine BMD according to calcium intake.

These findings demonstrated that the polymorphisms of both candidate genes have an important effect on BMD only when calcium intake is low, while higher calcium intake has a protective effect on BMD against genetic determination. These results also highlighted the importance of taking into account the nutritional intake and interactions with genetic polymorphisms when assessing the risk of chronic multifactorial diseases like osteoporosis. The related results were published in two original publications (*original publications 34 and 35*)^{16, 17}. In this work, I was involved in the study design, recruitment of the population, data

collection, BMD measurements, DNA samples preparation, dietary analysis, databases preparation, statistical analysis and drafting of the manuscripts. They were presented in 5 conferences (*conferences 24, 25, 26, 27, 28*) and I have received the award for Best Research Study in 2009 from the Hellenic Society for Research of Metabolic Bone Diseases (*award 4*). My doctoral studies were funded by a scholarship from the State Scholarships Foundation in Greece (*award 7*) following written examinations that ranked me 2nd in a national competition in 2005 and 2 research findings from the Hellenic Society for Research of Metabolic Bone Diseases (*award 6 and 9*) on 2007 and 2004.

Furthermore, the results of the bibliographic research that I had prepared during my doctoral studies were presented in two reviews focused on nutritional aspects of skeletal health, genetics and gene × environment interactions (*reviews 7 and 8*).

During this period, I participated in the educational programme of the doctoral seminars of the Laboratory for the Research of the Musculoskeletal System (LRMS) of the Medical School of National and Kapodistrian University of Athens. I had given lectures about the links between nutrition, genetics, their interactions and skeletal health (*teaching experience 2 and 3*). Furthermore, I have participated in the post-graduate course « Nutrition and development » of the Faculty of Dietetics-Nutrition of the Harokopio University of Athens 2010-2011 with a lecture on « Genetic and skeletal health and development » (*teaching experience 1*).

The recruitment of the post-menopausal population used in my doctoral project resulted in the establishment of the “OSTEOS-Osteoporosis: SNPs To Environment Study”. This population participated in the GEFOS/GENOMOS consortium which was a European Union (EU) 7th Framework Package funded project. The originality of this study, in which I was the principal researcher, included the large sample size (among the largest populations of Greek origin) and the assessment of interactions with nutrition, which were limited up to that time. With this population, I have participated in another International Consortium, the ReproGen consortium, where I acted as an analyst and author of one meta-analysis concerning the genetic regulation of menopause age (*original publication 30*)¹⁸. I was also among the initiators of the OSTEOS II study and population in 2010, which received a funding from the Hellenic Society for Research of Metabolic Bone Diseases (*award 3*) and the initial results were presented as poster presentations in 2 international conferences (*conferences 16 and 17*). Moreover, as part of my research training, I participated for 1 year in the recruitment, data collection and analysis of the FP6 Project “ZINCAGE. Nutritional zinc, oxidative stress and immune system: biochemical, genetics and life style factors in healthy aging” which was funded by the EU. Although I was not the principle investigator, my participation offered me

the experience of working in a multicentre research program on nutritional and genetic epidemiology.

In conclusion, this thesis brought together clinician and genetic research scientists and allowed the establishment of strong collaborations in national and international levels. It enforced my skills in laboratory work, genetic epidemiology of human chronic diseases, assessment of genetic interactions with environment, linking of genetics with clinical research as well as efficient group working tasks. It also enforced my skills in genetic epidemiology study designs, statistical analysis, drafting of manuscripts and participating in multicenter projects and international collaborations and consortia.

Post-doctoral researcher (2010-2014)

Université de Lorraine, Inserm, IGE-PCV « Interactions Gène-Environnement en Physiopathologie Cardio-Vasculaire» U1122 (previously EA 4373 « Génétique Cardiovasculaire »).

Project 1 : Study of vascular endothelial growth factors (VEGF) and its involvement in cardiovascular diseases (CVD)

The VEGF-A (most commonly VEGF) is one of the most important factors associated with angiogenesis and is considered a key regulatory molecule for both physiological and pathological angiogenesis^{19, 20}. Physiological angiogenesis occurs during fetal development. In the adult, angiogenesis is involved in skeletal growth, pregnancy, wound healing and also in inflammation, tissue graft and ischemia²¹. VEGF, through its two receptors VEGFR-1 and VEGFR-2 (vascular endothelial growth factor receptor 1 and 2) and related signaling pathways, exerts a variety of functions. These include the activation, migration and proliferation of endothelial cells (ECs), the survival of ECs (through endothelial nitric oxide (NO) production) and the mediation of vascular permeability, thus resulting in angiogenesis and other hemodynamic effects^{22, 23} (figure 3). Blood vessels are beneficial for normal tissue growth and regeneration but deviations in angiogenesis are connected to various disease. Insufficient vessel growth results in CVD (stroke, myocardial infarction), ulcerative disorders and neurodegeneration, while increased angiogenesis has been observed in cancer, and inflammatory disease²⁴. Especially for cancer, angiogenesis is a fundamental requirement for

tumor growth as oxygen and nutrients supply is required for tumor progression ²⁵. It has been shown that up to 60% of human tumors express the *VEGF* gene ²⁶. Due to its important role in angiogenesis, VEGF has been extensively studied and has been consequently associated with a variety of diseases such as those mentioned above. An important application of VEGF biology studies is the production of VEGF inhibitors as therapeutic approach in cancer. However, non-responsiveness in some type of cancers poses questions concerning VEGF mode of action. Furthermore, significant side effects have been observed with anti-VEGF treatment including severe cardiological effects such as hypertension, cardiac ischemia, arterial thromboembolic events (e.g cerebral and myocardial infarction) and left ventricular dysfunction. Even if the mechanisms for the cardiological side effects of these therapies are not yet well-defined, this observation further supports the connection of VEGF with CVD ²¹.

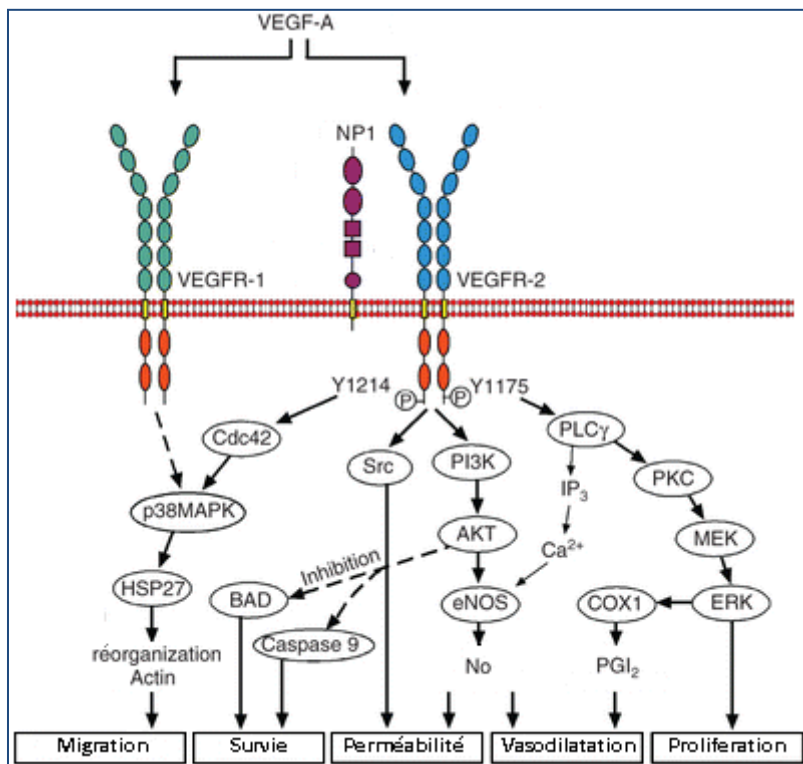


Figure 3: VEGF, its receptors and their roles. (modified by Vuorio et al, 2012 ²⁷)

VEGF therapies have been tested in randomized clinical trials, particularly in patients with coronary artery disease, with low clinical effects ²⁸. Higher levels of VEGF have been observed 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 ²⁹⁻³⁶. Also, the involvement of VEGF in atherosclerotic process is conflicting. A possible neo-vascularization and proinflammatory effect of VEGF has been identified in animal models and *in vitro* studies, which leads to progression of atherosclerosis and plaque instability ³⁷⁻⁴². In contrary, clinical trials using VEGF in humans ²⁸ and gene therapy in animal studies ^{43,44} do not support a positive effect of VEGF on atherosclerosis progression. It seems that the relationship between VEGF and CVD is complicated due to the complexity of these multifactorial diseases but also due to pleiotropic effects of VEGF.

VEGF gene gives rise to more than 14 isoforms grouped in two major families due to differential pre-mRNA splicing (figure 4). The VEGF_{xxx} family results from splicing in exons 6, 7 and the proximal splice site in exon 8 (exon 8a), while the VEGF_{xxx}b family isoforms differ in the selection of a distal splice site at exon 8 (exon8b) located 66bp downstream of the proximal splice site. Thus, the two families have the same number of amino-acids, but differ in amino-acid sequence of 6 amino-acids at the C-terminus ^{45, 46}. This variation results in significant differentiation in structure, receptor interaction and function ⁴⁷. As a consequence, while the VEGF_{xxx} isoforms have pro-angiogenic actions (promotion of angiogenesis), the VEGF_{xxx}b isoforms are anti-angiogenic ^{45, 47}. In normal conditions in adults the anti-angiogenic isoforms are expressed in non-angiogenic tissues and represent >50% of total VEGF protein ²⁵. In contrary, many cancers are characterized by a switch from the anti-angiogenic to pro-angiogenic isoforms which leads to neo-vascularization and consequently to tumor growth ^{25, 46, 47}. Also, it has been suggested that the isoform profile of different tumors may affect the responsiveness to anti-VEGF therapies ^{46, 47}. Another interesting point is that the alternative splicing leading to the different isoforms can be dependent on environmental factors. For example, it is known that VEGF gene is regulated by inflammation molecules (e.g. IGF-1, TNF- α , TGF- β) but there are no data concerning the type of isoforms produced ²⁵. The understanding of the differential and tissue-specific functions of VEGF related to the different isoforms production in normal and pathological tissues is expected to allow the development of personalized therapies ⁴⁸.

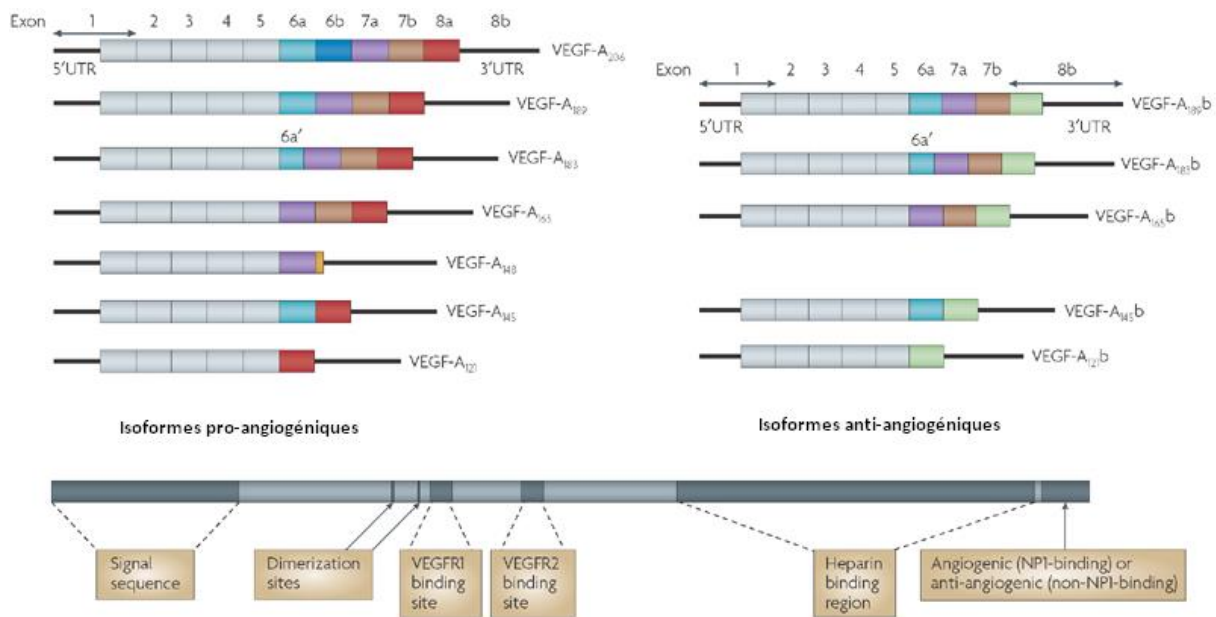


Figure 4. VEGF gene, isoforms and protein structure ⁴⁷

Given the complexity of VEGF biology and its importance to the development and therapy of complex diseases including CVD and cancer, this molecule has been investigated by the IGE-PCV; U1122 unit by a detailed integrative approach. In fact, it has designed and implemented a strategy for the study of VEGF and its links with CVD, which uses genetic and transcriptomics methodologies. The additive genetic heritability of VEGF plasma levels has been estimated in a sample of families from the STANISLAS Family Study (SFS) to be 60.5% of the total variance of the trait ⁴⁹. Basic biological determinant and reference values have also been established, indicating the age, the platelet count and the alanine aminotransferase activity as important factors in children and adults ⁵⁰. Furthermore, a study on pre-analytical variations influence on VEGF blood levels and gene expression has been performed in order to specify the optimal conditions and biological samples for the investigation of VEGF biology in blood ⁵¹. The following step included a large GWAS, where a sample of families from the SFS participated for the identification of genetic determinants of VEGF blood levels. Four single nucleotide polymorphisms (SNPs) in 3 chromosomes were significantly associated with VEGF concentrations and explained ~ 50% of the heritability of this phenotype ⁵². In the same study, a transcriptomics approach performed by IGE-PCV; U1122 unit with peripheral blood mononuclear cells (PBMCs) of the SFS individuals identified also six SNPs significantly associated with VEGF mRNA₁₂₁ levels, while SNPs

annotation and a biological pathway analysis software produced a putative gene network that describes the functional relationships between *VEGF* gene and the identified SNPs⁵².

Given the important proportion of explained VEGF heritability by the above identified SNPs, in my post-doctoral project we have selected these polymorphisms for the in-depth investigation of possible molecular links between VEGF and other CVD endophenotypes in order to examine physiological pathways that jointly regulate CVD development. I was the principal researcher of these projects.

In the first study, we have assessed the direct effects, gene \times gene and gene \times environment interactions of the 4 GWAS-identified SNPs on blood lipid levels (apolipoprotein E (apoE), triglycerides, total cholesterol, low- and high-density lipoprotein cholesterol levels (LDL and HDL)]. The study was performed on a discovery population (n=1006) from SFS and a replication population (n=1145) of European origin that makes part of the BRC IGE-PCV (BB-0033-00051). Meta-analysis of the 2 populations was performed using a weighted inverse normal method with a fixed effect using the “META” R package. We have demonstrated that the SNP rs6921438 was associated with HDL (beta = -0.08, P_{overall} = 1.2×10^{-7}) and LDL (beta = 0.13, P_{overall} = 1.5×10^{-4}). We also identified a significant association between the interaction rs4416670 \times hypertension and apoE levels (P_{overall} = 1.7×10^{-5}). The SNP rs6921438 has been previously associated negatively with levels of VEGF and is the variation that explains 41% of VEGF levels⁵². Therefore, we proposed that the rs6921438 could negatively affect the cardiovascular system through a decrease in HDL-C levels, an increase in LDL-C levels and a decrease in VEGF levels. These results were presented in one original publication⁵³ (*original publication 28*).

In a second study, we have assessed the associations between levels of VEGF and cellular adhesion molecules (CAMs) and inflammation markers, between their expression levels in PBMCs and possible genetic links (direct effects and gene \times gene interactions) between the 4 VEGF-related SNPs and CAM and inflammation molecules levels in a discovery population of 403 unrelated individuals from SFS. The assessed CAM and inflammation molecules included interleukin 6 (IL6), tumor necrosis factor α (TNF- α), E-selectin, L-selectin, P-selectin, c-reactive protein (CRP) and intercellular adhesion molecule-1 (ICAM-1). Four mRNA isoforms of *VEGF* gene were quantified (VEGF-121, -145, -165 and -189) and tested for associations with mRNA levels of IL6, TNF- α , L-selectin, P-selectin and ICAM-1. VEGF levels were associated with ICAM-1 (beta = 0.043, P = 0.023) and with E-selectin (beta = 0.085, P = 0.039). The mRNA levels of VEGF-145 isoform were associated with ICAM-1 mRNA (beta = 0.353, P = 0.021), L-selectin mRNA (beta = 0.611, P = 0.001) and TNF- α

mRNA (beta = 0.616, P = 0.001). Furthermore, significant epistatic interactions were identified for plasma VEGF, E-selectin, TNF- α , ICAM-1 and IL6 levels, while the SNP rs4416670 was associated with mRNA levels of L-selectin (beta = 0.196, P = 0.039). These results supported the hypothesis of complex and multilevel relationships between VEGF and CAM and inflammation molecules that are present even in healthy states. This work was presented in one original publication ⁵⁴ (*original publication 29*).

My contribution in these studies involved the studies design, the statistical analysis, the interpretation of the results and the drafting of the original research papers. These two studies were part of the doctoral thesis of Mohsen Azimi-Nezhad, which I co-supervised with Dr Siest (*doctoral student 6*) and of Marc Rancier (*doctoral student 4*) and Abdelsalam Saleh (*doctoral student 5*).

In a third study, the associations of the rs6921438 and rs10738760 VEGF-related SNPs with the risk of type 2 diabetes, nephropathy and retinopathy were assessed. Two case-control populations for type 2 diabetes were used and one case-control population for nephropathy and one for retinopathy were also assessed. No significant associations were identified thus indicating that the links between diabetes and its comorbidities with VEGF are mediated through other mechanisms. This work is presented in one original publication ⁵⁵ (*original publication 26*).

Two more doctoral theses co-supervised by me and Dr Siest were initiated during my post-doctoral period on VEGF projects (*doctoral students 4 and 5*). Their results will be developed in the next section (Research Engineer Inserm).

These results and the developed strategies were at the origins of the “Vascular endothelial growth factor’s European Genomic Federation (VEGF)” project that was submitted in 2014 under the HORIZON 2020 PHC-05-2014 «Health promotion and disease prevention: translating “omics” into stratified approaches» call. The project was coordinated by Dr Siest and I was the project manager. The VEGF Project included 12 participants coming from 6 European Universities and one from the USA. This project aimed to develop and assess VEGF-based personalised prevention programs for CVD integrating “-omics” profiles of individuals, complemented by environmental and lifestyle factors. The project was selected for a second phase evaluation but did not finally receive a funding.

Project 2: Assessment of gene × gene and gene × environment interactions for the regulation of blood pressure

It is well-accepted that hypertension is a major risk factor for CVD with a significant worldwide burden⁵⁶. Although large studies have tried to identify the most significant genetic components of blood pressure, only a very small variance of the relative traits is explained⁵⁷. The IGE-PCV; U1122 unit has set a strategy for the assessment of blood pressure genetic variants using a combination of candidate genes and GWASs methodologies, also taking into account gene × gene and gene × environment interactions with the application of functional approaches through transcriptomic analyses.

In one study, we have assessed the epistatic interactions between several candidate genes for blood pressure in a discovery population of 3600 individuals not treated with hypertensive therapies and a replication cohort of 4620 subjects. We have identified that the interaction between rs5355 of *SELE* gene and rs6046 of *F7* gene were significantly associated with systolic blood pressure levels in both populations (P= 0.047 and P= 0.03 respectively). An investigation of the underlying mechanisms for these associations was performed through transcriptomic analyses in PBMCs of 90 individuals. The rs6046A allele was associated with increased *NAMPT* gene mRNA levels (P≤9.2×10⁻⁵). *NAMPT* gene mRNA levels were also positively correlated with *ICAM-1*, *SELL*, *FPRI*, *DEFA1-3* and *LL-37* genes expression (P≤5×10⁻³), while *DEFA1-3* and *LL-37* gene expression levels were associated with systolic blood pressure (P=3×10⁻³ and P=0.01 respectively). These results suggest an indirect link between *NAMPT* gene expression and blood pressure through the expression of adhesion and innate immune system molecules, where the identified epistasis plays a role. The findings are presented in one publication⁵⁸ (*original publication 32*).

In a second study, we assessed the epistatic interactions between 100 SNPs 65 candidate genes for blood pressure in a discovery population of 1912 French unrelated adults and an independent replication population of 1755 unrelated European adults. Nine SNPs in 8 genes were significantly associated with blood pressure: *LPL* and *LDLR* (lipid metabolism), *F7* (coagulation factor), *AGTIR* (BP regulation), *CSF2*, *IL1B*, *TGFBI* and *VDR* (inflammatory pathways) genes. Two significant epistatic interactions were identified for both systolic and diastolic blood pressure in the discovery set: *VCAMI* (rs1041163) × *APOB* (rs1367117) and *SCGB1A1* (rs3741240) × *LPL* (rs1800590) (P≤0.04 and P≤0.03 respectively), while only the effects on diastolic blood pressure were significant in the replication population (P=0.045 and P=0.05 respectively). This study provided novel original results based on epistasis between

known candidate genes for blood pressure regulation. These results were presented in one publication ⁵⁹ (*original publication 27*) and in the 7th Santorini Conference Biologie Prospective as a poster presentation (*conference 11*).

My participation for these projects was on the design of the study, statistical analysis and the interpretation of the results as well as the critical correction of the manuscript.

Project 3: A genome-wide association study for the genetic determinants of circulating haptoglobin levels

Haptoglobin is an acute phase inflammatory molecule, whose physiological role is the scavenging of circulation haemoglobin and its degradation in the liver, and it serves as a reverse transporter of cholesterol to the liver. Its levels as well as its polymorphisms have been associated with CVD, other non-communicable diseases and infectious diseases.

In a GWAS study we assessed the genetic variants influencing the levels of haptoglobin in unrelated children. A discovery population of 631 French children from the SFS and 3 replication cohorts (total n=4391) were used. The polymorphism rs2000999 on *HP* gene was strongly associated with haptoglobin levels in the meta-analysis ($P=8.09 \times 10^{-59}$). This SNP was significantly associated also with total cholesterol ($\beta=-0.011$, $P=0.002$) and LDL levels ($\beta=-0.017$, $P=0.0008$) in 5 independent children cohorts (total n=8789), thus linking haptoglobin levels with CVD risk. These results were published as an original article ⁶⁰ (*original publication 31*) and in the 6th Santorini Conference Biologie Prospective as a poster presentation (*conference 21*). My participation in this project was focused on data interpretation and review of the manuscript and it was an excellent occasion for further training in the GWAS methodology and analysis.

Project 4: Telomere length genetic determinants in adults and children

Telomeres are structures made of tandem repeats of hexanucleotide units with numerous telomeric-interacting proteins that are present at the ends of chromosome. They are responsible for the protection of genomic integrity. The loss of several base pairs from telomeres during each mitotic division leads to telomere shortening which, after a critical limit, results to telomere dysfunction that triggers cell cycle arrest, apoptosis and/or cellular senescence ^{61, 62}. The telomere length has been linked with a variety of human diseases,

ranging from rare monogenic disorders to age-related complex diseases such as cancer, CVD and CVD risk factors such as obesity, hypertension and diabetes^{63, 64}. An important number of studies have identified genetic variants associated with leukocyte telomere length (LTL)⁶⁵⁻⁷¹. These studies are focused on adult populations, even though childhood is an extremely crucial period for the determination of LTL and the assessment of age-specific genetic determinants could be of great importance.

I have designed and analysed the data analysis in a study focused on genetic determinants of LTL in children. The effect of 19 previously identified polymorphisms of LTL in adults in GWAS studies was assessed in a sample of 322 unrelated normal weight children from the SFS. None of these variants were significant in our paediatric population, thus a discovery only GWAS approach was used and 6 novel variants, specific for children, reached the levels of suggestive association ($P \leq 5 \times 10^{-5}$). This study pointed out the importance of studying the genetic background of LTL in children and the preliminary results were presented in one publication⁷² (*original publication 22*) and in 2 international conferences as poster presentations (*conferences 13 and 10*). This work was included in the master thesis of Alexandros Petrelis, which I co-supervised (*master student 1*).

Furthermore, apart from the observed relationships with CVD endophenotypes (e.g. inflammation markers, glucose metabolism, oxidative stress) telomere length has been identified as an independent predictor of heart disease related events⁷³ and thus suggesting its possible use as new biomarker for CVD⁶⁴. It is accepted that telomere length reflects the accumulative burden of inflammation⁷⁴. Since 2013, I have participated in the “ANR Programme de Recherche Translationnelle en Santé”, entitled “TELARTA (Telomeres and Arterial Aging)”, where the IGE-PCV; U1122 unit was responsible for the task 5: “Characterization of inflammation markers in blood samples” for the assessment of plasma inflammation markers’ relationships with telomere length of muscular cells, white blood cells and endothelial progenitor cells from CVD patients and healthy subjects. Among the inflammation markers, CRP, TNF- α and IL6 are those most significantly associated with telomere length⁷⁵. However, a wider range of pro- and anti-inflammatory markers have been quantified including VEGF, interleukins 1b, 2, 4, 8, 10 and 12, interferon- γ (IFN- γ), monocyte chemotactic protein-1 (MCP-1), ICAM-1 and vascular adhesion molecule-1 (VCAM-1). My participation included the selection of inflammation molecules and the interpretation of the results, which are ongoing. The assessment of these relationships will increase our knowledge on inflammation and VEGF role in telomere dynamics, as the selected tissues reflect them.

Project 5: Pharmagenomic projects of clopidogrel

Another subject that gathers great attention in the application of genomics in diseases is the field of pharmacogenomics, which studies the effect of genetic variations to drug response ⁷⁶. This field, more than any other, is aiming to personalised medicine. Concerning cardiovascular medications, warfarin and clopidogrel ⁷⁷ as well as statins ⁷⁸ are the most commonly studied medications for possible gene × drugs interactions. The obtained results of pharmacogenomics in CVD have led to clinical considerations. For example, the Food and Drug Administration (FDA) officially suggests that genotypes for *CYP2C9* and *VKORC1* and for *CYP2C19* genes should be taken into account when prescribing warfarin and clopidogrel respectively ⁷⁶. Although the progress is slow, the application of genomics to clinical medicine is encouraging ⁷⁶. Furthermore, the implementation of “-omics” technologies is expected to provide further important information concerning the activity of implicated mechanisms and pathways and the responses to the environment ^{79,80}.

I have realised a pharmacological study dealing with the effect of clopidogrel on blood pressure levels, its interaction with calcium-channel blockers (CCB) and the effect of *CYP2C19* gene polymorphisms on these interactions in a population of myocardial infarction patients coming from Coimbra University hospital in Portugal (n=2322). Clopidogrel use was shown to be significantly associated with a lower diastolic blood pressure (beta = - 1.47, P = 0.034). Following stratification of the population based on CCB use, the association of clopidogrel use with low diastolic blood pressure was observed only in the CCB users group. Thus we have demonstrated that clopidogrel seems to improve the diastolic blood pressure lowering effect of CCB. In a population of healthy unrelated individuals from SFS (n=2155), we have assessed the association of the rs4244285 polymorphism of *CYP2C19* on blood pressure and significant associations were observed for both systolic and diastolic blood pressure (beta: - 1.69, P = 0.008 and beta: - 1.28, P = 0.003 respectively). This loss-of-function polymorphism of *CYP2C19* gene could be implicated in aldosterone regulation and thus in blood pressure levels and could be mediating the relation between clopidogrel and blood pressure. This work was presented in one original publication ⁸¹ (*original publication 25*), in 2 international conferences as oral presentations (*conferences 18 and 20*) and in one international conference as poster presentation (*conference 19*).

In another study, we have assessed whether interactions between *CYP2C19* polymorphisms (loss-of-function (*CYP2C19*2*) and gain-of-function (*CYP2C19*17*)) and smoking are associated with the levels of platelet activation and CRP level in a post-PCI (percutaneous

coronary intervention) population from the Centre Hospitalier Universitaire Timone in Marseille, after one month treatment with aspirin and either clopidogrel or prasugrel (n=1128). We have identified significant interactions between *CYP2C19**2 polymorphism and CRP levels (P=0.005) and between metabolising status and smoking for CRP in the way that among smokers the highest CRP levels were observed in poor metabolisers. We have thus demonstrated that the interaction between smoking and *CYP2C19* poor metabolising status is associated with higher CRP levels in post-PCI patients using thienopyridines. This work was presented in one original publication ⁸² (*original publication 21*) and in two international conferences as poster presentation (*conferences 9 and 14*). In this study I have participated in data analysis and interpretation and reviewing of the manuscript.

Other projects

During the post-doctoral period I have participated also in other projects linked with the thematic of the IGE-PCV; U1122 unit.

In one nutritional study, we have assessed the associations of alcohol consumption and alcoholic-beverage preferences with dietary patterns in a sample of SFS (n=423 unrelated middle-aged males). We demonstrated a negative association between increased alcohol intakes and consumption of milk, yogurt and fresh/uncured cheese, sugar and confectionery, pastries and cookies, vegetables and fruit (P=0.002), and a positive relationship with cheese, meat and organs, pork-butcher's meat, and potatoes (P=0.011). Thus, we have verified that the associations of poorer dietary practice with alcohol consumption found in different populations around the world were observed also in a French male population. This work was published in one original publication ⁸³ (*original publication 33*).

My working experience in the research field of genetic epidemiology of CVD in the IGE-PCV; U1122 unit has resulted also in the drafting of several scientific reviews focused on this field. One review was focused on the challenges of GWAS in CVD ⁸⁴ (*scientific review 9*). A second review was related to an update on genetics of adverse cardiovascular events ⁸⁵ (*scientific review 6*).

Participation in research contracts

As a post-doctoral research in the IGE-PCV; U1122 unit, I have participated in numerous research contracts of the unit:

1. “Les Interactions Gène-Environnement dans l’era des analyses pangénomiques (genome-wide scan, GWS)”. The project was a fellowship for my post-doctoral research funded by the Lorraine Region. My work in the previously detailed projects was included in this contract (2012-2013).
2. “BioIntelligence” project. This was a national French project coordinated by Dassault-Systèmes, with a great number of research and industrial partners: EPI Orpailleur, Sobios, Aureus pharma, Ipsen, Pierre Fabre, Sanofi-Aventis, Servier, Bayer CropScience, INSERM, Genopole Evry. It aimed to the design, development and implementation of clinically useful softwares for life science field and for multifactorial diseases. The research projects of VEGF and gene \times gene and gene \times environment interactions were used in this project to facilitate the development of metabolic pathways software. Apart from my participation in the research work, I also participated and represented the unit in the frequent meetings of the project group and drafted the annual reports of the team (2010-2014).
3. “Gene-Environment Interactions in Cardio-Vascular Physiopathology” project which was funded by the infrastructure BIOBANQUES Inserm under the call « Thematic networks for biobanks ». This project aimed at the development of an informatics platform named “Omics_BioBook” that would be able to manage, store and transfer with safety “-omics” data coming from the BRC IGE-PCV through the collaborating network of the unit IGE-PCV; U1122. I was among the principal researchers participating in this project and I have contributed in the development of this platform with the collaboration of the infrastructure BIOBANQUES and the Inserm Grand Est (2013-2015).
4. ““Omics” data of SFS” project. This project was based on contract between the unit IGE-PCV; U1122 and the Wellcome Trust Sanger Institute in UK that included the generation of GWAS data, epigenome-wide data (whole genome DNA methylation assay) and RNA sequencing data for a selected familial sample of SFS. I was involved in this project in the selection of the individuals, in transfer of biological material and data and in data analysis and interpretation (2013-2014). The results of this collaboration will be developed in the next section (Research Engineer Inserm).
5. “TELARTA (Telomeres and Arterial Aging)” project. As mentioned before, this was an “ANR Programme de Recherche Translationnelle en Santé”, where the unit IGE-PCV; U1122

was responsible for a work package. My participation was detailed above in the project “Telomere length genetic determinants in adults and children” (2014-2017).

Supervision of students and participation in evaluation committees

I have co-supervised the thesis of Mohsen Azimi Nezhad: « VEGF-A et phénotypes intermédiaires des maladies cardiovasculaires : une approche de génomique fonctionnelle » (*doctoral student 6*), whose results were detailed above in the Project 1 : Study of VEGF and its involvement in CVD. The thesis defense was on Septembre 2012.

The aim of this thesis was the investigation of the genetic determinants of VEGF levels along with the pre-analytical and analytical variation factors. The identified polymorphisms that determine VEGF levels were tested against metabolic syndrome, blood lipids, adhesion and inflammation molecules, while associations with *VEGF* gene expression isoforms were also assessed.

The publications related to this thesis are:

1. Debette S*, Visvikis-Siest S*, Chen MH, Ndiaye NC, Song C, DeStefano AL, Safa R, Azimi-Nezhad M, Sawyer D, Marteau JB, Xanthakis V, Siest G, Sullivan L, Pfister M, Smith H, Choi SH, Lamont J, Lind L, Yang Q, Fitzgerald P, Ingelsson E, Vasani RS**, Seshadri S**. Identification of cis and trans acting genetic variants explaining up to half the variation in circulating VEGF levels. *Circ Res.* 2011; 109(5): 554-63. * equal first authors, ** equal last authors.
2. Azimi-Nezhad M, Lambert D, Ottone C, Perrin C, Chapel C, Gaillard G, Pfister M, Masson C, Tabone E, Betsou F, Meyronet D, Ungeheuer MN, Visvikis-Siest S. Influence of pre-analytical variables on VEGF gene expression and circulating protein concentrations. *Biopreservation and Biobanking* 2012; 10(5):454-61.
3. Azimi-Nezhad M, Herbeth B, Siest G, Dade S, Ndiaye NC, Esmaily H, Hosseini SGhayour-Mobarhan M, Visvikis-Siest S. High Prevalence of Metabolic Syndrome in Iran in Comparison with France: What Are the Components That Explain This ? *Metab Syndr Relat Disord.* 2012. Jan 27; 10(3): 181-8
4. Stathopoulou MG*, Bonnefond A*, Ndiaye NC*, Azimi-Nezhad M, El Shamieh S, Saleh A, Rancier M, Siest G, Lamont J, Fitzgerald P, Visvikis-Siest S. A common variant highly associated with plasma VEGFA levels also contributes to the variation of both LDL-C and HDL-C. *J Lipid Res.* 2013; 54(2): 535-41. *equal first authors

5. Azimi-Nezhad M*, Stathopoulou MG*, Bonnefond A, Rancier M, Saleh A, Lamont J, Fitzgerald P, Ndiaye NC, Visvikis-Siest S. Associations of vascular endothelial growth factor (VEGF) with adhesion and inflammation molecules in a healthy population. *Cytokine*. 2013. 61: 602-607. *equal first authors
6. Ndiaye NC*, Azimi Nezhad M*, El Shamieh S*, Stathopoulou MG*, Visvikis-Siest S*. Cardiovascular diseases and genome-wide association studies. *Clin Chim Acta*. 2011. 412(19-20): 1697-701. *equal contribution

In the same period, I co-directed the thesis of Marc Rancier : « Intérêt de la génomique du VEGF en endocrinologie clinique » and Abdelsalam Saleh : « VEGF: Un biomarqueur potentiel dans la physiopathologie cardiovasculaire » which were presented on March 2015 (*doctoral students 4 and 5*). Their results and my implication will be detailed in the next section (Research Engineer Inserm).

The aim of the thesis of Marc Rancier was to identify the VEGF related polymorphisms and the *VEGF* expression isoforms that could be useful for personalised medicine. Thus, we have studied the effect of the identified VEGF determinants on diseases intermediate phenotypes such as adhesion, and inflammation molecules, metabolic syndrome and lipid, while studies in auto-immune thyroid diseases were performed aiming to investigate links with thyroid hormones and thyroiditis.

The publications related to this thesis are:

1. Stathopoulou MG*, Bonnefond A*, Ndiaye NC*, Azimi-Nezhad M, El Shamieh S, Saleh A, Rancier M, Siest G, Lamont J, Fitzgerald P, Visvikis-Siest S. A common variant highly associated with plasma VEGFA levels also contributes to the variation of both LDL-C and HDL-C. *J Lipid Res*. 2013; 54(2): 535-41. *equal first authors
2. Azimi-Nezhad M*, Stathopoulou MG*, Bonnefond A, Rancier M, Saleh A, Lamont J, Fitzgerald P, Ndiaye NC, Visvikis-Siest S. Associations of vascular endothelial growth factor (VEGF) with adhesion and inflammation molecules in a healthy population. *Cytokine*. 2013. 61: 602-607. *equal first authors
3. Zaaber I*, Rancier M*, Stathopoulou MG, Saleh A, Marmouch H, Masson C, Murray H, Kurth MJ, Lamont J, Fitzgerald P, Mahjoub S, Said K, Bel Hadj Jrad Tensaout B, Mestiri S, Visvikis-Siest S. Plasma VEGF- related polymorphisms are implied in autoimmune thyroid diseases. *Autoimmunity* 2016 Jun;49(4):229-35. * equal first authors
4. Rancier M*, Zaaber I*, Stathopoulou MG, Chatelin J, Saleh A, Marmouch H, El Shamieh S, Masson C, Murray H, Lamont J, Fitzgerald P, Mahjoub S, Said K, Tensaout BB,

Mestiri S, Visvikis-Siest S. Pro- and anti-angiogenic VEGF mRNAs in autoimmune thyroid diseases. *Autoimmunity*. 2016 Aug 5:1-7. * equal first authors

The aim of the thesis of Abdelsalam Saleh was to investigate the associations between the VEGF related variants and its expression isoforms with its plasma levels and with cell adhesion molecules, inflammatory molecules, plasma lipids, candidate genes (NOS3, CD14, MMP3 and IL-4) and with cardiovascular risk factors (obesity and blood pressure) in healthy individuals.

The publications related to this thesis are:

1. Stathopoulou MG*, Bonnefond A*, Ndiaye NC*, Azimi-Nezhad M, El Shamieh S, Saleh A, Rancier M, Siest G, Lamont J, Fitzgerald P, Visvikis-Siest S. A common variant highly associated with plasma VEGFA levels also contributes to the variation of both LDL-C and HDL-C. *J Lipid Res*. 2013; 54(2): 535-41. *equal first authors
2. Azimi-Nezhad M*, Stathopoulou MG*, Bonnefond A, Rancier M, Saleh A, Lamont J, Fitzgerald P, Ndiaye NC, Visvikis-Siest S. Associations of vascular endothelial growth factor (VEGF) with adhesion and inflammation molecules in a healthy population. *Cytokine*. 2013. 61: 602-607. *equal first authors
3. Saleh A*, Stathopoulou MG*, Dadé S, Ndiaye NC, Azimi-Nezhad M, Murray H, Masson C, Lamont J, Fitzgerald P, Visvikis-Siest S. Angiogenesis related genes NOS3, CD14, MMP3 and IL4R are associated to VEGF gene expression and circulating levels in healthy adults. *BMC Med Genet*. 2015;16:90. * equal first authors

Also, I co-supervised the master II thesis of Alexandros Petrelis : « Investigations biostatistiques/bioinformatiques des interactions gène x gène impliquées dans le risque cardiovasculaire - la longueur des télomères leucocytaires chez les enfants » (*master student I*). The defense was on September 2014 and I have participated in the evaluation committee.

The aim of this report was to assess the epistatic interactions that are implicated in the determination of telomere length of children through GWAS and candidate gene approaches.

One publication was produced by this work:

1. Stathopoulou MG, Petrelis, AM, Buxton JL, Froguel P, Blakemore AIF, Visvikis-Siest S. Genetic Determinants of Leucocyte Telomere Length in Children: a Neglected and Challenging Field. *Paediatr Perinat Epidemiol* 2015;29(2):146-150

Organisation of conferences

I have participated in the organisation of the following conferences as members of the organising committee:

- 7th Santorini Conference Biologie Prospective, Santorini, Greece, 2014.
- Second ESPT Conference Pharmacogenomics: From Cell to Clinic, Lisbon, Portugal, 2013.
- 6th Santorini Conference Biologie Prospective, Santorini, Greece, 2012.

My tasks included the communication of the conferences, the organisation of poster sessions, on site organisation and the drafting of the post-conference scientific manuscripts. In the 7th Santorini Conference Biologie Prospective, I was also member of the Poster Committee that was responsible for the evaluation of posters and selection of award winners.

Research Engineer Inserm, IR2 (2015 - today)

Université de Lorraine, Inserm, IGE-PCV; U1122

Project 1 : The study on VEGF

Following up the projects that were done during the post-doctoral period on VEGF, we aimed to continue our studies on the links between VEGF and CVD intermediate phenotypes, but also to further assess the role of VEGF on disease risk and its usefulness as a biomarker for personalised medicine.

In one study, we have assessed possible genetic connections between different angiogenesis-related genes, including *NOS3*, *CD14*, *MMP3*, *IL4R* and *IL4* with *VEGF* gene expression and plasma levels. The study was performed in 403 unrelated adults of SFS, where the variants rs2569190, rs2243250, rs2010963, rs3918226, rs1799983, rs1800779, rs3025058, rs1805015 and rs1801275 of the *NOS3*, *CD14*, *MMP3*, *IL4R*, and *IL4* genes were selected to be genotyped on the basis of their functionality on the related genes. Furthermore, the 4 VEGF-related SNPs were also assessed (rs6921438, rs10738760, rs6993770, rs4416670). Four mRNA isoforms of *VEGF* gene (VEGF-121, VEGF-145, VEGF-165, VEGF-189) were quantified in PBMCs of 65 individuals. We found that the SNP rs1799983 of *NOS3* gene was associated with VEGF-145 mRNA levels ($\beta = 0.17$, $P = 0.033$). Five variants in *NOS3*, *CD14*, *IL4R*, *MMP3* genes and one VEGF-related SNP were associated with VEGF plasma levels through epistatic interactions ($P < 0.05$). Furthermore, significant gene \times environment interactions were identified for the rs1800779 SNP of *NOS3* with levels of HDL, triglycerides and with obesity for VEGF plasma levels ($P < 0.05$). This study demonstrated interactions between SNPs of angiogenesis-related genes with VEGF plasma levels and gene expression, especially for *NOS3* gene, thus supporting the hypothesis that VEGF plays a central role in linking different angiogenetic mechanisms related to chronic diseases. These results were presented in one original publication⁸⁶ (*original publication 19*) and as a poster presentation in the 7th Santorini Conference Biologie Prospective (*conference 12*). This was the work of Abdelsalam Saleh thesis (*doctoral student 5*), which I co-supervised. For this project, I performed the study design, the analysis and interpretation of data and the drafting of the manuscript.

In the next step, we worked on a project that aimed to assess the links between VEGF and auto-immune thyroid diseases (AITD). A case control study was performed with 389 patients

with AITD (297 with Hashimoto thyroiditis (HT) and 92 with Graves' disease (GD)) and 364 healthy controls recruited at the University Hospital Fattouma Bourguiba, Monastir, Tunisia. The 4 VEGF-related SNPs were genotyped (rs6921438, rs10738760, rs6993770, rs4416670), and five VEGF mRNA expression isoforms were quantified in 71 patients and 86 controls (VEGF-121, VEGF-165, VEGF-145, VEGF-189 and VEGF165b) after 6 months of treatment. VEGF plasma levels were measured in 110 patients after 6 months of treatment. We demonstrated that the rs10738760 was significantly associated with an increased risk of GD compared with HT ($\beta = 0.54$, $P = 0.003$) and controls ($\beta = 0.46$, $p = 0.006$). A significant association between rs6921438 and risk of GD compared with controls was also observed ($\beta = 0.324$, $P = 0.05$). The rs4416670 polymorphism was associated with an increased risk of hyperthyroidism in 6 months treated patients ($\beta = 1.82$, $P = 0.049$), independently of their initial diagnosis, while a significant positive association between the rs10738760 and plasma levels of FT3 ($\beta = 0.716$, $P = 0.024$) after 6 months treatment was observed. No significant differences were found between the groups for plasma VEGF levels. After 6 months of treatment VEGF-165 mRNA levels were higher in GD ($P = 0.040$), while the VEGF-189 was lower in GD ($P = 10^{-3}$), in HT ($P = 10^{-3}$) and in all individuals with AITD compared to controls ($P = 10^{-3}$). However, VEGF-165b was higher in HT compared to GD ($p = 0.017$). VEGF-165b was more increased in individuals with hypothyroidism compared to euthyroid subjects ($p = 0.043$) after treatment. This study demonstrated for the first time associations between specific VEGF isoforms and different AITD after treatment and a common genetic link between VEGF and AITD is supported. These results were presented in 2 original publications^{87,88} (*original publications 13 and 18*). This was the work of Marc Rancier thesis (*doctoral student 4*) and of Jerome Chatelin thesis (*doctoral student 2*), which I co-supervise. For this project, I performed the study design, the analysis and interpretation of data and the reviewing of the manuscript.

The next study on VEGF was focused on a new GWAS study aiming to identify novel polymorphisms that explain more of the individual variability of VEGF levels. A greater sample was used in this effort with 6 discovery cohorts ($n = 13312$) and *in silico* and *de novo* replication in $n = 2800$ individuals. Six novel variants were identified that together with the 4 previously found (figure 5) explain 52% of the variability of VEGF levels. The novel variants are linked with known and novel pathways associated with VEGF actions. This is the biggest GWAS study on VEGF levels today. These results are presented in one original publication⁸⁹ (*original publications 17*) and in one conference as a poster presentation

(*conference 15*). In this work I have performed the design of the replication, the analysis of SFS data, the interpretation of the results and the reviewing of the manuscript.

In the following study, we aimed to assess the genetic effect of the 4 VEGF-related polymorphisms (rs6921438, rs10738760, rs6993770, rs4416670) on the risk of depression. A case-control population of 437 patients diagnosed with depressive disorders and 477 healthy controls was recruited in the Mental Health Centre of Extremadura University Hospital, Badajoz, Spain. We have identified a direct effect of rs4416670 on depression risk (OR: 1.60, P=0.010). This study demonstrated genetic links between VEGF and depression for the first time. As we have previously associated the rs4416670 with gene expression of L-selectin⁵⁴, we hypothesised that this SNP association with depression could be mediated by VEGF and L-selectin. These results are presented in an original publication⁹⁰ (*original publication 11*). This work was part of the thesis of Ting Xie (*doctoral student 3*), which I co-supervised. For this work, I have performed the study design, the interpretation of the results and the drafting of the manuscript.

In a collaborative study, we have assessed the effect of the 4 VEGF-related polymorphisms on the risk of metabolic syndrome in an Iranian population (n=336) (rs6921438, rs10738760, rs6993770, rs4416670). We have demonstrated that rs6993770 was associated with an increased risk for metabolic syndrome (P = 0.007). This work was published in one original publication⁹¹ (*original publication 2*), and my work was focused on study design, data interpretation and reviewing of the manuscript. Studies on the VEGF-related SNPs in French populations have been performed and replications are ongoing, where I am implicated in the study design, the data analysis and interpretation. The first results indicate an association of the rs10738760 with decreased risk for metabolic syndrome (OR=0.47, P=0.010). These results have not been published yet and represent a part of the work of the theses of Mohsen Azimi-Nezhad, Abdelsalam Saleh, Marc Rancier and Jerome Chatelin (*doctoral students 2, 4, 5 and 6*), which I co-supervised. Furthermore, this subject was the focus of a scientific review, where we have participated in the reviewing of the manuscript⁹²(*scientific review 4*).

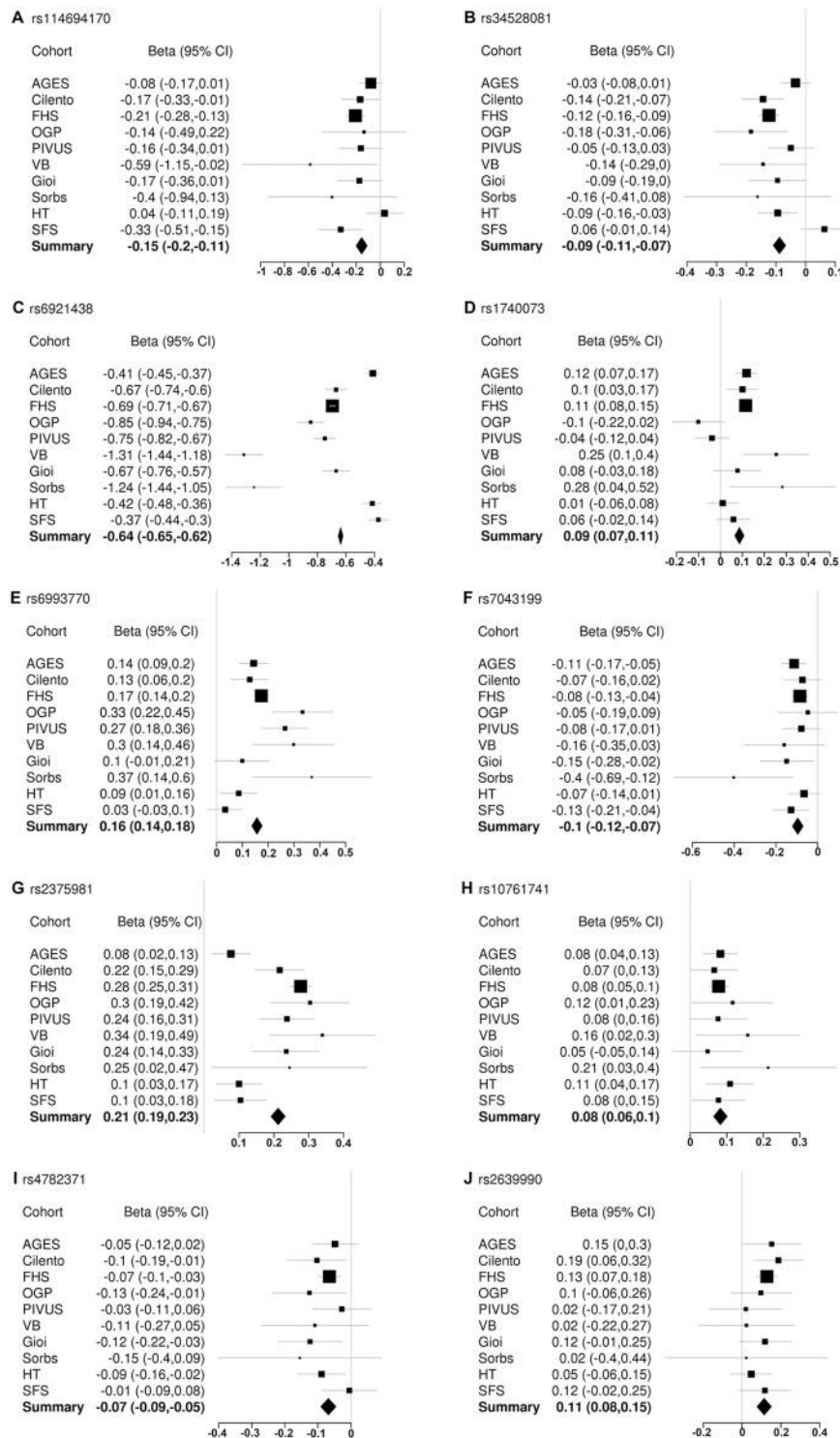


Figure 5: Panels A-J. The effect size (Beta) and the 95% Confidence Interval (CI) in all discovery and replication studies and the cumulative effect in the inverse-variance meta-analysis for the 10 replicated SNPs (87)

Two more studies on VEGF “-omics” determinants are ongoing and are part of the thesis of Vesna Gorenjak, which I co-supervise (*doctoral student 1*). The first study aims to assess the epigenetic determinants of VEGF levels in 201 healthy individuals of the SFS through a discovery only epigenome-wide association analysis (EWAS). Twenty CpG sites within 13

genes reached EWAS significance ($P = 1.08 \times 10^{-07}$ to $P = 5.64 \times 10^{-15}$). Methylation detected in promoter regions of *TPX2* and *HAS-1* genes could explain previously reported associations with *VEGF-A* gene. In the second study, we have assessed associations between VEGF levels and inflammation molecules (IL1 α , IL1 β , IL2, IL4, IL6, IL8, IL10, INF- γ , TNF- α , MCP-1 and epidermal growth factor (EGF)) levels measured in PBMCs extracts. Associations with *VEGF* gene expression isoforms (VEGF-121, VEGF-145, VEGF-165, VEGF-189) were also tested. We have identified significant associations between VEGF levels and levels of IL4 ($\beta=0.028$, $P=0.013$), MCP-1 ($\beta=0.015$, $P<0.0001$) and EGF ($\beta=0.017$, $P<0.0001$). Furthermore, mRNA isoform VEGF-165 was associated with MCP-1 and IL1 α ($P=0.002$ and $P=0.008$, respectively); and mRNA isoform VEGF-189 was associated with IL4 and IL6 ($P=0.019$ and $P=0.034$, respectively) levels. This study provided insights of mechanisms implicated in VEGF regulation in PBMCs. The related manuscripts have been submitted for publication. My involvement included the conception and design of the studies, the analysis and interpretation of the results and the reviewing of the manuscript.

This extended research on VEGF has led in the development of the Vascular endothelial growth factor European Genomic Federation Consortium (VEGF Consortium) by Sofia Siest as its leader and by me as a project manager (www.vegfconsortium.org) in 2015. The consortium aims to develop a transnational collaborative network dedicated to large integrative and multidisciplinary genomic studies of the VEGF in order to generate applicable knowledge for medical practice. Its objectives are:

- To combine data from multiple cohorts in order to identify VEGF “-omics” profiling in health and non-communicable diseases
- To elucidate the pivotal role of VEGF in the pathophysiology of non-communicable diseases
- To demonstrate the patients’ stratification potential of VEGF “-omics” profiling
- To implement the research results into clinical practice and establish the role of VEGF as a predictive, preventive, diagnostic and prognostic biomarker
- To provide information on the effect of VEGF “-omics” profiling in side effects and response to therapy through pharmacogenomics studies
- To propose implementation strategies and European guidelines involving VEGF “-omics” profiling for the management of non-communicable diseases
- To share methodologies, data and knowledge in the field of “-omics” management and innovative statistics

- To develop standardized teaching and evaluation methods practiced and validated by the Consortium

Today, it comprises 22 participants, from 11 different countries including the USA and Canada. It brings together an important network of academic and industrial organisations with highly complementary skills and experiences to successfully fulfil the objectives identified.

I have drafted the manuscript that describes the development and the objectives of the Consortium ⁹³ (*original publication 7*). This work was also presented in 2 international conferences as poster presentations (*conferences 5 and 7*). Three of the doctoral students co-supervised by me are involved in the development and the projects of the VEGF Consortium: Ting Xie, Jerome Chatelin and Vesna Gorenjak (*doctoral students 3, 2 and 1 respectively*).

I have also drafted two proposals entitled “The VEGF Project” on behalf of the VEGF Consortium that were submitted under the calls H2020-SC1-PM-02-2017: New concepts in patient stratification in 2016 and H2020-SC1-BHC-25-2019: Demonstration pilots for implementation of personalised medicine in healthcare in 2018. The first project did not receive the funding, while the second project is under revision by the European Commission.

Within the frames of the consortium, we have designed two projects that are ongoing and make part of the thesis of Jerome Chatelin, whose thesis I co-supervise (*doctoral student 2*). One project is focused on the assessment of the role of the 4 VEGF-related SNPs (rs6921438, rs10738760, rs6993770, rs4416670) with BMD, age of menopause and risk of osteoporosis in a sample of 3 populations of post-menopausal women (OPG n= 52, SBCE n= 91, ARTEOS n= 97). We have identified a significant interaction between rs6921438 x rs6993770 and increased femoral neck BMD (b= 0.478, P= 1.52x10⁻⁷).

In a second study we are assessing the effect of the 10 VEGF-related SNPs with levels of thyroid hormones and risk of hyperthyroidism and hypothyroidism in LifeLines cohort (LLs) (n= 4826), in Cilento Study (n= 2100), in CHRIS study (n= 3850) and in MICROS study (n= 873). Analysis results are currently being completed for the last two studies and a meta-analysis will reveal the significant results.

In these 2 studies, I am involved in the conception and design, in the selection of population and data preparation, in the analysis and interpretation of the results.

Project 2 : Effects of *LSR* Polymorphisms on lipidemia and Alzheimer's disease (AD)

The disturbances of lipid homeostasis in peripheral tissues, named dyslipidemias, have been clearly shown to be significant risk factors for atherosclerosis, the underlying cause of CVD⁹⁴. The apolipoprotein E (ApoE) plays an important role in lipid homeostasis by acting as a ligand for lipoprotein receptors. It is involved in particular in the hepatic clearance of lipoproteins rich in triglycerides⁹⁵. The *APOE* gene is located on chromosome 19q13.1, a locus related to hyperlipidemia as indicated by GWAS⁹⁶. The *APOE* gene has a well-characterised three-allele polymorphism ($\epsilon 2$, $\epsilon 3$ and $\epsilon 4$) that determines the variability of an individual's lipid profile. The $\epsilon 3$ allele is the most common allele and is considered the wild-type allele. The $\epsilon 4$ variant is linked to high levels of plasma lipids. In addition, the common polymorphism of the *APOE* gene is related to type III hyperlipidemia (type III HLP, OMIM 107741), a familial dyslipidemia characterised by the accumulation of TG-rich lipoprotein residues, leading to an increased risk of atherosclerosis.⁹⁷ Carriers of the *APOE* $\epsilon 2 / 2$ genotype show the highest probability of having a type III HLP. On the contrary, as indicated previously^{98,99}, we found significant associations of the *APOE* $\epsilon 2$ allele with reduced levels of triglycerides, cholesterol and LDL and increased levels of ApoE in adults^{100, 101}. This paradox is further complicated by the fact that even if the clearance of dietary lipids in type III HLP patients is less effective in individuals with the *APOE* $\epsilon 2 / 2$ genotype, only a few patients $\epsilon 2 / 2$ actually develop the disease¹⁰², indicating that environmental factors and other genetic factors must contribute to Type III HLP¹⁰³.

Furthermore, *in vivo* studies have pointed towards potential links between dyslipidemias, age-related cognitive decline and Alzheimer's disease (AD)¹⁰⁴. However, results from epidemiological studies comparing lipid levels in controls and AD or dementia patients have not been conclusive, or have even shown contradictory results¹⁰⁴. This may be due to the complexity of dyslipidemias which result from perturbations in lipid homeostasis resulting in hypercholesterolemia, hypertriglyceridemia, or both (mixed hyperlipidemia) with changes in lipoprotein profiles that are not taken into account in the analyses. Studies evaluating efficacy of cholesterol lowering treatments such as statins to treat AD have proven inconclusive, suggesting that hypercholesterolemia is not the only risk factor involved¹⁰⁵. It is therefore necessary to identify the key pathways regulating peripheral lipid homeostasis that influence risk for cognitive decline and AD. ApoE is the strongest risk factor for the sporadic form of AD, which represents > 95% of AD cases. The $\epsilon 4$ variant is a strong AD risk factor¹⁰¹ and $\epsilon 2$ may lower the risk of AD and age of onset¹⁰⁶, but the involved mechanisms remain as yet

unclear. Studies have focused particularly on ApoE's role in β -amyloid peptide (A β) deposition and clearance in the brain ¹⁰⁶, but little information is available on peripheral effects of ApoE isoforms on cognitive decline and AD. The ability of plasma ApoE to restore synaptic function despite lack of ApoE expression in the brain was recently reported ¹⁰⁷, suggesting a potential link between ApoE-dependent regulation of peripheral lipid homeostasis, cognitive function and AD.

The lipolysis stimulated lipoprotein receptor (LSR) is involved in the regulation of lipid distribution between different tissues. It recognizes ApoB and ApoE and plays an important role in the elimination of triglycerides-rich lipoproteins during the postprandial phase ^{108, 109}. LSR represents a potential link between dyslipidemia and atherosclerosis. Although the absence of both *LSR* alleles is lethal at the embryonic stage, studies in the *LSR* +/- heterozygous mice revealed that reduced LSR levels led to high postprandial lipemia, moderate hypercholesterolemia, and hypertriglyceridemia. ¹⁰⁸. *LSR* +/- heterozygous mice placed on a cholesterol-rich "Western" diet show an accumulation of atherogenic lipoproteins such as LDL and triglycerides-rich lipoproteins, which is accompanied by an increase in lipid deposition in the aorta ¹⁰⁸. Hepatic LSR knockout results in both hypertriglyceridemia and hypercholesterolemia in mice, confirming that LSR contributes significantly to the regulation of lipid status ¹¹⁰. Cell culture studies (fibroblasts, hepatocytes) have demonstrated the ability of lipid particles containing ApoE to bind to LSR, leading to their internalization and degradation in cells ^{111, 112}. In addition, the binding affinity seems different according to the *APOE* variants: the very low density lipoproteins (VLDL) of a patient $\epsilon 2 / 2$ with HLP type III are unable to bind to the LSR ¹¹². The low affinity of LSR for VLDL of a patient $\epsilon 2 / 2$ could contribute to a decrease in hepatic clearance of triglycerides-rich lipoproteins, leading to an increase in plasma triglycerides observed in this particular class of hyperlipidemia.

Moreover, dyslipidemic *LSR*+/- mice display age-dependent modified brain cholesterol trafficking, increased levels of membrane cholesterol ¹¹³ along with increased susceptibility to amyloid stress ¹¹⁴. LSR is important for blood brain barrier (BBB) integrity ^{115, 116}, which may explain why hypertriglyceridemia has been associated with BBB impairment ^{117, 118} often observed in AD. Taken together, these results suggest that LSR pathway may represent an important molecular link between dyslipidemias and risk for cognitive decline and AD.

The *LSR* gene that encodes LSR is also located on chromosome 19q13.1 just upstream of *APOE*. Little information is currently available on the role of *LSR* polymorphisms in the regulation of lipid levels. SNP rs916147 (A / G), located in a splice junction between intron 5 and exon 6, was associated with lipid levels in a small population of obese adults ¹¹⁹. In

addition, this SNP is a quantitative expression locus (eQTL) for LSR and can alter the expression of LSR in different tissues (subcutaneous and visceral adipose tissue, tibial artery, coronary artery and skeletal muscle), thereby indicating its potential functionality (www.gtexportal.org). The SNP rs34259399 is located on exon 6 and preliminary unpublished data suggest that it is involved in the regulation of LSR expression and activity. The role of these two functional SNPs on AD risk is unknown. We have thus hypothesized that APOE variants could interact with LSR polymorphisms to modify the effect of APOE on blood lipid regulation and AD risk.

In the first study, we have used a discovery population of healthy adults and children coming from the SFS (n=432 unrelated adults and n=328 children (<18 years old)) and a replication population of n = 1744 unrelated adults to test the association of the direct and epistatic effects of LSR and APOE variants on blood lipids levels (cholesterol, triglycerides, HDL, LDL, ApoB and ApoE). We have found significant epistatic interactions between the LSR rs916147 and APOE ϵ 2 allele in adults for cholesterol (b=0.114, P=0.810 \times 10⁻⁸), ApoE (b=0.125, P=0.625 \times 10⁻³) and LDL levels (b=0.059, P=0.120 \times 10⁻³). These results replicated for cholesterol and ApoE. The interaction of the LSR rs916147 with APOE ϵ 2 reversed the direct effect of APOE ϵ 2 on cholesterol, ApoE and LDL levels. We could therefore suggest that for APOE ϵ 2 subjects, the interaction with the rs916147 could lead to an increased risk for APOE ϵ 2/2 subjects to develop type III HLP. These results were presented in one original publication ¹²⁰ (original publication 6) and were presented as a poster presentations in 2 international conferences (*conference 1 and 8*).

In a second publication, we investigated the effects of the LSR and APOE polymorphisms on AD risk. A case-control population of 142 patients diagnosed with AD and a control group of 63 healthy volunteers of European origin was used. We identified significant epistatic interactions between the 2 functional LSR SNPs and APOE ϵ 4 allele for AD: rs34259399 \times APOE ϵ 4 (beta= -0.95, P=0.00002) and rs916147 \times APOE ϵ 4 (beta=-0.83, P=0.0068). In more details, all non- ϵ 4 allele carriers had increased risk of AD when combined with the with the LSR polymorphisms. These results were published in one original publication ¹²¹ (*original publication 3*).

These two studies were parts of the thesis of Ting Xie (*doctoral student 3*), whom I co-supervised. My involvement included the study conception and design, data analyses and interpretation and drafting of the manuscripts.

Project 3: Triggering Receptors Expressed on Myeloid cells (TREM) and inflammation

Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) is a member of a recently discovered immunoglobulin family^{122, 123}. It is implicated in an increase of the inflammatory responses of neutrophils and monocytes¹²⁴ and is related to the production of pro-inflammatory cytokines (TNF- α and IL1b)¹²⁵. TREM-1 apart from its role during the pathophysiology of septic shock¹²², it is also involved in other acute and chronic inflammatory diseases of non-infectious etiology, such as rheumatoid arthritis¹²⁶, atherosclerosis¹²⁷, acute myocardial infarction¹²⁸ and critical limb ischemia¹²⁹. The mechanisms that explain this action are not yet clearly described. Adhesion molecules like selectins have an important role during this process¹³⁰⁻¹³². In the one study, we have investigated the genetic links between variants within or near the *TREM-1* gene and soluble TREM-1 (sTREM-1 and TREM-1sv) serum levels and with the expression levels of two TREM-1 splicing isoforms in PBMCs, the membrane form (mbTREM-1) and the soluble form (TREM-1sv). Further, we have tested the associations between the significant these SNPs and levels of soluble E-selectin, L-selectin and P-selectin, which are involved in the early stages of atherosclerotic processes. A discovery cohort with 351 individuals from SFS and an independent replication population of 80 subjects was used. We have included 10 SNPs in the analysis from which the rs2234246 was associated with increased sTREM-1 levels in the discovery population ($b = 0.3$, $P = 0.003$), and with increased levels of mRNA of mbTREM-1 isoform ($b = 0.49$, $P = 0.007$). The same SNP was associated with increased soluble L-selectin levels ($b = 0.05$, $P = 0.011$). The associations of the SNP with sTREM-1 and L-selectin levels were confirmed in the replication population ($b = 0.13$, $P = 0.0007$ and $b = 0.03$, $P = 0.018$ respectively). In this study, we identified one polymorphism affecting sTREM-1 levels and mbTREM-1 isoform in PBMCs, which is also associated with L-selectin levels suggesting that TREM-1 could act in the trans-epithelial migration process of leukocytes and more specifically of monocytes through expression of L-selectin. This work was presented in one publication¹³³ (*original publication 10*) and was presented in one international conference as a poster presentation (*conference 3*).

Triggering Receptor Expressed on Myeloid cells-2 (TREM-2) is expressed on the cell surface of macrophages and dendritic cells derived from monocytes, as well as in microglia and osteoclasts. TREM-2 plays a role in a series of tyrosine phosphorylation reactions that regulate various inflammatory responses^{125, 134}. It exerts anti-inflammatory properties during the immune response^{135, 136}, including stimulation of phagocytosis and suppression of

cytokine production like the TNF- α ^{137, 138} and it is thus linked with atherosclerosis. We have therefore assessed the effect of *TREM-2* polymorphisms on TNF- α levels and intima media thickness of the femoral artery in 809 children and adults from the SFS and in 916 unrelated adults of an independent replication cohort. We have demonstrated significant associations between the SNP rs6918289 with plasma levels of TNF- α in discovery and replication populations ($b=0.13$, $P=0.0026$, and $b=0.20$, $P=0.023$, respectively), and with femoral artery thickness in the discovery cohort ($b= 0.02$, $P=0.026$). Thus, this SNP could be a risk factor for inflammatory diseases and atherosclerosis. These results are presented in one publication¹³⁹ (*original publication 4*) and in 2 international conferences as poster presentations (*conference 4 and 6*). This work was part of the thesis of Vesna Gorenjak, which I co-supervise (*doctoral student 1*).

For both these projects I participated in the data interpretation and the reviewing of the manuscripts.

Project 4: Telomere length as a biomarker for personalised medicine

Continuing the research activities on TL genetics and its use as a biomarker in personalised medicine, we have designed different projects, which make part of the thesis of Vesna Gorenjak, which I co-supervise (*doctoral student 1*). A scientific review underlining the importance of telomere length in personalised medicine has been published with my participation in the data collection and drafting of the manuscript¹⁴⁰ (*scientific review 2*).

In a candidate gene study, we are assessing the effect of previously identified genetic determinants of LTL and of novel candidate genes including the 10 VEGF-related SNPs with muscle TL (MTL) in the case-control population TELARTA (ANR Programme de Recherche Translationnelle en Santé) ($n= 451$). MTL is reflecting the TL at the time of birth, as muscle cells do not undergo cell divisions. Thus, the study will identify whether the candidate genes assessed affect the TL at birth or they are implicated in TL attrition. Statistical analysis is ongoing.

Other projects on TL include new GWAS and EWAS efforts including novel bioinformatics approaches in collaboration with international consortia such as the CHARGE consortium Aging Working Group, which are under development. All these projects and the preliminary results were presented as poster presentation in the 9th Santorini Conference in 2018 (*conference 2*).

Project 5: Novel genetic determinants of inflammation molecules

Inflammation is now considered a basic biological process implicated in atherosclerosis as its role is central to all stages of atherosclerosis. As a result, inflammation biomarkers have been extensively studied in relation to CVD traits, especially CRP, which has also been included in recent risk prediction algorithms¹⁴¹. Inflammation represents one the basis axes of research of the IGE-PCV; U1122 unit.

In one recent study, we have investigated the associations between *IL6R* polymorphisms with CRP and lipids levels in a healthy population. We have used 368 unrelated adults from SFS as discovery cohort and 995 adults as an independent replication population. The variants rs4845625 and rs4537545 of *IL6R* were selected based on their contradictory role on IL6 and blood lipids levels. Both polymorphisms were significantly associated with CRP levels (rs4845625 beta =0.182, P=0.011 and for s4537545 beta =-0.189, P=0.009). The rs4845625 was associated with LDL in both populations (beta =0.052, P=0.007, and beta =0.024, P=0.035). Haplotypes analyses confirmed these results. *In silico* analysis revealed that these SNPs are linked with genes involved in the regulation of the JAK-STAT pathway cascade that affects lipid metabolism. This work was presented in one original publication¹⁴² (*original publication 9*)

Aiming to increase the impact of our studies in the inflammation field, the unit has developed collaboration with the CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology) consortium Inflammation Working Group (CIWG), which I lead as a principal investigator for SFS.

I have participated in the data interpretation and the drafting of the manuscript (member of the writing group) of the most recent and largest GWAS on CRP¹⁴³ (*original publication 1*). The study included 88 cohorts with 204402 European individuals. We have identified 58 distinct variants significantly associated with levels of CRP, including 16 previously identified CRP loci. The associations are explained by 55 genes that are involved in the biology of the immune system and liver. Mendelian randomisation analyses reported causal associations between genetically increased CRP and a protective effect on schizophrenia and increased risk of bipolar disorders.

Two more projects on GWAS meta-analysis for IL6 and TNF- α levels are being completed within the CIWG. For these projects; I have performed the data preparation and analysis for SFS and we are currently in the phase of reviewing the related manuscripts.

My participation in these projects has led to the design of a project on childhood inflammation within the frames of the CIWG consortium. Inflammation in childhood has been mostly linked with adiposity, obesity and obesity-related CVD¹⁴⁴. In children, CRP levels have been associated with CVD risk factors, such as adiposity, obesity, and blood pressure, even in very young ages and these associations are most probably mediated by abdominal obesity (measured with waist circumference)^{145, 146}. Furthermore, there are indications that CRP is independently associated with advanced atherosclerosis in youth¹⁴⁷ and that CRP levels in childhood predict their values in adulthood¹⁴⁸. Apart from CRP, TNF- α and interleukin 6 (IL6) have been associated to adiposity, visceral fat, insulin resistance and other CVD risk factors.¹⁴⁹ The identification of genetic determinants of inflammation markers in adults and in children could contribute significantly in the discovery of new genetic biomarkers of CVD risk and also in the identification of new biological pathways implicated in these associations. The published GWAS are mainly focused on CRP while TNF- α and IL6 are not well represented¹⁵⁰⁻¹⁶⁵. Furthermore, the studies have been performed in adult populations (> 18 years old), thus excluding the investigation of children-specific genetic determinants. Additionally, given the strong correlations between inflammation markers and adiposity, the use of this factor as a covariate in the regression models and the study of genes x adiposity interactions are likely to reveal genetic determinants that their effect could be masked by these interactions and which have not been previously discovered in GWAS.

I have proposed to lead a new project focused on inflammation in childhood within the CIWG. This project aims to identify novel childhood-specific genetic variants for CRP, TNF- α , IL6 and other traits (such as white blood cells count) and to assess their interactions with abdominal obesity. GWAS on discovery and replication cohorts of healthy children (<18 years old), followed by meta-analysis will be performed. The proposal has been discussed and approved by the partners of the consortium, the analysis plan design has been circulated and the discovery populations' selection is ongoing.

Other projects

My participation in the project “Omics” data of SFS Project (described in the previous section Post-doctoral researcher), in the databases development and statistical analysis of the produced data has resulted in my inclusion in 3 original publications¹⁶⁶⁻¹⁶⁸ (*original publications 23, 24 and 20*). These were large collaborative studies, where a large number of

cohorts were analysed including SFS. The first study was a GWAS meta-analysis of 339224 individuals from 125 studies that resulted in identification of 56 novel BMI-associated loci, implicated in pathways related to synaptic function, glutamate signalling, insulin secretion/action, energy metabolism, lipid biology and adipogenesis¹⁶⁶. The second study was GWAS meta-analysis of 224459 individuals that identified 33 novel waist/hip circumference associated variants and 19 novel SNPs associated with waist and hip circumference measures involved in pathways of adipogenesis, angiogenesis, transcriptional regulation, and insulin resistance. In these studies I was the analyst of the SFS study data and reviewed the related manuscripts. The third study assessed the genome-wide burden of runs of homozygosity on 16 complex traits in 354224 subjects from 102 studies and demonstrated the existence of directional dominance on four complex traits (stature, lung function, cognitive ability and educational attainment). In this study my contribution was on databases preparation and sharing.

Furthermore, I have participated as the analyst of the SFS in multiple projects of the Global Burden of Metabolic Risk Group Factors of Chronic Disease Collaborating Group (NCD Risk Factor Collaboration - NCD-RisC). I have performed descriptive analysis on height, weight, BMI, blood pressure, blood lipids levels and insulin levels in SFS as indicated by the analysis plan of each study shared within the consortium. The 6 produced publications are presenting the trends of these traits in very large samples across the globe¹⁶⁹⁻¹⁷⁴ (*original publications 5, 8, 12, 14, 15, 16*).

Moreover, I have contributed in the data collection and drafting of 3 scientific reviews: one on personalised medicine major achievements, one on pharmacogenomic challenges in CVD and one on genetics beyond GWAS, based on the experience and expertise of the IGE-PCV; U1122 Unit on the field of genetics of CVD¹⁷⁵⁻¹⁷⁷ (*scientific reviews 1, 3 and 5*). Two of them are parts of the thesis of Jerome Chatelin and Vesna Gorenjak, which I co-supervise (*doctoral students 2 and 1*).

Participation in international consortia

1. VEGF Consortium: I am the project manager of the consortium and member of the Steering Committee. I am participating in the conception and design of multiple projects (details and analyses of publications in the Project “Study on VEGF” above). Since 2015, I have drafted two H2020 proposals within the frames of the calls H2020-SC1-PM-02-2017: New concepts

in patient stratification in 2016 and H2020-SC1-BHC-25-2019: Demonstration pilots for implementation of personalised medicine in healthcare in 2018.

2. CHARGE Consortium: I am the main analyst and contact person for the IGE-PCV; U1122 unit. I have participated in 4 projects:

- Meta-GWAS CRP project, where apart from analyses, I was member of the writing committee and I participated in the design of methodology, coordination of communication and data centralization of > 100 cohorts participating (details on publication of Project “Novel genetic determinants of inflammation molecules” above).
- Meta-GWAS IL6 project, where I have realised the statistical replication analyses in SFS and other populations of the BRC IGE-PCV, and I have participated in the drafting and reviewing of the manuscript.
- Meta-GWAS TNF- α project, where I have realised the statistical replication analyses in SFS and other populations of the BRC IGE-PCV, and I have participated in the drafting and reviewing of the manuscript.
- Inflammation project on childhood, which is an ongoing project where I am the leader and principal investigator within the CHARGE consortium (details on Project “Novel genetic determinants of inflammation molecules” above).

3. Global Burden of Metabolic Risk Group Factors of Chronic Disease Collaborating Group (NCD Risk Factor Collaboration - NCD-RisC): I am the main analyst and contact person for the IGE-PCV; U1122 unit. I have participated in data analysis included in 6 publications (details on publications on Other projects above).

Participation in research contracts and contracts as PI

Since 2015, I have participated in the following contracts of the IGE-PCV; U1122 unit:

1. “Mastic treatment for Healthy obese with NAFLD diagnosis-MAST4HEALTH”, which is funded by the European Community within the frames of the project H2020-MSCA-RISE-2015: Marie Skłodowska-Curie Actions Research and Innovation Staff Exchange. This European projects is a multicenter double blind randomised clinical trial, where a Mastiha or a placebo treatment is used in patients with non-alcoholic fatty liver diseases (NAFLD). Mastiha is a natural product of Greece, produced in Chios island, with antioxidant/anti-inflammatory and lipid lowering properties. The aim of the intervention is to test the effectiveness of Mastiha supplement as a new non-pharmacologic strategy for NAFLD. The

investigation of Mastiha effect will be performed through testing of gene-diet interactions, correlation of genetic and epigenetic markers with metabolomic and intestinal microbiota profiles pre- and post-intervention, while the effectiveness of the intervention will be evaluated via clinical and laboratory markers. The consortium of this European project consists of 13 academic and industrial partners from 7 European countries. I have drafted the participation of the IGE-PCV; U1122 unit in the proposal document and reviewed all related parts. The unit is leader of the work package 4: “Effects of Mastiha on inflammation and adhesion biomarkers and on oxidative stress status indexes”. I am responsible for the tasks of our team that includes the development of databases with IL6, IL1A, TNF- α and VEGF gene expressions data pre and post intervention. Within the frames of this project, I have worked as secondee for 9 months in the Chios Mastic Growers Association, in Greece, where I worked on coordinating the randomisation of treatments for the clinical trial and the design and development of the trial and 3 months in BIOTECH VANA, in Spain, where I have worked on data preparation for the development of a software for integration of metadata and “-omics” profiles for indexing, management and interpretation. I have participated in the training of 4 secondees that have worked in the IGE-PCV; U1122 unit as detailed in the co-supervision of students section above (2016-2020).

2. “Fight Heart Failure, FIGHT-HF”, which is funded by ANR within the frames of the projects “Hospital-University health research” (Recherche Hospitalo-Universitaire en santé, RHU), ANR-15RHU-0004 (2015-2020). This project aims to generate new heart failure classifiers, and to improve patient outcomes with the use of a strategic approach that combines knowledge based on i) underlying mechanisms, ii) “-omics” and imaging bioprofiling, iii) co-morbidities and iv) holistic disease management to define mechanistically relevant and clinically actionable bioprofiles. The consortium is consisted of 8 academic and industrial partners. The IGE-PCV; U1122 unit is responsible for the task 2.3: “TREM-1: inflammation in HF post-myocardial infarction” of work package 2 and more specifically the study of genetic links between TREM-1 with inflammation molecules and for the task 3.1: “-Omics biomarker prediction of transition to heart failure and mechanistic profiling task” of work package 3 and most specifically the study of genetics of TL as a marker of vascular aging. My participation on this contract is detailed in the project “TREM and inflammation” and “Telomere length as a biomarker for personalised medicine” above (2015 - 2020).
3. “TELARTA” project, which is funded by ANR within the frames of the project “ANR Programme de Recherche Translationnelle en Santé”. Details on this contract have been

- given in the previous section (Post-doctoral research) but novel actions were taken as described in the project “Telomere length as a biomarker for personalised medicine” above (2014 - 2017).
4. “GEENAGE project (Functional Genomic, Epigenomic and ENvironment interplay to IMPACT management of healthy and pathological AGEing)” funded within the frames of the LUE project (Lorraine University of Excellence). The aim of this contract is to propose new strategies of diagnosis and management of healthy and pathological ageing by targeting the functional consequences of the interplay between genes, epigenome and environment. The IGE-PCV; U112 unit is involved in the scientific research axe 3 : “Regulation of telomere length and impact on the accelerated cardiovascular ageing and relevant co-morbidities”, which aims to study the genetic and environmental factors that are important in the regulation of telomeres early in life. The work on TL genetics described in the project “Telomere length as a biomarker for personalised medicine” above is my participation in this contract (2016 – 2026).
 5. “Development of a system for the management and analysis of biological samples and “-omics” data related to VEGF” project which is funded by Pôle BMS « Biologie, Médecine, Santé » within the frames of the project AAP-BMS interne “Projets incitatifs - Crédits CS-UL 2018”. For this project I am the Principal Investigator. This scientific project aims, through a network composed by 1 biological resource center (BRC IGE-PCV), 1 Research Unit (IGE-PCV; U1122) and 1 International Consortium (VEGF Consortium), the development of a system for management and analysis of biological samples and associated "-omics" data to elucidate factors that are linked with VEGF and which are involved in the development of cardiovascular pathologies. The project is linked with “Omics_BioBook” platform, which is a biostatistical/bioinformatics platform where associated data are stored and managed. Management software for samples and data are under development in order to facilitate and amplify the interconnections and exchanges between network partners (2018-2019).

Supervision of students and participation in evaluation committees

Since my nomination as a research engineer in Inserm in 2015, I have co-supervised 3 doctoral students having an official authorization (ACT) from the doctoral school BioSE. These were:

1. Ting Xie: « Interactions épistatiques pour le développement des algorithmes de prédiction des maladies cardiovasculaires: De l'approche gène-candidat au séquençage de prochaine génération ». The defense took place on December 2017 and I have participated in the evaluation committee (*doctoral student 3*).

The aim of this thesis was the use of novel methods for the study of epistatic interactions and epigenetic modifications of key molecules like the VEGF, the ApoE and the LSR on intermediate phenotypes of CVD and the risk of chronic diseases like AD, depression and osteoporosis.

The publications related to this thesis are:

1. Xie T*, Akbar S*, Stathopoulou MG*, Oster T, Masson C, Yen FT, Visvikis-Siest S. Epistatic interaction of apolipoprotein E and lipolysis-stimulated lipoprotein receptor genetic variants is associated with Alzheimer's disease. *Neurobiol Aging*. 2018 May 3. pii: S0197-4580(18)30147-7. * equal first authors
2. Xie T*, Stathopoulou MG*, Akbar S, Oster T, Siest G, Yen FT, Visvikis-Siest S. Effect of LSR polymorphism on blood lipid levels and age-specific epistatic interaction with the APOE common polymorphism. *Clin Genet*. 2018 Apr;93(4):846-852. * equal first authors
3. Stathopoulou MG, Xie T, Ruggiero D, Chatelin J, Rancier M, Weryha G, Kurth MJ, Aldasoro Arguinano AA, Gorenjak V, Petrelis AM, Dagher G, Dedoussis G, Deloukas P, Lamont J, Marc J, Simmaco M, Van Schaik RHN, Innocenti F, Merlin J-L, Schneider J, Alizadeh BZ, Ciullo M, Seshadri S, Visvikis-Siest S - The VEGF Consortium. A transnational collaborative network dedicated to the study and applications of the Vascular Endothelial Growth Factor–A in medical practice: The VEGF Consortium. *Clin Chem Lab Med*. 2018 Mar 28;56(4):83-86.
4. Xie T*, Stathopoulou MG*, de Andrés F, Siest G, Murray H, Martin M, Cobaleda J, Delgado A, Lamont J, Lledó E P, LLerena A, Visvikis-Siest S. VEGF-related polymorphisms identified by GWAS and risk for major depression. *Transl Psychiatry*. 2017 Mar 7;7(3):e1055. * equal first authors
5. Chatelin J*, Stathopoulou MG*, Aldasoro Arguinano AA*, Xie T*, Visvikis-Siest S*. Pharmacogenomic challenges in cardiovascular diseases: examples of drugs and considerations for future integration in clinical practice. *Curr Pharm Biotechnol*. 2017;18(3):231-241. * equal first authors

2. Jérôme Chatelin: « Interactions gène x gène, gène x environnement et Médecine Prédictive, Préventive, Personnalisée et Participative (4P) dans l'inflammation ». The defense will take place at the beginning of 2019 and I will participate in the evaluation committee (*doctoral student 2*).

This thesis is aiming to study the effect of genetic variants related to VEGF and its expression isoforms with the risk factors and the risk of developing chronic diseases including CVD, osteoporosis, and thyroid diseases in order to demonstrate their clinical utility in personalised medicine.

The so far accepted publications related with this thesis are:

1. Stathopoulou MG, Xie T, Ruggiero D, Chatelin J, Rancier M, Weryha G, Kurth MJ, Aldasoro Arguinano AA, Gorenjak V, Petrelis AM, Dagher G, Dedoussis G, Deloukas P, Lamont J, Marc J, Simmaco M, Van Schaik RHN, Innocenti F, Merlin J-L, Schneider J, Alizadeh BZ, Ciullo M, Seshadri S, Visvikis-Siest S - The VEGF Consortium. A transnational collaborative network dedicated to the study and applications of the Vascular Endothelial Growth Factor–A in medical practice: The VEGF Consortium. *Clin Chem Lab Med*. 2018 Mar 28;56(4):83-86.
2. Chatelin J*, Stathopoulou MG*, Aldasoro Arguinano AA*, Xie T*, Visvikis-Siest S*. Pharmacogenomic challenges in cardiovascular diseases: examples of drugs and considerations for future integration in clinical practice. *Curr Pharm Biotechnol*. 2017;18(3):231-241. * equal first authors
3. Rancier M*, Zaaber I*, Stathopoulou MG, Chatelin J, Saleh A, Marmouch H, El Shamieh S, Masson C, Murray H, Lamont J, Fitzgerald P, Mahjoub S, Said K, Tensaout BB, Mestiri S, Visvikis-Siest S. Pro- and anti-angiogenic VEGF mRNAs in autoimmune thyroid diseases. *Autoimmunity*. 2016 Aug 5:1-7. * equal first authors

3. Vesna Gorenjak: « Outils biostatistiques /bioinformatiques pour une recherche de médecine personnalisée : télomères, facteur de croissance de l'endothélium vasculaire (VEGF) et molécules associées ». The defense is planned for the end of 2019 I will participate in the evaluation committee (*doctoral student 1*).

This thesis aims to study the telomere length and the VEGF as biomarkers for personalised medicine. Modern approaches will be used like mendelian randomisation and the study of epigenetic modifications and will be combined with classical approaches of GWAS and

candidate genes in order to clarify the determinants of telomere length, VEGF and associated molecules and their association with intermediate phenotypes of CVD.

The so far accepted publications related with this thesis are:

1. Gorenjak V*, Aldasoro Arguinano AA*, Dadé S, Stathopoulou MG, Vance DR, Masson C, Visvikis-Siest S. The polymorphism rs6918289 located in the downstream region of the TREM2 gene is associated with TNF- α levels and IMT-F. *Sci Rep.* 2018 May 8;8(1):7160. * equal first authors
2. Stathopoulou MG, Xie T, Ruggiero D, Chatelin J, Rancier M, Weryha G, Kurth MJ, Aldasoro Arguinano AA, Gorenjak V, Petrelis AM, Dagher G, Dedoussis G, Deloukas P, Lamont J, Marc J, Simmaco M, Van Schaik RHN, Innocenti F, Merlin J-L, Schneider J, Alizadeh BZ, Ciullo M, Seshadri S, Visvikis-Siest S - The VEGF Consortium. A transnational collaborative network dedicated to the study and applications of the Vascular Endothelial Growth Factor–A in medical practice: The VEGF Consortium. *Clin Chem Lab Med.* 2018 Mar 28;56(4):83-86.
3. Visvikis-Siest S, Gorenjak V, Stathopoulou MG. Personalised Medicine: The Odyssey from Hope to Practice. *J Pers Med.* 2018 Sep 21;8(4).
4. Gorenjak V, Akbar S, Stathopoulou MG, Visvikis-Siest S. The future of telomere length in personalized medicine. *Front Biosci (Landmark Ed).* 2018 Mar 1;23:1628-1654.

Furthermore, I was the professional tutor of the trainee of License Professionel of Chloé Hattrisse : « Certification d'un Centre de Ressources Biologiques » (*trainee 2*).

The aim of this internship was the support of the procedures for the development and the quality certification of the Quality Management System of the BRC IGE-PCV.

Also, during the project « Mastiha treatment for obese with NAFLD diagnosis - MAST4HEALTH », within the framework of the HORIZON 2020 Marie Skłodowska-Curie actions - Research and Innovation Staff Exchange (MSCA RISE), I was the responsible for the training of 4 secondees in the fields of genetic epidemiology and related statistical analyses: Dwaine Vance, Randox Laboratories, 6 months, Lauren Doherty, Randox Laboratories, 3months, Eirini Chroni, Chios Mastiha Growers Association, 5 months and Georgia Maurikou, Chios Mastiha Growers Association, 5 months (*trainnees 1*).

Organisation of conferences

I have participated in the organisation of the following conferences as member of the organising committee:

- The 9th Santorini Conference, Santorini, Greece, 2018.
- 8th Santorini Conference, Santorini, Greece, 2016.
- Third ESPT Conference: Integration of pharmacogenomics in clinical decision support, Budapest, Hungary, 2015.

My tasks included the communication of the conferences, the organisation of poster sessions, on site organisation and the drafting of the post-conference scientific manuscripts. In the 8th Santorini Conference I was also member of the Poster Committee that was responsible for the evaluation of posters and selection of award winners. I was President of this committee in the 9th Santorini Conference.

Other activities linked to research

1. BRC IGE-PCV

I am member of the Steering Committee of the BRC IGE-PCV (BB-0033-00051) located in the IGE-PCV; U1122 unit. I am Manager and Coordinator of Collaborations and Responsible for the transfert of data and biological material. I ensure all the processes concerning the inputs and outputs, the transfer and the quality of the samples and stored data. I coordinate the release and the entry of data and the development and management of the related databases. I am also Quality advisor and I participate in the implementation of the quality approach for the quality certification according to the standard NF S 96-900. I participate in the development of the Quality Management System, in the meetings and the audits related to the quality certification.

I am also member of the emergency standby personnel for the security of biological material stored in the freezers of the BRC IGE-PCV. I have the responsibility of a mobile phone that informs of the slightest problem with the temperature of the freezers and I must intervene on the spot in case of need on a plan of 24h / 24h and 7 days / 7 days.

2. Administrator of the “Omics_BioBook” platform

As mentioned before, this platform is a Biostatistical / Bioinformatics platform, developed in collaboration with the Inserm Grand Est, the University of Lorraine and the infrastructure BIOBANQUES. The platform is installed in the Interuniversity Center of Informatics Resources of Lorraine - C.I.R.I.L (Centre interuniversitaire de ressources informatiques de Lorraine). It is a platform of high-performance with high storage capacity (60 TB for storage and 80 To in the automatic replication server) that is designed so as to meet the methodological and technological advances of “-omics” data storage and analysis, to ensure the security of the data of the BRC IGE-PCV, and to ensure the speed and performance of the large-scale analyses of the international consortia in which the IGE-PCV; U1122 unit participates. I have participated very actively in its development and I am the platform administrator concerning the management of user sessions. I participate in the development of a customized version of the software TumoroteK® which is used for the management of biological samples in the "Omics_BioBook" platform. An interface is also being developed with the aim of linking clinical data, biological data, “-omics” data and sample data for BRC IGE-PCV populations. This interface will facilitate the search for populations according to project-specific selection criteria and the creation of the databases that will be used for statistical analysis. An SFTP server has also been installed to ensure the safe transfer of big data between the unit and our collaborators and consortia.

Administrative activities

I am a member of the laboratory council of the unit IGE-PCV; U1122. I participate in the annual meetings that have as aim to:

1. Give an opinion on the main scientific policy decisions of the laboratory, changes in its structure, human resources management, research contract policy, technology transfer policy, training program, budget and finances
2. To make reflections on the main strategic directions of the unit on the medium and long term.

Projet de Recherche: L'étude des déterminants « -omiques » du VEGF dans la médecine personnalisée des maladies chroniques

Scientific Project: The study of « -omics » determinants of VEGF in the personalised medicine of chronic diseases

Introduction

My scientific interest and my research experience on the field of VEGF as a biomarker useful in personalised medicine has led to the development of this research project that will be developed during the following years within the frames of the IGE-PCV ; U1122 unit and the VEGF Consortium.

According to the World Health Organization (WHO), non-communicable diseases are responsible for >71% of all deaths worldwide. Among them, CVD (like heart attacks and stroke), cancers, chronic respiratory diseases (such as chronic obstructive pulmonary disease and asthma) and diabetes account for over 80% of all premature non-communicable diseases deaths ¹⁷⁸. CVD, including all the diseases of the heart and circulation, represent the primary cause of death worldwide ¹⁷⁹. CVD have high prevalence and economic impact: overall cost of CVD to the EU economy is €210 billion a year ¹⁸⁰. They require tailored management strategies, thus personalised early risk prediction is an urgent need for European citizens and healthcare systems.

Angiogenesis is a complex process promoting the development and growth of new vascular structures in contact with pre-existing vasculature. As mentioned before, it plays a significant role in physiological conditions but it is also involved in a wide-range of pathological conditions including vascular diseases and cancer. Induction of angiogenesis is regulated by a variety of molecules such as the VEGF family, TNF- α , interleukins, chemokines, and growth factors ¹⁸¹. However, VEGF is the most potent factor associated with angiogenesis and is considered a key regulatory molecule for both physiological and pathological angiogenesis ¹⁹. Atherosclerosis is a chronic inflammatory condition characterized by lipid-containing inflammatory lesions of large and medium-sized arteries. These lesions cause a focal thickening of the intima resulting in local hypoxia, which is enhanced by the inflammatory cell content of the lesions and the produced inflammatory factors ¹⁸². Hypoxia activates a metabolic pathway mediated mostly by the transcriptional factor HIF-1 alpha (hypoxia-inducible factor 1-alpha), which up-regulates the expression of various genes including

nitrous-oxide synthetase (*NOS*) and *VEGF*. The effect of *VEGF* actions in the vasculature can have a positive and a negative impact. The production of *NO* and prostacyclin (*PGI₂*) from *VEGF* activation has an important role in maintenance of endothelium integrity, in the lowering of vascular tonus and in the inhibition of platelet aggregation and thrombosis. It also induces collateral angiogenesis and thus restores blood circulation in infarcted areas ²⁷. However, the neovessels formed in the atherosclerotic plaques express adhesion molecules such as *VCAM-1*, *ICAM-1* and *E-selectin*, which allow the leukocyte infiltration in atherosclerotic plaque (figure 6). The recruited leukocytes produce inflammation molecules and various metalloproteinases (*MMPs*), which contribute to the instability of the plaque. Furthermore, *VEGF* cell activation leads to increased production of inflammation factors such as *TNF α* and *IL1b*. The increased vascular permeability and the *VEGF*-induced attraction result in increased macrophages infiltration. These inflammation mechanisms further enhance the *VEGF* expression and persistence of angiogenic stimuli, resulting in pathological angiogenesis. Moreover, the vascular permeability causes the extravasation of erythrocytes, which in combination with the fact that newly developed vessels are dysmorphic and immature, results in intraplaque hemorrhage ^{22, 23, 27}. These complex procedures finally result in plaque instability which is the major cause of most severe clinical events, as plaque rupture can occur and cause sudden thrombosis of the artery ¹⁸².

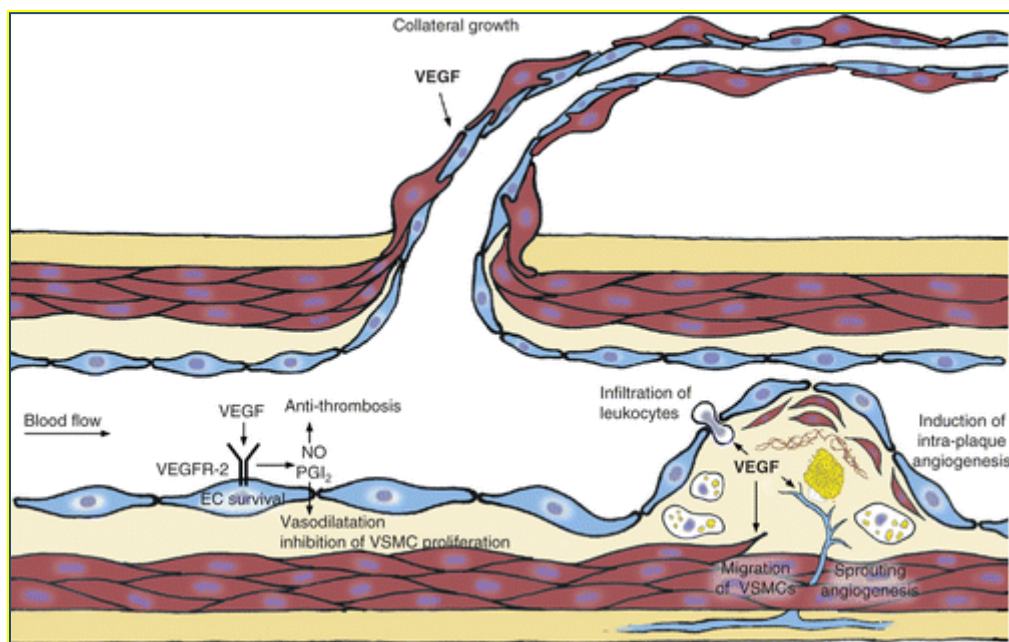


Figure 6: Role of VEGFs in the normal vessel wall and atherosclerotic lesions.

Therefore, angiogenesis, through VEGF and inflammation, plays a powerful role in atherosclerosis development and adverse clinical events. Due to this dual role of VEGF in cardiovascular system, pro-angiogenic therapeutic approaches based on VEGF have been tested as an alternative treatment for ischaemic diseases. More than 25 clinical trials have been conducted using recombinant VEGF protein or gene therapy but have shown low clinical effects, probably due to the transient nature of the treatments. Protein therapy was associated with an elevated risk of side-effects such as hypotension, while gene therapy was better tolerated¹⁸³. Moreover, as mentioned before, the anti-VEGF factors used in cancer lead to a range of cardiovascular side-effects including hypertension, cardiac ischemia, arterial thromboembolic events (e.g. cerebral and myocardial infarction) and left ventricular dysfunction²⁷. Furthermore, it seems that the relationship between VEGF and CVD is complicated also due to pleiotropic effects of VEGF. Apart from its angiogenic functions, VEGF exerts a variety of actions in other organs, through its complicated signaling pathways in ECs and other cells, which explains its implication in a wide range of conditions such as inflammation, vascular diseases, hypertension, cardiac, liver and kidney function¹⁸⁴. Angiogenesis and VEGF are considered as a common pathway linking different non-communicable diseases that also represent a huge burden for public health.

Neurodegenerative diseases such as AD and osteoporosis are common non-communicable diseases with a high burden for the society and healthcare systems. Neurodegenerative diseases are major challenge for public health, are mostly incurable and have a high prevalence and cost in Europe: dementias alone are estimated to affect 7 million people and their cost is €130 billion a year¹⁸⁵. Osteoporosis is also a disease with great prevalence and importance in public health. In Europe 200 million women worldwide are affected. Nevertheless, it is a disease poorly identified and treated (80% of individuals at risk are not identified or treated)¹⁸⁶.

VEGF seems to play a complex role in the central nervous system, and in dementia and AD development. VEGF is produced and secreted by astrocytes and through its receptors it activates pathways of cell survival, angiogenesis, and vascular cell permeability in the central nervous system¹⁸⁷. Increased VEGF production after an ischemic stroke leads to cerebral angiogenesis and increased BBB permeability¹⁸⁸. In brain tissue of AD patients, reduced *VEGF* gene expression has been detected, an indication of pathological angiogenesis^{189, 190}. Increased serum VEGF levels in AD patients with cerebral microbleeds¹⁹¹, while lower VEGF levels have been observed in AD patients in another study¹⁹². Recently, VEGF levels in cerebrospinal fluid of AD patients show no differences compared to healthy controls¹⁹³.

However, other studies demonstrate increased intrathecal levels of VEGF in AD patients¹⁹⁴, and higher VEGF expression in microvessels isolated from AD patients brain tissues compared to controls¹⁹⁵, thus indicating a complex role of VEGF in AD pathophysiology. Genetic studies have also given controversial results concerning the role of *VEGF* polymorphisms and risk for AD¹⁹⁶⁻¹⁹⁸.

Concerning the link between VEGF and osteoporosis, this is also well documented. Vasculature is important for osteogenesis¹⁹⁹, the development of bones and for bone remodeling and fracture healing, as it is important for the transfer of precursors of osteoclasts and osteoblasts, nutrients (calcium, phosphorus, protein), vitamin D, growth and differentiation factors^{200, 201}. VEGF receptors on ECs are involved with the activation of expression of cell-adhesion molecules, release of cytokines and chemokines resulting in degradation of the vessel's basement membrane, allowing cells to migrate to the extracellular matrix²⁰². VEGF receptors are present on primary osteoblasts²⁰³ and VEGF promotes bone formation, acting directly on survival, chemotactic migration²⁰⁴ and the activity²⁰⁵ of osteoblasts. Mice that do not express VEGF in bone progenitor cells present an osteoporosis phenotype with decreased trabecular bone, thickening of bone cortices and increased adipocytes associated with trabecular bone⁶⁹. All of these observations provide evidence of the major role VEGF-A plays in bone development and remodeling.

The IGE-PCV; U1122 unit's main objective is to determine the respective roles of genetic and environmental factors (and their interactions) involved in the development of and predisposition to CVD. In order to identify early, specific biomarkers, the unit has set up a scientific strategy based on an intermediate phenotype-genotype approach and used family, cross-sectional and longitudinal study designs. This methodological strategy is unique in integrating genomics, transcriptomics and proteomics (systems biology), thus facilitating the identification of functional polymorphisms with significant use as predictive and/or preventive markers. VEGF is one of the major axes of the research work of the unit. The majority of the work completed so far on VEGF by the unit, including the studies developed in the section above where I have participated, are summarized in the figure 7.

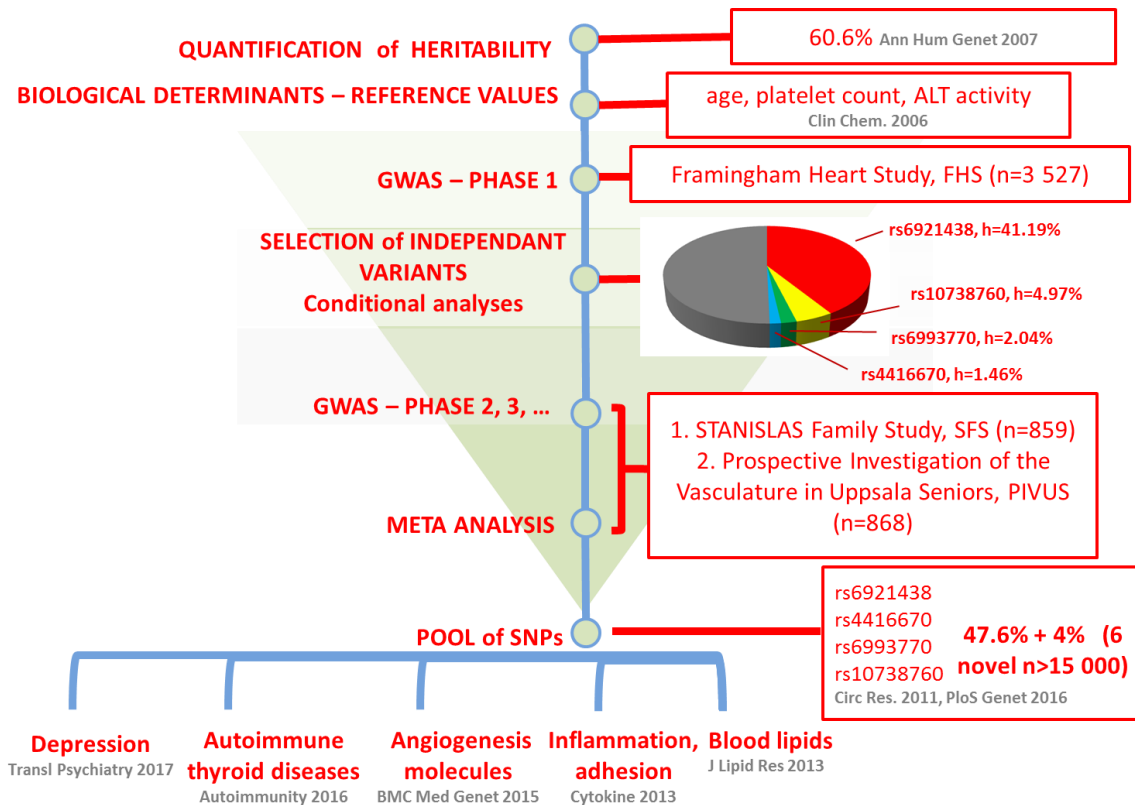


Figure 7. Realisations of the IGE-PCV; U1122 unit with my contribution on VEGF.

They have originally identified the high heritable component of VEGF in SFS, which is more than 60%⁴⁹. This finding has been also verified by two partners of the VEGF Consortium^{206, 207}. This is an extreme genetic component and this makes VEGF unique among other biomarkers of chronic diseases. Up to half of this heritability is explained by 4 very common variations (frequency between 35-50%) identified by a GWAS, where the unit participated with SFS population⁵², while 6 more variants have been identified in a more recent VEGF GWAS⁸⁹. This is also an exception in the field of genetic studies of complex diseases, as for most markers, the variability explained by identified variants does not reach levels higher than 5-10%. It is therefore a biomarker ideal for population stratification. Significant associations have been identified between these polymorphisms and risk factors of chronic diseases (cholesterol levels, blood pressure and inflammation molecules)^{53, 54}, with angiogenesis molecules⁸⁶, with risk for depression⁹⁰ and with thyroid hormones levels in patients with autoimmune thyroid diseases^{87, 88}. Furthermore, as discussed before, VEGF is implicated in the pathophysiology of CVD and other chronic diseases, while the existence a large number of transcription isoforms (and related protein products) from the *VEGF* gene can allow the

study of VEGF specificity for CVD and other diseases. These arguments demonstrate that VEGF represents an ideal biomarker for the application of “-omics” methodologies in personalised medicine of chronic diseases. Therefore, the in-depth investigation of VEGF and its clinical use in chronic diseases risk prediction can have an important impact in public health.

In line with this and using my past experience and expertise on VEGF and public health research field, the purpose of the proposed project is the development of VEGF-based CVD risk prediction model based on the validity and utility of its molecular determinants as CVD biomarkers. The most recent “-omics” methodologies will be applied for the identification of molecular determinants of VEGF that explain the largest part of its inter-individual variability.

Objectives

The general aim of the project is to develop risk prediction algorithms for the stratified risk prediction of CVD and other chronic diseases based on VEGF “-omics” determinants. Big data from “-omics” determinants (genetics, transcriptomics and epigenetic) of VEGF and VEGF-related molecules will be combined with environmental factors and epistatic interactions with other molecules involved in pathophysiological processes common to the assessed diseases (angiogenesis, inflammation, lipid metabolism). The project’s specific objectives are:

1. The identification of VEGF “-omics” determinants and their interactions with environmental/lifestyle factors in health through robust and valid multi-disciplinary “-omics” assays (including genetics, epigenetics, and transcriptomics) in large familial, longitudinal populations of healthy individuals of European ancestry.
2. The identification of VEGF “-omics” determinants that affect CVD, AD and osteoporosis risk factors, as well as their interactions with environmental/lifestyle factors in healthy populations.
3. The identification of VEGF “-omics” determinants that affect CVD, AD and osteoporosis risk in case-controls and prospective studies. This study will allow to test the specificity of different “-omics” determinants on different diseases.
4. The development of VEGF “-omics”-based computational risk prediction models and algorithms. Their predictive value will be assessed in prospective studies aiming to identify

at-risk groups among individuals throughout their lives. Their utility and stratification efficacy will be compared to conventional risk prediction algorithms, taking into account both age and sex.

The project will use the resources available in the IGE-PCV; U1122 unit and the VEGF Consortium that is coordinated by the unit. Resources in population include recognised cohorts (SFS, Framingham Heart Study (FHS); Cilento study, LLs, ORIGIN), case-controls populations, large populations from biobanks (BRC IGE-PCV, BBMRI-ERIC a pan European infrastructure that regroups more than 250 biobanks and UK Biobank) and collaborations with other consortia such as CHARGE, METASTROKE and MEGASTROKE for which the desired “-omics” data are available. The “Omics_BioBook” platform will be used for data storage and analyses.

Implementation of the project

The project will be based on 4 axes, one for each specific objective.

Axe 1: Identification of VEGF molecular determinants in health

The main objective of axe 1 is to identify the genetic, transcriptomic and epigenetic determinants of VEGF biology in health with the use of robust “-omics” assays validated in large familial, longitudinal cohorts of healthy individuals.

Genetic determinants of VEGF levels

The results of the recent GWAS study on VEGF levels will be expanded with the addition of new populations that are available through CHARGE consortium and the ORIGIN Study and new GWAS meta-analyses. The aim is to gather more than 20000 individuals thus increasing the power of the study and the potential for identification of novel SNPs that will increase the percentage of variability of VEGF levels explained. A list of GWAS-level significant independent SNPs will be developed, while SNPs reaching a level of significance of 0.05 by false discovery rate correction (less strict criterion compared to classical Bonferroni correction applied in GWAS) can also be added in the list of candidate genetic determinants of VEGF. Novel discoveries will also be included by gene \times environment interactions studies,

where environmental factors known to affect the risk of CVD, AD and osteoporosis will be selected: BMI, levels of blood lipids, inflammation molecules, blood pressure etc. Large scale replications will be performed in UK Biobank. A genetic score of all the independent markers will be developed.

Transcriptomic determinants of VEGF levels

RNA sequencing through TrueSeq (Illumina) and sequencing on HiSeq2500 instruments (75 bp paired end reads) has been performed for 220 individuals from 70 families of SFS. These data and other RNA sequencing and qPCR methods data available in the healthy populations participating in the VEGF Consortium will be combined so as to quantify the expression of pro- and anti- angiogenic isoforms of *VEGF* gene in PBMCs. Following their quantification, association analysis and meta-analyses will be performed in order to identify the isoforms that affect more the levels of VEGF.

Epigenetics determinants of VEGF levels

DNA methylation profiling with the Infinium Methylation450K beadchip (Illumina) that can interrogate 450,000 CpG sites and quantify DNA methylation levels is available for 220 individuals from SFS and the other healthy populations participating in the VEGF Consortium. EWAS meta-analyses will be performed to identify methylation probes that are significantly associated with VEGF levels.

Axe 2: Identification of VEGF molecular determinants associated with chronic diseases risk factors

The main objective of the axe 2 is to establish and quantify the effect of VEGF “-omics” determinants on common to the assessed chronic diseases risk factors and their interactions with environmental/lifestyle factors. These disease risk factors include blood lipids, blood pressure, glucose metabolism, obesity and inflammation traits such as systolic blood pressure, diastolic blood pressure, mean arterial blood pressure, CRP, haptoglobin, IL-6, TNF- α , fasting blood glucose, fasting insulin levels, BMI, waist circumference, waist/hip ratio, HDL, LDL, ApoE, triglycerides, lipid accumulation product index (LAP) and visceral adiposity index

(VAI). Ten-year risk of a first fatal atherosclerotic event, such as heart attack, stroke, aneurysm of the aorta, or other calculated by SCORE system will also be used as a phenotype of interest^{208, 209}. These phenotypes are available in SFS, LLs, Cilento, FHS but also in many studies of the BRC IGE-PCV, of CHARGE consortium and of UK Biobank, gathering a powerful sample size. Direct, gene × gene and gene × environment interactions will be tested for the identified “-omics” determinants of the axe 1. This axe will identify specific VEGF “-omics” determinants that affect specific pathways and diseases risk factors.

Axe 3: Identification of VEGF molecular determinants associated with chronic diseases risk

The main objective of the axe 3 is to identify the disease-specific VEGF “-omics” determinants that could be used for disease risk prediction. Case-controls populations with CVD (coronary artery disease and stroke), AD and osteoporosis will be used. These populations are available in the BRC IGE-PCV, The VEGF, CHARGE, METASTROKE and MEGASTROKE consortia, the BBMRI-ERIC and the UK biobank. In these populations, the identified from axe 1 and axe 2 VEGF “-omics” determinants will be tested for associations with increased risk for the assessed diseases. We expect to identify specific “-omics” determinants for each disease and lead to the selection of the strongest biomarkers to be added in the risk prediction models of the next axe.

Axe 4: Development and assessment of predictive value of personalised risk prediction algorithms based on VEGF “-omics” determinants

The main objective of the axe 4 is to develop and demonstrate the independent and additive predictive value of specific to CVD, AD and osteoporosis VEGF “-omics” determinants, identified in the previous axes, in computational prediction algorithms for the assessment of disease risk. The predictive value will be assessed in large prospective cohorts of European ancestry with the aim to identify at-risk groups among individuals throughout their lives. The incremental predictive utility and stratification efficacy of the new algorithms will be compared to conventional risk prediction algorithms such as the SCORE system²⁰⁸, the Framingham General Cardiovascular Risk Profile (FGCVDRP)²⁰⁹ for CVD and the FRAX WHO Fracture Risk Assessment Tool for osteoporosis²¹⁰.

The steps that will be followed for the development and the testing of the new personalised “-omics” algorithms are ²¹¹⁻²¹³:

1. Population and end-points: prospective cohorts like FHS, LLs, ORIGIN, and others available in CHARGE consortium and the BBMRI-ERIC infrastructure. End points will include severe events of CVD, fractures and AD diagnosis.
2. Predictors: Defined in axe 2 and 3.
3. Model estimation: Sex-specific Cox proportional hazards regression is most commonly used for time-to-event data, such as long-term mortality (following testing of proportionality of hazards). The estimation of the regression coefficients for Cox regression models will be performed with maximum likelihood methods. To limit overfitting of models to the available data, techniques such as statistical shrinkage techniques, penalised maximum likelihood estimation, and the least absolute shrinkage and selection operator will be used.
4. Assessment of the model performance and validity: internal validation (reproducibility) will be performed using methods of cross-validation. External validation (transportability between the two cohorts) will also be performed. Calibration of the models (agreement between observed outcomes and predicted) will be assessed with goodness of fit methods and predicted-to-observed ratios. Discrimination is commonly quantified with a concordance (c) statistic. For a binary endpoint, c is identical to the area under the receiver operating characteristic (ROC) curve, which plots the sensitivity (true-positive rate) against specificity (false-positive rate) for consecutive cut-offs for the predicted risk. For a time-to-event endpoint, such as survival, the calculation of c may be affected by the amount of incomplete follow-up (censoring). We note that the c statistic is insensitive to systematic errors in calibration, and considers the rather artificial situation of classification in a pair of patients with and without the endpoint. The clinical usefulness will be assessed by the net benefit. This measure is consistent with the use of an optimal decision threshold to classify patients. The relative weight of harms and benefits is used to define the threshold, and is used to calculate a weighted sum of true-minus false-positive classifications. For the comparison of additive value and the performance between the novel and the conventional algorithms, methods such as continuous net reclassification improvement (NRI) and integrated discrimination improvement (IDI) will be used.

Expected results

The major expected results are:

- VEGF “-omics” determinants to be used for molecular make-up of CVD, AD and osteoporosis.
- Novel VEGF “-omics”-based risk prediction models – algorithms for CVD, AD and osteoporosis.

These results will have an important impact in the knowledge of VEGF biology and its implication in the assessed diseases, but also, will demonstrate its utility as a biomarker for personalised medicine through early risk prediction.

The knowledge generated by the project will be made available to the scientific community and the public following several publications in high impact factor international peer-reviewed journals in the medical, genetics and epidemiology fields and with presentations in scientific conferences.

The VEGF consortium website will be used for the communication of the results within the consortium through a private area and with the general public through a public interface.

Summary of valorisation and transfer activities and scientific visibility

Total number of publications (peer-review journals): 66

- ✓ Number of original publications (peer-review journals): 44
- ✓ Number of scientific reviews (peer-review journals): 9
- ✓ Number of conference publications (peer-review journals): 13

Lectures-conferences-symposia: 34

From the 53 original publications and scientific reviews, 48 were found in the Web Of Science database on November 2018. The analyses of these 48 publications revealed the following:

Sum of the citations: 3528

Sum of citations excluding self-citations: 3482

Average citations per item: 73.5

h-index: 16

The figure 8 demonstrates the numbers of publications by year.

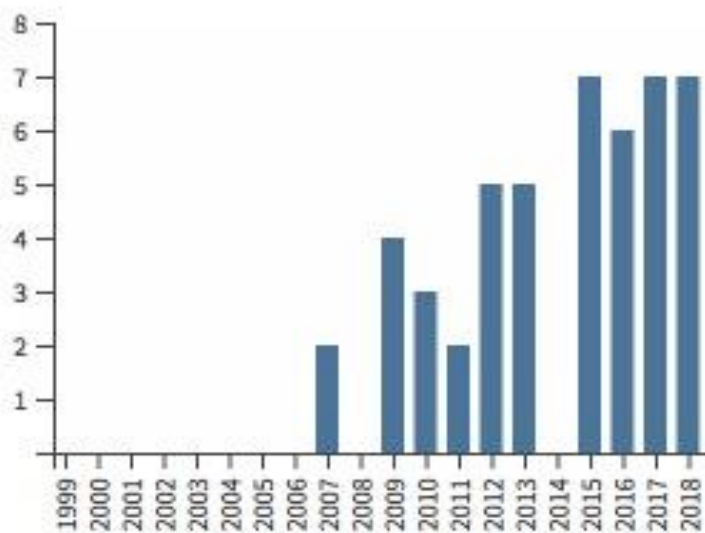


Figure 8. Total publications by year

The figure 9 demonstrates the citations by year.

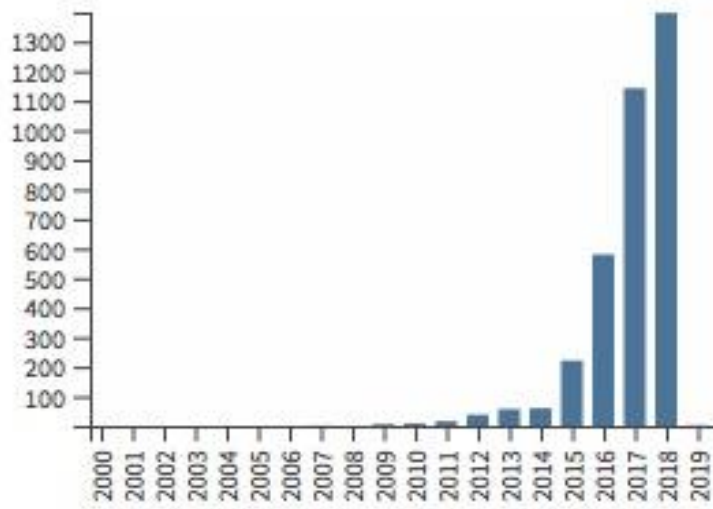


Figure 9. Number of citations by year

The figure 10 demonstrates the 25 first research areas of my publications

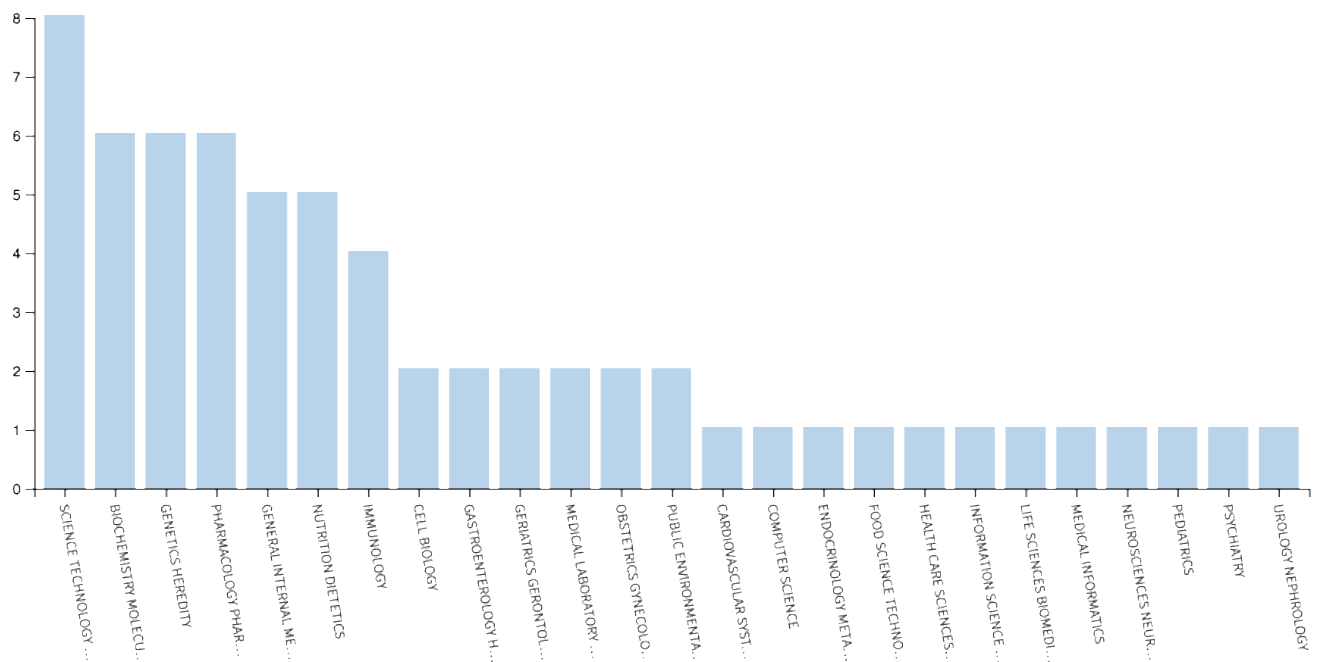


Figure 10. Research areas of publications

My scientific visibility is also strengthened by my participation in international consortia as participant but also as leader of projects and as project manager of the VEGF Consortium. My participation in national and european projects is also demonstrating my scientific visibility.

Five major publications related to the scientific project

Letter to the Editor

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A transnational collaborative network dedicated to the study and applications of the vascular endothelial growth factor-A in medical practice: the VEGF Consortium

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Keywords: collaborative network; multidisciplinary genomic studies; personalized medicine; VEGF-A.

To the Editor,

The Vascular Endothelial Growth Factor European Genomic Federation (VEGF) Consortium (www.vegfconsortium.org) was founded in June 2014 by Sophie Visvikis-Siest (chair of the consortium) and an international group of researchers with an interest focused on VEGF-A and its implications in personalized medicine.

Here, we present the VEGF Consortium and describe its objectives and ambitions, its structure and its components together with the methodologies used in projects and preliminary results.

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The VEGF Consortium aims to develop a transnational collaborative network dedicated to large integrative and multidisciplinary genomic studies of the VEGF-A in order to generate applicable knowledge for medical practice thanks to the following specific objectives:

- to combine data from multiple cohorts in order to identify VEGF-A ‘-omics’ profiling in health and non-communicable diseases
- to elucidate the pivotal role of VEGF-A in the pathophysiology of non-communicable diseases
- to demonstrate the patients’ stratification potential of VEGF-A ‘-omics’ profiling
- to implement the research results into clinical practice and establish the role of VEGF-A as a predictive, preventive, diagnostic and prognostic biomarker
- to provide information on the effect of VEGF-A ‘-omics’ profiling in side effects and response to therapy through pharmacogenomics studies

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- to propose implementation strategies and European guidelines involving VEGF-A ‘-omics’ profiling for the management of non-communicable diseases
- to share methodologies, data and knowledge in the field of ‘-omics’ management and innovative statistics
- to develop standardized teaching and evaluation methods practiced and validated by the consortium.

It comprises 11 working groups, where the partners of the consortium are participating based on their expertise:

1. VEGF-A ‘-omics’ profiling in health
2. VEGF-A ‘-omics’ profiling in diseases
3. ‘-Omics’ technologies
4. Methodological aspects
5. VEGF-A clinical implementation
6. Pharmagenomics
7. Endothelins and endothelial factors
8. VEGF-A basic research (cancer cell lines, animal models)
9. VEGF-A and inflammation
10. Communication and scientific/educational meetings
11. Raising awareness of populations

Prospective, longitudinal, family-based or population-based cohorts of healthy individuals are the core of the consortium. These are the following:

1. The STANISLAS family study: This is a longitudinal family structure cohort of community-based population of French origin recruited in 1993–1995, followed up for 15 years. A number of 1006 nuclear families comprising two parents and at least two biological children over 6 years old are included in the study [1].
2. The Framingham Heart Study: It is an ongoing, longitudinal, community-based, observational cohort study that was initiated in 1948 to prospectively investigate the risk factors for cardiovascular disease (CVD). The original cohort enrolled 5209 men and women, the offspring cohort enrolled 5124 participants (including 3514 biological offspring) and Gen 3 included 4095 individuals [2–4].
3. Cileto study: This is a population-based study that aims at identifying genetic risk factors for common diseases and traits. The sample includes isolated populations from three villages (Campora, Giot and Cardile). The overall sample size of individuals participating to the study is 2100 [5].
4. The LifeLines Cohort Study and Biobank (LLs): LLs is a multidisciplinary prospective population-based cohort study examining the health and health-related behaviors of 167,000 persons living in the north east

region of The Netherlands in a three-generation design [6].

Furthermore, cohorts of patients and case-control studies are also included in the consortium:

5. The Hellenic Study of Interactions between SNPs and Eating in Atherosclerosis Susceptibility: case-control study of coronary artery disease.
6. Ljubljana patients: case-control studies recruited from Ljubljana, Slovenia that include cases of osteoporosis, osteoarthritis, CVD and diabetes [7–9].

In addition, partners of the consortium have access to large biobanks such as the UK Biobank (Panagiotis Deloukas), Biobanking and Biomolecular Resources Research Infrastructure – European Research Infrastructure Consortium BBMRI-ERIC (including Sophie Visvits-Siest with the Biological Resources Center IGE-PCV – BB-0033-00051) and the Alliance for Clinical Trials in Oncology (Federico Innocenti).

Given the wide range of the research field and the need for application of different methodologies in order to successfully achieve its objectives, the consortium is composed of scientists with different and complementary expertise and with a large range of resources such as large study populations, research materials and harmonized data.

Although the VEGF Consortium was officially founded on 2014, it was based on the long-term collaboration between some of its founding partners. Therefore, a number of significant results have been published already, and many projects are ongoing, with promising preliminary results.

Among the most basic steps was the identification through two genome-wide association studies (GWAS) of 10 genetic variants that explain >50% of the circulating VEGF-A levels variability [10, 11]. This is a unique finding among GWAS. In most GWAS, the identified variants do not explain >10% of the individual variability of the assessed traits. This finding has strengthened our belief that VEGF-A will indeed be used as strong biomarker for personalized medicine. Significant associations between some of these polymorphisms and intermediate phenotypes of chronic diseases have been identified since then: high-density and low-density lipoprotein [12], L-selectin gene expression [13] and free tri-iodothyronine (FT3) levels [14]. Significant epistatic interactions between these variants were observed for intercellular adhesion molecule 1 (ICAM-1), E-selectin, interleukin 6 and tumor necrosis factor α (TNF- α) plasma levels [13]. Concerning specific disease risk, we have shown

that these polymorphisms and/or their epistatic interactions can affect the risk for depression [15], and for autoimmune thyroid diseases [14], whereas no associations were found for diabetes type 2 [16].

Concerning the expression isoforms of *VEGF-A* gene, we have shown that these are significantly associated with ICAM-1, L-selectin and TNF- α expression [13] and with specific autoimmune thyroid diseases [17].

Furthermore, we have also identified associations between VEGF-A circulating levels and thyroid hormones levels [17] and with ICAM-1 and E-selectin levels [13].

Through a candidate gene approach of polymorphisms in genes involved in angiogenesis, we have identified direct and epistatic effects of variants on nitric oxide synthase 3 (*NOS3*), *CD14*⁺ monocytes, matrix metalloproteinases (*MMPs*) and interleukin 4 receptor (*ILR4*) genes with levels of VEGF-A and VEGF-A expression isoforms, but also gene \times environment interactions [18].

An important result is also the production of two patents based on the results of studies performed by partners of the consortium [19, 20].

Several projects are ongoing and focused on CVD intermediate phenotypes, thyroid diseases, cancer and stroke.

Three face-to-face meetings have been organized to date: Paris (2014), Budapest (2015) and Santorini (2016).

The VEGF Consortium is an ambitious international collaboration that aims to pave the way for the implementation of VEGF-A in personalized medicine and routine clinical practice. The designed projects take advantage of the wide expertise of its partners, the large infrastructures of cohorts and biobanks and a combination of the most up-to-date “-omics” approaches for generating multidimensional data, as well as ‘systems medicine’ approaches, network analysis and computational modeling methodologies.

Among its major originalities is that it integrates commercialization and communication platforms, targeting the valid and easy measurement of the identified biomarkers in large-scale settings but also the education of the general population, patients, scientists and health practitioners.

The VEGF Consortium is a novel consortium with an innovating structure and original goals. The ultimate goal is to ameliorate life expectancy, quality of life for individuals and financial benefits for health systems.

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Honorarium: None declared.

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ORIGINAL ARTICLE

VEGF-related polymorphisms identified by GWAS and risk for major depression

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Depression is a common, severe, disabling mental disease that affects millions of people of all ages worldwide. Various studies have shown that neurotrophic/growth factors have a key role in depression and, more specifically, vascular endothelial growth factor (VEGF) is implicated in the pathogenesis of depression. The purpose of this study was to investigate the potential links between four VEGF-related single-nucleotide polymorphisms (SNPs), previously identified through a genome-wide association study (GWAS) and depression. The direct effects and epistatic interactions of the four VEGF-related SNPs (rs10738760, rs6921438, rs6993770 and rs4416670) on depression were investigated through a case-control study including 437 individuals diagnosed with depression and 477 healthy volunteers as controls. Gender, age and body mass index influence was additionally analyzed. The SNP rs4416670 was associated with increased risk for depression (OR: 1.60, *P*: 0.010). This result demonstrates the existence of relationships between VEGF genetic determinants and depression. This novel association reveals new molecular mechanisms suggesting the potential role of VEGF in depression development that could help to promote a personalized prediction for this severe common disease.

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INTRODUCTION

Depression is a common, severe, disabling mental disease that affects millions of people of all ages. Depression is also a leading cause of mortality worldwide, as it can lead to suicide and causes a high burden for patients, their families and the healthcare system. In addition population studies have shown that women are affected by depression more than men, but no study clearly explains the causes of this observation.¹

Various studies have shown that neurotrophic/growth factors are key factors in depression. One of the most important is brain-derived neurotrophic factor (BDNF) and several studies have focused also on vascular endothelial growth factor (VEGF), which is recognized as a major inducer of angiogenesis and vasculogenesis.² However, VEGF is a multifunctional molecule that has effects also in the central nervous system.³ VEGF has been shown to have effects on specific areas of the brain, such as hippocampus,⁴ and neurogenesis has been shown to be stimulated *in vitro* and *in vivo* by VEGF.⁵ Proinflammatory cytokines have been implicated in depression,^{6,7} while VEGF is regulated by proinflammatory cytokines in depression.⁸ Taking together the above information, it has been suggested that interaction between VEGF and proinflammatory cytokines could influence depression and/or the potential efficacy of the treatments with antidepressants.⁹

Numerous studies have investigated the peripheral/serum levels of VEGF in depressed patients and compared them with a control group of healthy subjects; however the results are unclear and ambiguous yet, due to differences in the populations studied. Increased VEGF levels have been observed in depressed patients

when compared to healthy controls,¹⁰ but no differences have been reported in other studies.^{11,12} In contrast to these results, one study revealed a decrease in plasma and serum VEGF levels in depressed patients.¹³ However, environment factors like gender, age and body mass index (BMI) have not been adequately assessed in the aforementioned association studies and they could potentially explain the controversy observed.¹⁴

Given the associations between VEGF and depression, a common genetic regulation could be hypothesized. Depression is a complex disease, and it has a heritable background.¹⁵ Few studies have focused on VEGF gene polymorphisms role in depression. Among them, Viikki *et al.*¹⁶ have shown that one VEGF polymorphism, rs699947, is associated with treatment resistant depression. Recently we have identified through a genome-wide association study (GWAS) four single-nucleotide polymorphisms (SNPs) in three chromosomes, which explain almost 50% of the inter-individual variability of VEGF circulating levels.¹⁷ Therefore, we hypothesize that these strong genetic determinants of VEGF could also be implicated in depression. Thus, the aim of this study was to investigate the direct effects and epistatic interactions of the 4 VEGF-related polymorphisms (rs10738760, rs6921438, rs6993770 and rs4416670) on depression by a case-control study, aiming to detect potential genetic biomarkers between VEGF and depression.

MATERIALS AND METHODS

Population

Four hundred thirty seven unrelated Spanish Caucasian patients diagnosed with depressive disorders (132 males/305 females, mean age: 51.49 ± 14.04

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years, mean BMI: 28.56 ± 6.56 kg/m²) who were attending a Mental Health Centre, were included. The descriptions of depressive disorders and any psychiatric condition, in which depression was a primary symptom and therefore were under antidepressant treatment, were taken from the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV criteria).²³ A group control of 477 (187 males/290 females, mean age: 66.24 ± 15.01 years, mean BMI: 29.33 ± 11.41 kg/m²) Caucasian healthy volunteers from the same geographical region was also studied. The individuals of the control group were selected by psychiatrists who performed a mental and physical revision, as well as revised medical history to check health state, in order to consider about inclusion as healthy individuals. First-degree relatives were also assessed by psychiatrists regarding potential psychiatric disorders. However, to avoid familial stratification issues, only unrelated individuals have been included in the control group. Healthy volunteers with a previous history of adverse drug effects and those with any drug intake in the 2 weeks before the study were excluded from participation in the study. Women who reported or suspected pregnancy were also excluded from the study.

Written informed consent was obtained for all the participants of the study, and the study was performed according to the Helsinki Declaration. This study was approved by the Ethical Committee of Extremadura University Hospital, Badajoz, Spain and of Navarra Health Care System, Pamplona, Spain.

Genotyping

Five milliliters of peripheral blood were drawn in a tube with EDTA, and then the genomic DNA was isolated with the QIAGEN Blood DNA isolation kit (Qiagen, Hilden, Germany) and evaluated for integrity and concentration through 1% agarose electrophoresis and spectrophotometry. The SNPs rs6921438, rs4416670, rs6993770 and rs10738760 were genotyped using the competitive allele-specific PCR (KASP) chemistry coupled with a FRET-based genotyping system (<http://www.lgcgroup.com/services/genotyping/#.VmQwfr6GFI>), and genotyping was confirmed by Randox Laboratories (Crumlin, UK; Evidence Investigator) using an assay based on a combination of multiplex PCR and biochip array hybridization.

Statistical analysis

Hardy-Weinberg equilibrium was tested using a χ^2 -test. The analysis was carried out using the Generalized Multifactor Dimensionality Reduction (GMDR09) software (University of Alabama, Birmingham, AL, USA), which is a nonparametric and genetic model-free²⁹ alternative to linear or logPage Page 6/istic regression for detecting and characterizing nonlinear interactions among discrete genetic and environmental attributes. Age, gender and BMI were used as co-variables. The score of co-variables was calculated by logistic regression, and then the analysis was performed using the GMDR. Epistatic interactions were assessed only between SNPs with significant direct effects and all the others. The SNPs analyzed are located in different chromosomes; therefore haplotype analysis was not performed as no linkage between SNPs occurs. Direct associations were tested at two-sided $P < 0.012$ (corrected for 4 tests) and epistatic interactions were tested at two-sided $P < 0.05/\text{number of final interaction tests}$.

RESULTS

The characteristics of the SNPs are presented in Table 1. Hardy-Weinberg equilibrium was not observed in the SNP rs4416670 in the group of cases due to significant disagreement found between expected and observed frequencies, thus indicating a difference in the frequency of this SNP in depression patients.

Associations of the SNPs in cases-controls

Among the four SNPs studied, only the rs4416670 was significantly associated with depression. As shown in Tables 2A and 2B, this SNP was associated with an increased risk for depression (OR: 1.60; Table 2A), while the minor allele C was more frequent in patients (Table 2B). No association with depression was found for the other three SNPs.

Epistatic interactions between the 4 SNPs and depression

As only one SNP (rs4416670) was directly associated with depression, we have assessed only the epistatic interactions

Table 1. SNPs characteristics in cases and controls.

Chr	SNP	Minor allele	MAF patients	HWE patients	MAF control	HWE control
6	rs4416670	C	0.469	0.035	0.468	0.579
6	rs6921438	A	0.408	0.496	0.454	0.606
8	rs6993770	T	0.356	0.412	0.340	0.663
9	rs10738760	A	0.463	0.076	0.470	0.769

Abbreviations: Chr, chromosome; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; SNP, single-nucleotide polymorphism.

Table 2A. Associations between VEGF-related with depression

Marker	Allele Major/minor	OR (95% CI)	P	Chi-square
rs4416670	T/C	1.60 (1.03-2.46)	0.010	4.38 (P=0.036)

Abbreviations: CI, confidence interval; OR, odds ratio; VEGF, vascular endothelial growth factor.

Table 2B. Alleles investigation for the rs4416670 in patients versus controls

Marker/allele	Combination score ^a		Class level ^b
	Patient (n = 437)	Control (n = 477)	
rs4416670			
CC	56.39	47.00	1
TC	119.36	109.75	1
TT	41.93	58.79	0

^aProbability of being affected. ^b0—control; 1—patient.

involving this SNP. The significance cut off value was set at $P < 0.05/3$ (3 interaction tests) = 0.016.

No significant interaction was identified.

DISCUSSION

In this study, we identified links between VEGF-related genetic polymorphisms and depression. More precisely, one SNP (rs4416670) related to VEGF levels¹⁷ was associated with increased risk for depression. The minor allele of this polymorphism has been previously associated with decreased VEGF levels.¹⁷ As this polymorphism has a very high frequency (almost 50%), the effect of the minor allele can affect a large percentage of the population, thus having an important impact for public health.

Several studies revealed that proinflammatory cytokines are implicated in the etiology of depression.^{6,20} Miller *et al.*²⁰ have underlined the relationship between proinflammatory cytokines and depression in individuals with disorders that include chronic inflammation. We have previously shown that in supposed healthy individuals the C allele of rs4416670 was positively associated with L-selectin mRNA, and that VEGF₁₄₅ mRNA positively regulated L-selectin expression.²¹ At the same time, L-selectin has been shown to correlate with mental diseases such as the pathogenesis of schizophrenia²² and the severity of panic disorder,²³ thus indicating that this molecule can play a significant role in these conditions. Despite these associations have not been demonstrated yet in patients with depression, based on our results, we could propose the hypothesis that L-selectin could be implicated in depression development, and this effect could be additionally dependent on the genotype of rs4416670. The presence of the C

allele of this SNP is increasing expression of α -selectin, which could indicate an increased risk for depression. This hypothesis should be tested in future studies.

Concerning the role of VEGF, the C allele of the rs4416670 (currently linked with increased risk of depression) was associated with decreased levels of VEGF.¹⁷ Therefore, our present findings are consistent with a previous study showing a negative correlation between serum VEGF concentration and scores on a widely used diagnostic measure of depression, the Hamilton Depression Rating Scale.¹² Furthermore, a significantly decrease in plasma VEGF has been shown in individuals exposed to a stressful military training and environment.²⁴ Thus, it is possible that the present findings could be also explained in the frame of the diathesis-stress model of depression,^{25,26} making individuals carrying this VEGF SNP more likely to develop depression due to a significant downregulation of VEGF blood levels during or after exposure to stress, and/or to a greater vulnerability to stress. Another previous study also showed that the subset of patients who responded to antidepressant treatment were those with decreased plasma VEGF levels.²⁷ Therefore, future studies will have to test whether these SNPs show less tolerance to environmental stress, and/or decreases in plasma VEGF levels and whether they are overrepresented among depressive patients who respond to antidepressant treatment contrary to those who do not respond.

In conclusion, to our knowledge, this is the first study that has assessed the common genetic regulation between VEGF and depression with important original results that reveal novel molecular mechanisms for this common, severe and disabling mental disease. The identification of SNPs that may predict susceptibility to depression and/or response to treatment can have important implication in clinical practice. Further research on these mechanisms involved in depression development and response to treatment, as well as the functional role of these SNPs could allow the implementation of these findings in personalized risk prediction and hopefully targeted treatment of depression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.


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A common variant highly associated with plasma VEGFA levels also contributes to the variation of both LDL-C and HDL-C[§]

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Abstract Vascular endothelial growth factor A (VEGFA) is among the most-significant stimulators of angiogenesis. Its effect on cardiovascular diseases and on the variation of related risk factors such as lipid parameters is considered important, although as yet unclear. Recently, we identified four common variants (rs6921438, rs4416670, rs6993770, and rs10738760) that explain up to 50% of the heritability of plasma VEGFA levels. In the present study, we aimed at assessing the contribution of these variants to the variation of blood lipid levels (including apoE, triglycerides, total cholesterol, low- and high-density lipoprotein cholesterol levels (LDL-C and HDL-C)) in healthy subjects. The effect of these single-nucleotide polymorphisms (SNPs) on lipid levels was assessed using linear regression in discovery and replication samples ($n = 1,006$ and $n = 1,145$; respectively), followed by a meta-analysis. Their gene \times gene and gene \times environment interactions were also assessed. SNP rs6921438 was associated with HDL-C ($\beta = -0.08$ mmol/L, $P_{\text{overall}} = 1.2 \times 10^{-7}$) and LDL-C ($\beta = 0.13$ mmol/L, $P_{\text{overall}} = 1.5 \times 10^{-4}$). We also identified a significant association between the interaction rs4416670 \times hypertension and apoE variation ($P_{\text{overall}} = 1.7 \times 10^{-5}$). Therefore, our present study shows a common genetic regulation between VEGFA and cholesterol homeostasis molecules. The SNP rs6921438 is in linkage disequilibrium with variants located in an enhancer- and promoter-associated histone mark region and could have a regulatory effect in the expression of surrounding genes, including VEGFA.—Stathopoulou, M. G., A. Bonnefond, N. C. Ndiaye, M. Azimi-Nezhad, S. El Shamieh, A. Saleh, M. Rancier, G. Siest, J. Lamont, P. Fitzgerald, and S. Visvikis-Siest. A common variant highly associated with plasma VEGFA levels also contributes to the variation of both LDL-C and HDL-C. *J. Lipid Res.* 2013, 54: 535–541.

Supplementary key words vascular endothelial growth factor A polymorphism • lipid metabolism • cardiovascular disease • high density lipoprotein-C • low density lipoprotein-C • epistatic interaction • gene \times environment interaction

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The vascular endothelial growth factor (VEGF) family is one of the most important regulators of vascular biology. VEGFA, in particular, stimulates angiogenesis in a wide range of normal and pathological processes (1). Owing to this marked role in blood vessel homeostasis, the contribution of VEGFA to cardiovascular diseases (CVDs) and atherogenesis has been studied in recent years (2). However, the effect of VEGFA on CVDs is still unclear.

The use of anti-VEGF agents in cancer therapy has shown cardiovascular side effects such as hypertension, cardiomyopathy, and hemorrhagic events (3). Furthermore, VEGFA therapies have been tested in randomized clinical trials, particularly in patients with coronary artery disease, with results showing low clinical importance (2). Many studies have identified higher VEGFA levels in patients with vascular diseases; nevertheless, it is not clear whether the upregulation of VEGFA is an adaptation to ischemia or if it is, rather, causal in the onset of these diseases (4–11). Concerning the involvement of VEGFA in atherosclerosis, results seem to be conflicting. A possible neovascularization and proinflammatory effect of VEGFA, which leads to progression of atherosclerosis and plaque instability, in particular (12–17), has been identified in animal models and in vitro studies. In contrast, clinical trials using VEGFA in humans (2) and gene therapy in animal studies (18, 19) do not support a positive effect of VEGFA on atherosclerosis progression. The complexity of CVDs, combined with the pleiotropic effects of VEGFA, could partially explain the differences between studies.

Abbreviations: BMI, body mass index; BRC IGE-PCV, Biological Resources Bank "Interactions Gène-Environnement en Physiopathologie Cardiovasculaire"; CVD, cardiovascular disease; GWAS, genome-wide association study; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism; TC, total cholesterol; VEGF, vascular endothelial growth factor.

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An interesting point would be the assessment of the effect of VEGFA (which is expressed in almost all vascularized tissues, including the heart, the liver, and the kidney) on known cardiovascular risk factors in supposedly healthy populations, such as lipid parameters (20), inasmuch as this could partially explain the implications of VEGFA in the physiopathology of CVDs. In particular, high-density and low-density lipoprotein cholesterol levels (HDL-C, LDL-C, respectively) are considered independent risk factors for the development of CVD (20). Increased levels of circulating VEGFA have been found in subjects with uncomplicated hyperlipidemia in a small-sample-size study (21), and a similar finding has been shown in a pilot study in patients with hypercholesterolemia (22). Significant associations were found between HDL-C and VEGFA levels in a supposedly healthy population from Japan (23), whereas in a supposedly healthy population in the SAPHIR study, VEGFA was negatively correlated with LDL-C, total cholesterol (TC), and apoB only in women (24). Although the relation between lipid profiles and VEGFA levels is not yet clearly defined, these observations suggest an eventual implication of this molecule in lipid metabolism.

Genetic studies can be highly useful in the identification of strong associations between distinct metabolic pathways of risk factors involved in a common pathophysiology. Via a recent genome-wide association study (GWAS), we identified four single-nucleotide polymorphisms (SNPs) that explained up to 50% of the heritability of VEGFA circulating levels (25). The investigation of the effect of these new genetic variants, as well as the interactions between them and with environmental factors, on blood lipids levels might give some insight into the relation between VEGFA and blood lipids. Therefore, the aim of the present study is the assessment of genetic determinants of blood lipid levels using these four novel VEGFA-related SNPs in supposedly 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 nonoverlapping populations extracted from the Biological Resources Bank (BRC) "Interactions Gène-Environnement en Physiopathologie CardioVasculaire" (IGE-PCV) in Nancy, in northeast France. They consist of supposedly 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 were not included. Subjects taking blood lipid-lowering drugs or medications having an effect on cardiovascular function (including inotropic agents, β blockers, calcium-channel blockers, organic nitrates, anti-arrhythmics, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, diuretics, clot busters, anti-coagulants, anti-platelet drugs, anti-diabetic drugs, and insulin) were also excluded. The study protocols were approved by the local ethics committee of each recruitment center, and all subjects gave written informed consent for their participation in the study.

Data collection

For both populations, biological and clinical measurements and health and lifestyle information were collected using appropriate validated questionnaires and procedures as described previously (26, 27). Hypertension was defined as systolic blood pressure ≥ 140 mm/Hg, diastolic blood pressure ≥ 90 mm/Hg; 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 ≥ 30 kg/m².

ApoE serum levels were measured using a turbidimetric immunoassay method (28), and triglycerides, TC, and HDL-C plasma levels were measured as previously described (26, 27). In particular, TC was measured using a cholesterol oxidase-paraminophenazone method, triglycerides using a glycerophosphate oxidase/paraminophenazone alanyl-glycine method, and HDL-C levels using a phosphotungstate method. LDL-C levels were calculated using the Friedewald formula (29). VEGFA plasma levels were measured in a subsample of 403 individuals from the discovery population by Randox, Ltd. (Crumlin, UK) using a biochip array analyzer (Evidence $\text{\textcircled{R}}$) (25). Blood collection was performed after overnight fasting.

Genotyping

DNA was 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 $\text{\textcircled{R}}$ (<http://genoscreen.fr>) using a Sequenom $\text{\textcircled{R}}$ iPLEX Gold assay (Medium Throughput Genotyping Technology) (30) 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. For each SNP, 192 duplicate samples were used, and a concordance of 100% was found.

Statistical analysis

Continuous variables are presented as mean value \pm standard deviation, and categorical variables are given in percentages. Hardy-Weinberg equilibrium was tested using the χ^2 test. VEGFA 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 VEGFA 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 lipid 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 a case in which more than one SNP is 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 to clarify the independent determinants of the trait.

Concerning the use of BMI as a covariate in the regression models and before performing the analysis on SNP associations with lipid traits, we assessed the existence of direct effects of the SNPs on BMI, using linear regression models adjusted for age and gender. The results were not statistically significant (data not shown), thus allowing the use of BMI in the analyses models.



Supplemental Material can be found at:
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The environmental factors used for the gene \times environment interactions assessment were BMI, smoking, or hypertension. We assessed the contribution of these interactions using linear regression models adjusted for age, gender, BMI, the environmental factor, and the additional interaction term (SNP \times environmental factors). Significant results were considered those with $P < 0.004$. For the significant SNPs implicated in gene \times environment interactions, separate regression models using the environmental factor as the dependent variable were performed to control for a direct association between the SNPs and the factor.

The assessment of gene \times 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>) (31) and the SPSS statistical software version 16.0 (SPSS, Inc.; Chicago, IL).

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 with previous findings in the literature (previous GWAS). SNPs with a correlation coefficient $\geq 80\%$ were considered in linkage disequilibrium (LD). The GWAS investigator of HuGENavigator engine (32) and the NHGRI catalog of published GWAS (<http://www.genome.gov/gwasstudies>) (33) were used 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-C ($\beta = -0.09$ mmol/l, $P = 1.2 \times 10^{-4}$ and $\beta = -0.01$ mmol/l, $P = 8.3 \times 10^{-3}$, respectively; **Table 3**) and between rs6921438 with plasma LDL-C concentrations ($\beta = 0.14$ mmol/l, $P = 6.7 \times 10^{-5}$, **Table 3**). Specifically, the minor allele A of rs6921438 is associated with decreased HDL-C and increased LDL-C values, whereas the minor allele T of rs6993770 is associated with increased levels of HDL-C. These significant associations were only confirmed for SNP rs6921438 in the replication population ($\beta = -0.07$ mmol/l, $P = 2.7 \times 10^{-4}$ and $\beta = 0.12$ mmol/l, $P = 8.4 \times 10^{-3}$ for HDL-C and LDL-C, respectively; **Table 3**). Conditional analysis including both rs6993770 and rs6921438 revealed that rs6921438 was the only SNP with significant direct effect on HDL-C levels in the discovery and replication populations ($\beta = -0.09$ mmol/l, $P = 1.2 \times 10^{-4}$ and $\beta = -0.07$ mmol/l, $P = 2.7 \times 10^{-4}$, respectively; **Table 3**). Of note, these results remained significant in both populations after adjustments for both smoking and hypertension (data not shown). SNP rs6921438 explained 1% of the variability of HDL-C and 0.2% for LDL-C in both populations. Meta-analysis of the results of both populations gave highly significant associations between rs6921438 and both HDL-C and LDL-C levels ($\beta = -0.08$

mmol/l, $P = 1.2 \times 10^{-7}$ and $\beta = 0.13$ mmol/l, $P = 1.5 \times 10^{-4}$ for HDL-C and LDL-C, respectively; **Table 3**). SNP rs6993770 was not significantly associated with HDL-C 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-C levels ($\beta = -0.08$ mmol/l, $P = 1.2 \times 10^{-7}$; **Table 3**).

The nonsignificant associations between the four assessed SNPs and the other lipid traits are presented in supplementary Table I for the discovery and replication populations, as well as the results of the meta-analyses.

Among 10 previously published GWAS concerning lipid levels, rs6921438 (and SNPs in LD with rs6921438) was not reported in the lists of statistically significant SNPs (34–43). Supplementary Table II presents the nonsignificant associations of the four SNPs with the assessed lipid traits in a large GWAS meta-analysis (43). Therefore, rs6921438 is a novel SNP associated with both HDL-C and LDL-C levels.

A significant association between the interaction rs4416670 \times hypertension and apoE variation was also identified ($P = 3.5 \times 10^{-3}$, 1.6×10^{-3} , and 1.7×10^{-5} , respectively, in the discovery and replication populations and meta-analysis; **Table 4**). The minor allele of the polymorphism was associated with lower levels of apoE in hypertensive participants ($\beta = -0.71$ mg/l, -0.75 mg/l, and -0.73 mg/l, respectively; **Table 4**). To test for a possible direct association of rs4416670 in hypertension, further analyses were performed in both populations. No significant associations were observed between the same SNP and hypertension ($P = 0.576$ for discovery and $P = 0.592$ for replication population).

Regarding epistatic interactions, in the discovery set, we found that the SNP rs6921438 interacted with rs6993770 for HDL-C levels ($\beta = 0.05$ mmol/l, $P = 2.4 \times 10^{-5}$, **Table 4**). Although this finding was not significant in the replication population ($\beta = 0.02$ mmol/l, $P = 0.035$, **Table 4**), it was significant in the meta-analysis of the results ($\beta = 0.03$ mmol/l, $P = 2.6 \times 10^{-5}$, **Table 4**).

In a subsample of the discovery population ($n = 403$) with VEGFA plasma level measurements, no correlation was found between VEGFA plasma levels and any of the assessed lipid traits ($R = 0.022$, $P = 0.657$ for HDL-C, $R = -0.010$, $P = 0.845$ for LDL-C, $R = 0.017$, $P = 0.741$ for TC, $R = 0.069$, $P = 0.164$ for triglycerides and $R = 0.066$, $P = 0.184$ for apoE). Furthermore, VEGFA levels were not associated with lipid levels in regression models ($P > 0.05$) (see supplementary Table III).

DISCUSSION

The present study assessed the effect of VEGFA-related SNPs on blood lipid traits and found significant associations and gene \times environment interactions for HDL-C, LDL-C, and apoE levels in two independent populations of healthy unrelated adults of European origin.

In particular, SNP rs6921438 was robustly associated with both HDL-C and LDL-C plasma levels. This SNP is an

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TABLE 1. Characteristics of the study participants

Variable	Discovery (n = 1,006)		Subsample with VEGFA plasma-level 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	
HDL-cholesterol (mmol/l)	1.47	0.44	1.60	0.47	1.61	0.62
LDL-cholesterol (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
ApoE (mg/l)	42.68	15.14	41.84	17.63	47.39	18.33
Vascular endothelial growth factor A (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).

intergenic genetic variant located on chromosome 6p21.1, at 171 kb downstream of the *VEGFA* gene. More precisely, this SNP is located between LOC100132354 (a noncoding RNA) and the *C6orf223* gene (encoding an uncharacterized protein). To our knowledge, no expression data are available for the *C6orf223* gene. SNP rs6921438 is in linkage disequilibrium with a few SNPs close to it (<6 kb): rs4513773, rs7767396, and rs9472159 ($r^2 > 0.7$; $D' > 0.9$; in the HapMap CEU population), all located between LOC100132354 and *C6orf223*. SNP rs9472159 is located in an enhancer- and promoter-associated histone mark region according to ENCODE (<http://genome.ucsc.edu/ENCODE/index.html>). Therefore, it could regulate the expression of surrounding genes, including *VEGFA*. In a recent GWAS, we showed that the minor allele of rs6921438 strongly contributed to decreased VEGFA circulating levels (with an explained variance of VEGFA levels of 41%) (25). Subsequently, it appears that rs6921438 could have a negative effect in the cardiovascular system through a decrease in HDL-C levels, an increase in LDL-C levels, and a decrease in VEGFA levels.

It should be mentioned that to our knowledge, this is the first study that investigates the effects of VEGFA-related SNPs with blood lipid traits. Also, rs6921438 has not been identified in any previous GWAS concerning HDL-C and LDL-C levels (34–43). The existing GWASs have managed to explain a small percentage of the blood lipid trait variance (e.g., for HDL-C, it ranges from 0.6% to 10%); thus,

other genetic variants remain to be found. Nevertheless, in the present study, rs6921438 explained 1% of HDL-C variability and 0.2% of LDL-C variability (in both populations). A large meta-analysis of GWAS including more than 100,000 participants did not identify the genetic variants assessed in the present study as significant determinants of blood lipid levels (see supplementary Table II) (43). The reason for this discrepancy may be due to differences in the studied populations. Teslovich et al. (43) included in their study more than 45 populations of European descent corresponding also to family-based or case-control studies. These populations may be different from our healthy unrelated European adult populations.

In the present study, a candidate gene approach was used based on SNPs identified from a GWAS concerning VEGFA-level heritability. The use of GWAS-identified SNPs as candidate loci for other trait variations could help in 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 VEGFA, could support this methodology.

To further assess the effect of this common regulation, we tested the association between VEGFA and blood lipid levels, although the results were nonsignificant. Therefore, plasmatic levels of VEGFA may have an indirect implication in lipid metabolism, at least in supposedly healthy individuals. However, Blann et al. (21) demonstrated that

TABLE 2. Characteristics of the four studied genetic variants

Chr	SNP	Minor allele	Direction of effect on plasma VEGFA ^a	Variance of plasma VEGFA explained (%) ^b	Location		MAF ^c	
					Function	Closest genes	Discovery	Replication
6	rs6921438	A	-0.72	41.2	Intergenic	<i>LOC100132354/ C6orf223</i>	0.42	0.40
6	rs4116670	C	-0.13	1.5	Intergenic	<i>LOC100132354/ C6orf223</i>	0.47	0.45
8	rs6993770	T	-0.17	2.0	Intronic	<i>ZFPM2</i>	0.30	0.31
9	rs10738760	G	-0.28	5.0	Intergenic	<i>KCNV2/ VLDLR</i>	0.48	0.45

^a According to Ref. 25, effect size in the discovery cohort (VEGFA values in ng/l, log-transformed).

^b According to Ref. 25.

^c MAF, minor allele frequency.



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TABLE 3. Significant associations of SNPs with blood lipids

SNP	Traits/Study	Mean values level \pm SD ^a by genotype (mmol/l)			β [SE] ^d (mmol/l)	P
		XX	XY	YY		
rs6921438	HDL-C ^c / discovery	1.97 \pm 0.47	1.89 \pm 0.49	1.76 \pm 0.54	-0.09 [0.02]	1.2 \times 10 ⁻⁴
	HDL-C / replication	1.73 \pm 0.62	1.61 \pm 0.62	1.39 \pm 0.58	-0.07 [0.02]	2.7 \times 10 ⁻⁴
X-allele = G	HDL-C / meta-analysis	NA ^e	NA	NA	-0.08 [0.01]	1.2 \times 10 ⁻⁷
Vallele = A	LDL-C ^d / discovery	3.03 \pm 1.08	3.29 \pm 1.09	3.61 \pm 1.093	0.14 [0.05]	6.7 \times 10 ⁻³
	LDL-C / replication	3.50 \pm 1.19	3.54 \pm 1.17	3.84 \pm 1.06	0.12 [0.04]	8.4 \times 10 ⁻³
	LDL-C / meta-analysis	NA	NA	NA	0.13 [0.03]	1.5 \times 10 ⁻⁴
rs6993770	HDL-C / discovery	1.86 \pm 0.52	1.93 \pm 0.47	2.06 \pm 0.49	0.01 [0.02]	8.3 \times 10 ⁻³
X-allele = A	HDL-C / replication	1.61 \pm 0.62	1.61 \pm 0.63	1.61 \pm 0.64	-0.02 [0.02]	0.318
Vallele = T	HDL-C / meta-analysis	NA	NA	NA	0.01 [0.01]	0.298
rs6921438	HDL-C / discovery	NA	NA	NA	-0.09 [0.02]	1.2 \times 10 ⁻⁴
	conditioned for rs6993770	NA	NA	NA	-0.07 [0.02]	2.7 \times 10 ⁻⁴
	HDL-C / replication	NA	NA	NA	-0.08 [0.01]	1.2 \times 10 ⁻⁷
	HDL-C / meta-analysis	NA	NA	NA	-0.003 [0.01]	0.780
rs6993770	HDL-C / discovery	NA	NA	NA	0.005 [0.01]	0.58
	conditioned for rs6921438	NA	NA	NA	0.002 [0.008]	0.790
	HDL-C / replication	NA	NA	NA	0.002 [0.008]	0.790
	HDL-C / meta-analysis	NA	NA	NA	0.002 [0.008]	0.790

Associations that are not significant are reported in supplementary Table 1.

^aSD, standard deviation.

^b β , effect size; SE, standard error.

^cHDL-C, HDL-cholesterol.

^dLDL-C, LDL-cholesterol.

^eNA, not applicable.

subjects with hyperlipidemia have increased levels of VEGFA compared with healthy controls, even if the authors found no correlation of VEGFA with blood lipid levels. Additionally, similar results were observed in a small pilot study of hypercholesterolemia patients by Belgore, Lip, and Blann (22). Both of these studies were performed in small sample sizes, and they have included pathological populations in a case-control design. Thus, these results cannot be directly compared with the present study, where healthy populations have been used. In the study of Kimura et al. (23), serum VEGFA levels were negatively correlated with HDL-C levels in healthy adults; however, this correlation was observed only in male populations. Moreover, in this study, measurements were performed in serum samples, which demonstrate higher levels of VEGFA compared with plasma levels that were measured in the present study

(23, 25). Finally, Sandhofer et al. (24) have shown that plasma VEGFA levels were negatively associated with TC and LDL-C in a healthy female sample. Although the population of this study is larger than our discovery cohort, the female sample is significantly older compared with ours. As previously observed, VEGFA levels increase with age, especially in women (44). In the abovementioned study, VEGFA 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 supposedly healthy populations, VEGFA is only marginally or not associated with blood lipid levels. Thus, although a common genetic background between VEGFA and blood lipids, especially HDL-C and

TABLE 4. Significant gene \times environment and gene \times gene interactions with blood lipids

Interaction	Traits/Study	β [SE] ^a	P
rs4416670 \times hypertension	ApoE ^b /discovery	-0.71 [0.24]	3.5 \times 10 ⁻³
	ApoE/replication	-0.75 [0.23]	1.6 \times 10 ⁻³
	ApoE/meta-analysis	-0.73 [0.17]	1.7 \times 10 ⁻³
rs6921438 \times rs6993770	HDL-C ^c /discovery	0.05 [0.01]	2.4 \times 10 ⁻³
	HDL-C/replication	0.02 [0.01]	0.035
	HDL-C/meta-analysis	0.03 [0.01]	2.6 \times 10 ⁻³

^a β , effect size; SE, standard error; units for β coefficient are: mg/l for apoE and mmol/l for HDL-cholesterol.

^bApoE, apolipoprotein E.


^cHDL-C, HDL-cholesterol.



LDL-C, may exist, a clinical manifestation cannot 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.

We also analyzed the effect interactions of the four VEGFA-related SNPs between them and with environmental factors, on lipid parameters. It is currently widely accepted that gene×gene and gene×environmental interactions can explain a significant amount of genetic heritability and can help identify subgroups among populations with higher disease risk (45–47). Hypertension, BMI, and smoking were the “environmental factors” selected because these are known risk factors for CVD. Because these are factors commonly present in individuals with pathological blood lipid levels, the effect of their interactions with genetic variants could have significant clinical importance. We identified a significant effect of the interaction between rs4416670 and hypertension on decreased apoE levels. ApoE serum levels are known to be associated with the APO-E common polymorphism; in particular, we have previously shown that the APO-E2/2 genotype contributed to high apoE levels and APO-E4/4 with low apoE levels (48). Here, we have identified a new genetic variant that has an effect on apoE levels through an interaction with hypertension. The SNP×hypertension interaction that we demonstrated is not due to a direct relationship between the two components of the interaction. Hypertension is among the major risk factors for CVD (49). The presence of altered blood lipid levels and high blood pressure is a very common manifestation in subjects with CVD. 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 CVD.

This study was the first designed to assess common genetic regulation between VEGFA and blood lipids. The selection of healthy populations is important for the understanding of these relationships because in pathological situations like CVD, the clinical profile of both blood lipids and VEGFA 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 the findings. We acknowledge, however, the limited number of individuals with VEGFA plasma-level measurements.

In conclusion, we found a robust association between a common VEGFA-related genetic variant and both HDL-C and LDL-C. Furthermore, apoE variation was partly explained by interactions between another VEGFA-related SNP and hypertension, which is known to be related to CVD. The assessment of other CVD risk factor 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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What Is the Contribution of Two Genetic Variants Regulating VEGF Levels to Type 2 Diabetes Risk and to Microvascular Complications?

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Abstract

Vascular endothelial growth factor (VEGF) is a key chemokine involved in tissue growth and organ repair processes, particularly angiogenesis. Elevated circulating VEGF levels are believed to play a role in type 2 diabetes (T2D) microvascular complications, especially diabetic retinopathy. Recently, a genome-wide association study identified two common single nucleotide polymorphisms (SNPs; rs6921438 and rs10738760) explaining nearly half of the variance in circulating VEGF levels. Considering the putative contribution of VEGF to T2D and its complications, we aimed to assess the effect of these VEGF-related SNPs on the risk of T2D, nephropathy and retinopathy, as well as on variation in related traits. SNPs were genotyped in several case-control studies: French and Danish T2D studies ($N_{\text{cases}} = 6,920$ - $N_{\text{controls}} = 3,875$ and $N_{\text{cases}} = 3,561$ - $N_{\text{controls}} = 2,623$; respectively), two French studies one for diabetic nephropathy ($N_{\text{cases}} = 1,242$ - $N_{\text{controls}} = 860$) and the other for diabetic retinopathy ($N_{\text{cases}} = 1,336$ - $N_{\text{controls}} = 1,231$). The effects of each SNP on quantitative traits were analyzed in a French general population-based cohort ($N = 4,760$) and two French T2D studies ($N = 3,480$). SNP associations were assessed using logistic or linear regressions. In the French population, we found an association between the G-allele of rs6921438, shown to increase circulating VEGF levels, and increased T2D risk (OR = 1.15; $P = 3.7 \times 10^{-5}$). Furthermore, the same allele was associated with higher glycated hemoglobin levels ($\beta = 0.02\%$; $P = 9.2 \times 10^{-7}$). However, these findings were not confirmed in the Danes. Conversely, the SNP rs10738760 was not associated with T2D in the French or Danish populations. Despite having adequate statistical power, we did not find any significant effects of rs6921438 or rs10738760 on diabetic microvascular complications or the variation in related traits in T2D patients. In spite of their impact on the variance in circulating VEGF, we did not find any association between SNPs rs6921438 and rs10738760, and the risk of T2D, diabetic nephropathy or retinopathy. The link between VEGF and T2D and its complications might be indirect and more complex than expected.

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Introduction

Vascular endothelial growth factor A (VEGFA – also known as VEGF) is a key chemokine involved in tissue growth and organ repair processes. Primarily, the role of VEGF has been intensely investigated in the regulation of angiogenesis, namely the growth of novel vascular endothelial cells derived from arteries, veins or lymphatics, which is essential for organ development and tissue repair, but also for tumor growth [1,2]. VEGF mediates angiogenesis by increasing vascular permeability to water and proteins [3]. However, the excessive vascular permeability during pathological angiogenesis can contribute to both macro- and microvascular diseases [3]. In this regard, higher circulating VEGF levels have been detected in the serum of some patients presenting with various types of cancer [4], with cardiovascular diseases [5,6,7] or with diabetic microvascular complications [8]. Higher levels of serum VEGF play a central role in the development of diabetic retinopathy, and intravitreal anti-VEGF drugs are currently widely used in patients presenting with proliferative diabetic retinopathy or diabetic macular edema [8].

The heritability of circulating VEGF levels is very high, estimated at between 60 and 80% [9,10,11]. A recent genome-wide association study (GWAS) reported several common single nucleotide polymorphisms (SNPs) that were significantly associated with serum VEGF levels [12]. In particular, two SNPs explained a very large proportion of the heritability of circulating VEGF levels in the Framingham study: rs6921438 and rs10738760, explaining 41.2% and 5.0% of the VEGF variance, respectively [12]. SNP rs6921438 is located on chromosome 6p21.1, at 171 kb downstream of *VEGFA*, and close to the *C6orf223* gene (which encodes an uncharacterized protein); and SNP rs10738760 is located on chromosome 9p24.2, between the *VLDLR* and *KCNV2* genes (that encode the very low density lipoprotein receptor and the potassium voltage-gated channel subfamily V, member 2, respectively).

We postulated that if increased circulating VEGF is genuinely a causative risk factor for type 2 diabetes (T2D) and/or its microvascular complications, then the SNPs rs6921438 and rs10738760, which explain nearly half the variance in circulating VEGF, would also contribute to the genetic risk of T2D and its microvascular complications. Therefore, we aimed to assess the association of both these VEGF-related SNPs (rs6921438 and rs10738760) with the risk of T2D, diabetic nephropathy and retinopathy, and with variation in related metabolic traits, in European populations.

Materials and Methods

Study participants

Clinical characteristics and data available for the studied populations are reported in **Table S1**.

Genotyping of SNPs rs6921438 and rs10738760 was conducted in several study samples:

- *French T2D case-control study*. We analysed 6,920 unrelated French individuals with T2D ascertained from the French T2D family study collected by the CNRS-UMR8090 unit, from the Endocrinology-Diabetology Department of the Corbeil-Essonnes Hospital [13], from the Diabhycar/Diab2-Néphrogène study [14], from the D.E.S.I.R. cohort which is a longitudinal French general population sample [15], and from the S.U.VI.MAX study, which is fully described elsewhere [16]. We used 3,875 unrelated normoglycemic adults (age at exam ≥ 45 years), ascertained from the D.E.S.I.R. and the S.U.VI.MAX studies, as controls.

- *Danish T2D case-control study*. We analysed a total of 3,561 Danish patients with T2D recruited by the Steno Diabetes center [17], as well as the Inter99 [18] and ADDITION studies [19]. We used 2,623 unrelated normoglycemic adults (age at exam ≥ 45 years), recruited by the Steno Diabetes center and the Inter99 study, as controls.
- *D.E.S.I.R.* The Data from the Epidemiological Study on the Insulin Resistance Syndrome (D.E.S.I.R.) cohort is a longitudinal French general population sample, fully described elsewhere [15]. We assessed the association of SNPs rs6921438 and rs10738760 with variation in quantitative metabolic traits (fasting glucose, fasting insulin, glycated haemoglobin [A1c], homeostasis model of pancreatic beta-cell function [HOMA-B] and homeostasis model of insulin resistance [HOMA-IR]) in 4,760 non-diabetic D.E.S.I.R. participants.
- *Corbeil study*. We assessed the association of SNPs rs6921438 and rs10738760 with variation in quantitative traits related to T2D microvascular complications (estimated glomerular filtration rate [eGFR], urinary albumin/creatinine ratio [ACR]) in 1,970 participants with T2D from Corbeil [13].
- *Diab2-Néphrogène (D2NG) study*. We assessed the association of SNPs rs6921438 and rs10738760 with variation in quantitative traits related to T2D microvascular complications (eGFR and ACR) in 1,510 participants with T2D from the D2NG study [14].
- *Case-control study for diabetic nephropathy and retinopathy*. Participants with T2D from Corbeil or the D2NG study were included in this analysis.

In the Corbeil study, we defined patients with a stage of kidney disease higher than 2 as cases (see details below) in the analyses for association with diabetic nephropathy ($N_{\text{Corbeil}} = 689$). Normoalbuminuric controls were required to have a duration of diabetes ≥ 10 years ($N_{\text{Corbeil}} = 570$). For the analysis of retinopathy, 506 T2D individuals from Corbeil were included as cases, while controls ($N_{\text{Corbeil}} = 591$) were required to have a duration of diabetes ≥ 10 years and no signs of retinopathy. In cases, retinopathy was staged as background, severe non-proliferative or proliferative; and macular edema was staged as present/absent according to funduscopy by a trained ophthalmologist, and a retinal angiography or an optical coherence tomography (OCT) when clinically indicated.

In the D2NG study, diabetic retinopathy was considered for all participants with a similar ophthalmologist-based classification (cases: $N_{\text{D2NG}} = 838$; controls: $N_{\text{D2NG}} = 639$). All patients were considered for their retinopathy stage. With regards to renal status, cases were identified as patients presenting with different stages of renal involvement (see details below) and with retinopathy (to ensure the specificity of diabetic nephropathy) ($N_{\text{D2NG}} = 551$). Controls were normoalbuminuric and normal renal function patients with a known T2D duration ≥ 10 years ($N_{\text{D2NG}} = 287$). Furthermore, any subjects taking angiotensin-converting-enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs) were excluded from the control sample. This phenotype was not available in the Corbeil study, which constitutes a limitation of this study as ACE inhibitors or ARBs intake can influence the ACR and thus lead to a possible overestimation of the number of controls.

Glycemic status was defined according to 1997 American Diabetes Association criteria [20]: normal glucose, defined as fasting plasma glucose < 6.1 mmol/l without hypoglycemic

Table 1. Association of SNPs rs6921438 and rs10738760 with T2D risk in two European case-control studies.

SNPs	Studies	X frequency (%)				OR (95% CI)*	P	
		N	YY (%)	YX (%)	XX (%)			
rs6921438 (G-allele = X A-allele = Y)	French controls	525	3,745	861 (23.0)	1,834 (49.0)	1,050 (28.0)	-	-
	French cases	55.6	6,908	1,367 (19.8)	3,402 (49.2)	2,139 (31.0)	1.15 (1.07;1.22)	3.7 × 10⁻⁵
	Danish controls	50.8	2,600	590 (22.7)	1,324 (50.9)	686 (26.4)	-	-
	Danish cases	51.8	3,524	857 (24.3)	1,753 (49.7)	914 (25.9)	1.02 (0.94;1.10)	0.66
	Combined analysis	-	-	-	-	-	-	0.28
rs10738760 (A-allele = X G-allele = Y)	French controls	50.4	3,756	916 (24.4)	1,896 (50.5)	944 (25.1)	-	-
	French cases	50.6	6,914	1,733 (25.1)	3,362 (48.6)	1,819 (26.3)	0.98 (0.91;1.06)	0.63
	Danish controls	51.2	2,592	611 (23.6)	1,308 (50.5)	673 (26.0)	-	-
	Danish Cases	50.9	3,512	827 (23.5)	1,796 (51.1)	889 (25.3)	1.04 (0.96;1.12)	0.40
	Combined analysis	-	-	-	-	-	-	0.93

*OR from additive logistic regression models adjusted for age and gender.
T2D, type 2 diabetes; OR, odds ratio; CI, confidence interval; P, P-value.
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treatment; and T2D, defined as fasting plasma glucose ≥ 7.0 mmol/l and/or treatment with hypoglycaemic agents.

Clinical data relating to nephropathy were defined as follows: i/ Stage 2: microalbuminuria defined as ACR ≥ 30 mg/g [3.5 mg/mmol] and < 300 mg/g [35 mg/mmol] (at three consecutive measurements); ii/ Stage 3: macroalbuminuria defined as ACR ≥ 300 mg/g [35 mg/mmol] (at three consecutive measurements); and iii/ Stages ≥ 4 : eGFR < 30 ml/min/1.73 m², or patients undergoing dialysis or having received a kidney transplant.

All studies were approved by the local ethical committees (from France: CPP [Comité de Protection des Personnes] of Lille, Kremlin-Bicêtre, Corbeil-Essonnes, Poitiers and Paris; and from Denmark [Copenhagen]: ClinicalTrials.gov-Identifier

#NCT00289237) as being in accordance with the Declaration of Helsinki. All study participants also gave written informed consent.

Genotyping

All DNA samples used for the present study were extracted from blood. Genotyping of SNPs rs6921438 and rs10738760 was performed using TaqMan assays according to the manufacturer's instructions (Applied Biosystems; AB assay IDs C-11542106-10 and C-11257266-10, respectively). A genotyping success rate of at least 97% and no deviation from Hardy-Weinberg equilibrium ($P > 0.05$) were observed in all the study populations. Furthermore, a total of 182 samples were also assessed by Sanger sequencing,

Table 2. Effect of SNPs rs6921438 and rs10738760 on variation in quantitative metabolic traits in nondiabetic participants from D.E.S.J.R.

SNPs	Metabolic traits	Mean/median data level by genotype				β (SE)*	P
		N	YY	YX	XX		
rs6921438 (G-allele = X A-allele = Y)	Fasting glucose (mmol/l)	4,600	5.29 \pm 0.55	5.27 \pm 0.53	5.30 \pm 0.54	0.012 (0.010)	0.24
	Fasting insulin (pmol/l)	4,600	39.5 (29.4;53.9)	39.2 (28.4;56.5)	39.7 (28.7;56.1)	0.003 (0.011)	0.81
	HOMA-B	4,600	67.2 (51.0;92.9)	69.1 (48.4;96.2)	67.3 (48.2;93.9)	-0.009 (0.011)	0.38
	HOMA-IR	4,600	9.2 (6.5;13.2)	9.1 (6.4;13.6)	9.3 (6.5;13.6)	0.005 (0.012)	0.66
	A1c (%)	4,600	5.41 \pm 0.41	5.42 \pm 0.39	5.45 \pm 0.40	0.020 (0.007)	9.2 × 10⁻³
rs10738760 (A-allele = X G-allele = Y)	Fasting glucose (mmol/l)	4,602	5.28 \pm 0.53	5.28 \pm 0.54	5.29 \pm 0.54	0.009 (0.010)	0.39
	Fasting insulin (pmol/l)	4,602	38.5 (28.6;55.2)	38.9 (28.5;55.7)	41.4 (29.7;57.2)	0.018 (0.009)	0.053
	HOMA-B	4,602	66.9 (48.4;94.5)	67.9 (48.1;94.3)	70.0 (51.8;97.5)	0.017 (0.011)	0.11
	HOMA-IR	4,602	8.9 (6.5;13.2)	9.1 (6.4;13.6)	9.8 (6.6;13.8)	0.020 (0.010)	0.051
	A1c (%)	4,602	5.43 \pm 0.39	5.43 \pm 0.40	5.43 \pm 0.40	0.006 (0.008)	0.47

*Per X-allele effect size: coefficient β from additive linear regression models adjusted for age and gender.
Data are presented as mean \pm standard deviation or median (interquartile range). Data for fasting serum insulin, HOMA-B, and HOMA-IR were logarithmically transformed before statistical analysis.
HOMA-B, homeostatic model of pancreatic beta cell function; HOMA-IR, homeostatic model of insulin resistance; A1c, glycated hemoglobin; SE, standard error; CI, confidence interval; P, P-value.
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Table 3. Association of SNPs rs6921438 and rs10738760 with the risk of T2D micro-vascular complications in the Corbeil and Diab2-Néphrogène (D2NG) studies.

SNPs	Studies	N	YY (%)	YX (%)	XX (%)	OR (95% CI)*	P	
rs6921438 (G-allele = X, A-allele = Y)	Nephropathy	Controls (Corbeil)	561	126 (22.5)	279 (49.7)	156 (27.8)	-	-
		Cases (Corbeil)	683	139 (20.4)	327 (47.9)	217 (31.8)	1.14 (0.97;1.34)	0.10
		Controls (D2NG)	286	66 (23.1)	129 (45.1)	91 (31.8)	-	-
		Cases (D2NG)	547	108 (19.7)	250 (45.7)	189 (34.6)	1.12 (0.92;1.37)	0.26
		Combined analysis	-	-	-	-	-	0.064
	Retinopathy	Controls (Corbeil)	581	117 (20.1)	288 (49.6)	176 (30.3)	-	-
		Cases (Corbeil)	500	110 (22.0)	236 (47.2)	154 (30.8)	0.97 (0.82;1.15)	0.75
		Controls (D2NG)	632	123 (19.4)	318 (50.4)	191 (30.2)	-	-
		Cases (D2NG)	833	174 (20.8)	383 (46.0)	276 (33.2)	1.08 (0.93;1.27)	0.32
		Combined analysis	-	-	-	-	-	0.63
rs10738760 (A-allele = X, G-allele = Y)	Nephropathy	Controls (Corbeil)	559	145 (25.9)	269 (48.1)	145 (25.9)	-	-
		Cases (Corbeil)	680	184 (27.1)	321 (47.2)	175 (25.7)	0.96 (0.82;1.12)	0.61
		Controls (D2NG)	284	71 (25.0)	151 (53.2)	62 (21.8)	-	-
		Cases (D2NG)	543	144 (26.5)	249 (45.9)	150 (27.6)	1.07 (0.88;1.31)	0.48
		Combined analysis	-	-	-	-	-	0.97
	Retinopathy	Controls (Corbeil)	580	166 (28.6)	262 (45.2)	152 (26.2)	-	-
		Cases (Corbeil)	501	121 (24.1)	255 (50.9)	125 (25.0)	1.06 (0.90;1.25)	0.49
		Controls (D2NG)	629	150 (23.8)	323 (51.4)	156 (24.8)	-	-
		Cases (D2NG)	827	210 (25.4)	407 (49.2)	210 (25.4)	1.02 (0.87;1.20)	0.78
		Combined analysis	-	-	-	-	-	0.51

*OR from additive logistic regression models adjusted for T2D duration and gender.
OR, odds ratio; CI, confidence interval; P, P-value.
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and a concordance of more than 99% was observed for both SNPs.

Indices calculation

The eGFR was calculated using the modification of diet in renal disease (MDRD) equation as follows:

$eGFR = 175 \times (\text{creatinine}/88.4)^{-1.154} \times \text{age}^{-0.203} \times (0.742 \text{ if female})$, where creatinine is in $\mu\text{mol/l}$ [21].

The HOMA-B was calculated as:

$HOMA-B = (20 \times \text{fasting serum insulin}) / (\text{fasting plasma glucose} - 3.5)$, where fasting serum insulin is in mU/l and fasting plasma glucose is in mmol/l [22].

The HOMA-IR was calculated as:

$HOMA-IR = (\text{fasting plasma glucose} \times \text{fasting serum insulin}) / 22.5$, where fasting plasma glucose is in mmol/l and fasting serum insulin is in $\mu\text{mol/l}$ [22].

Statistical analyses

We analyzed the effect of SNPs rs6921438 and rs10738760 on quantitative traits (fasting plasma glucose, fasting serum insulin, HOMA-B, HOMA-IR and A1c) using linear regression models under an additive model, adjusted for age and gender, or for age and duration of T2D (eGFR and ACR). Data for fasting serum insulin, HOMA-B, HOMA-IR and ACR were logarithmically transformed before statistical analysis.

The effect of SNPs rs6921438 and rs10738760 on risk of T2D, retinopathy or nephropathy was assessed using a logistic regression

model adjusted for age and gender (T2D) or for T2D duration and gender (retinopathy and nephropathy).

The combined analyses were performed using a weighted inverse normal method via the function "metagen", with a fixed effect, in the "META" R package. No heterogeneity was observed ($P > 0.1$).

All statistical analyses were performed using SPSS (version 14.0 for Windows), except combined analyses and statistical power calculation which were performed using R (version 2.15.1 for Windows) and Quanto, respectively.

Results

Effect of SNPs rs6921438 and rs10738760 on the risk of T2D and variation in related metabolic traits

We firstly assessed the association between both SNPs rs6921438 and rs10738760, and T2D, in a large T2D French case-control study including 6,920 T2D patients and 3,875 normoglycemic controls.

Using a logistic regression adjusted for age and gender (under an additive model), we identified a significant association between the G-allele of rs6921438 (increasing circulating VEGF levels [12]) and increased T2D risk (odds ratio [95% confidence interval]: OR = 1.15 [1.07;1.22]; $P = 3.74 \times 10^{-3}$; **Table 1**). Of note, a meta-analysis of GWAS reported a significant association between a SNP located close to *VEGFA* and waist-hip ratio [23]. In order to assess whether the significant association between rs6921438 and T2D risk was driven by central obesity, the logistic regression was also adjusted for age, gender and BMI. We observed the same

Table 4. Effect of SNPs rs6921438 and rs10738760 on variation in quantitative traits related to T2D complications in type 2 diabetic participants from the Corbeil and Diab2-Néphrogène (D2NG) studies.

SNPs	Quantitative traits	Studies	N	Mean/median data level by genotype			β (SE)*	P
				YY	YX	XX		
rs6921438 (G-allele = X A-allele = Y)	eGFR (ml/min/1.73 m ²)	Corbeil	1,932	76.4±21.2	77.5±20.9	77.9±22.5	0.66 (0.67)	0.32
		D2NG	1,477	74.5±29.8	72.0±27.1	72.9±28.1	-0.89 (0.94)	0.35
		Combined analysis	3,409	-	-	-	-	0.80
	ACR (mg/mmol)	Corbeil	1,932	1.7 (0.8;5.0)	1.6 (0.8;5.6)	1.6 (0.8;5.4)	0.023 (0.047)	0.62
		D2NG	1,448	3.1 (0.9;21.3)	2.5 (1.0;16.0)	3.3 (1.0;15.1)	-5.8 (4.5)	0.20
Combined analysis		3,380	-	-	-	-	0.63	
rs10738760 (A-allele = X G-allele = Y)	eGFR (ml/min/1.73 m ²)	Corbeil	1,929	76.3±22.1	77.2±21.5	78.6±20.3	1.0 (0.7)	0.12
		D2NG	1,468	73.7±30.6	72.0±26.5	73.0±28.1	-0.28 (0.95)	0.77
		Combined analysis	3,397	-	-	-	-	0.28
	ACR (mg/mmol)	Corbeil	1,929	1.6 (0.8;5.5)	1.7 (0.8;5.4)	1.7 (0.8;5.0)	-0.018 (0.046)	0.70
		D2NG	1,438	3.5 (1.1;21.6)	2.6 (0.9;14.1)	2.8 (1.0;19.1)	-3.5 (4.7)	0.45
Combined analysis		3,367	-	-	-	-	0.69	

*Per X-allele effect size: coefficient β from additive linear regression models adjusted for T2D duration and gender.

Data are presented as mean \pm standard deviation or median (interquartile range). Data for ACR were logarithmically transformed before statistical analysis.

eGFR estimated glomerular filtration rate using modification of diet in renal disease (MDRD) formula; ACR, urinary albumin/creatinine ratio; T2D, type 2 diabetes; SE, standard error; CI, confidence interval; P, P-value.

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magnitude of association between rs6921438 and T2D risk after correction for BMI (OR = 1.17 [1.08;1.26]; $P = 7.25 \times 10^{-5}$; data not shown).

Furthermore, the same allele was associated with higher glycated haemoglobin (A1c) levels in 4,600 nondiabetic French participants from the D.E.S.I.R. study (effect size (standard error): $\beta = 0.020$ (0.007) %A1c; $P = 9.2 \times 10^{-5}$; **Table 2**). However, no association with fasting glucose, fasting insulin, HOMA-B or HOMA-IR was observed (**Table 2**).

We then aimed to replicate the significant association of rs6921438 with T2D in another European population. However, the association between SNP rs6921438 and T2D risk was not

significant in a T2D case-control study including 3,524 Danish T2D patients and 2,600 Danish controls (OR = 1.02 [0.94;1.10]; $P = 0.66$; **Table 1**). Furthermore, the same SNP did not significantly contribute to variation in fasting glucose, fasting insulin, HOMA-B, HOMA-IR or A1c in 5,621 nondiabetic Danish participants from the Inter99 study (data not shown).

We also did not observe any significant association between the A-allele of the second VEGF-associated SNP rs10738760 (reported to increase circulating VEGF levels [12]) and increased T2D risk (French study: OR = 0.98 [0.91;1.06]; $P = 0.63$; Danish study: OR = 1.04 [0.96;1.12]; $P = 0.40$; **Table 1**). No significant effect of

Table 5. Case numbers needed for reaching a statistical power of 80% according to the expected odds ratio or effect.

Study	Statistical Power	$ \beta $	OR	N_{case} [N_{control}]
Fasting glucose (continuous)	80%	0.03 mmol/l	NA	4,364 [4,760]
Fasting insulin (continuous)	80%	0.03 pmol/l	NA	4,364 [4,760]
Nephropathy (case-control)	80%	NA	1.18	1,157 [1,242]
Retinopathy (case-control)	80%	NA	1.17	1,285 [1,336]
eGFR (continuous)	80%	1.42 ml/min/1.73 m ²	NA	3,567 [3,480]
ACR (continuous)	80%	0.10 mg/mmol	NA	3,534 [3,480]

Only statistical power of association analyses with a P-value above 0.05 was analysed.

β , effect size; OR, odds ratio; NA, not applicable; eGFR, estimated glomerular filtration rate using modification of diet in renal disease (MDRD) formula; ACR, urinary albumin/creatinine ratio.

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the same allele on variation in fasting glucose, fasting insulin, HOMA-B, HOMA-IR or A1c was observed ($P > 0.05$; **Table 2**).

Effect of SNPs rs6921438 and rs10738760 on T2D-related microvascular complications and variation in related traits

We next assessed the effect of both SNPs on the presence of diabetic nephropathy in two French case-control studies (Corbeil and D2NG), including a total of 1,242 T2D patients with nephropathy and 860 T2D controls. We did not observe any significant association, either in the case-control analyses or in the combined analysis ($P > 0.05$; **Table 3**).

We also assessed the effect of both SNPs on the presence of diabetic retinopathy in the same French case-control studies, including a total of 1,336 T2D patients with a retinopathy and 1,231 T2D controls. Again, no significant association was observed, in either of the two case-control analyses or in the combined analysis ($P > 0.05$; **Table 3**). Furthermore, we did not observe any significant association between rs6921438 or rs10738760 and macular edema in patients from the D2NG study or Corbeil cohort (data not shown). Of note, use of subject age instead of T2D duration in the adjusted regression model did not modify the results for either diabetic retinopathy or diabetic nephropathy (data not shown).

Finally, we assessed the association of both SNPs and the variation in quantitative traits related to T2D microvascular complications (i.e. eGFR and ACR) in a total of 3,480 French T2D participants from Corbeil and D2NG. No significant association was observed in these analyses ($P > 0.05$; **Table 4**).

Discussion

We initially observed a strong association between the G-allele of rs6921438 (the allele increasing VEGF levels in the general population [12]), and increased T2D risk (BMI-adjusted or not) in the French population. This result was in line with some studies which reported that T2D patients show higher VEGF levels compared with normoglycemic individuals [12,24,25]. Furthermore, we found the same allele to be associated with increased A1c levels in a French general population sample.

However, we did not confirm these findings in the Danish population. Of note, the frequency of the G-allele was higher in the French population than in the Danes (52.5% versus 50.8% in controls/55.6% versus 51.8% in cases). Therefore, there may be a geographic effect on the frequency of this SNP. Nevertheless, in the international DIAGRAM consortium which performed meta-analysis of GWAS for T2D risk, the SNP rs6921438 was not significantly associated with T2D ($N_{cases} = 8,130$; $N_{controls} = 38,987$) [26]. Furthermore, no significant effect for rs6921438 on A1c ($N = 46,368$) [27] or fasting glucose levels ($N = 46,186$) [28] was observed by the analyses conducted by the MAGIC consortium which performed meta-analysis of GWAS for glucose- and insulin-related traits. Thus, the significant association in the French study is likely to be a false positive result. The second VEGF-associated SNP rs10738760 was not associated with T2D (or related metabolic traits) either in the French individuals or in the Danes.

Furthermore, we did not find any significant association of VEGF-related SNPs rs6921438 or rs10738760 on the presence of diabetic microvascular complications or variation in related traits. These results are in line with the data from the CKDGen and CARE Renal consortia which did not show any significant contribution of these SNPs to eGFR, ACR or microalbuminuria [29,30,31]. Of note, the CKDGen consortium identified a genome-wide significant effect of SNP rs881858 located close to

VEGFA (and SNP rs6921438) on eGFR variation [31]. However, SNPs rs881858 and rs6921438 were not in linkage disequilibrium in the HapMap European (CEU) population ($r^2 = 0.0$ / $D' = 0.1$).

Therefore, in the present study, we were unable to find a direct link between SNPs rs6921438 and rs10738760, which explain almost half of the variance in circulating VEGF [12], and risk of T2D, diabetic nephropathy or more importantly, diabetic retinopathy including macular edema. A limitation of this study which should be taken into consideration would be a lack of statistical power, to some extent (**Table 5**). However, even if the size of the present study (continuous or case-control) was relevant, we were not able to identify even marginally significant effects (**Table 5**).

Of note, the effect of VEGF was previously clearly demonstrated in diabetic macular edema [32]. Thus, our current negative result must be considered with caution. Factors driving VEGF locally may be different from those significantly associated with variation in plasma levels. Ultimately, VEGF might also be regulated in the retina by factors largely exceeding the impact of genetic determinants.

If confirmed in other studies with VEGF levels and clinical phenotypes related to diabetic complications, these findings would show that the link between VEGF and T2D and its complications might be indirect and more complex than expected.

Supporting Information

Table S1 Clinical characteristics of the population studies. Data are presented as mean \pm standard deviation or median (interquartile range). **NA**, not applicable or not available; **BMI**, body mass index; **HbA1c**, glycated hemoglobin; **eGFR**, estimated glomerular filtration rate using modification of diet in renal disease (MDRD) formula; **ACR**, urinary albumin/creatinine ratio; **T2D**, type 2 diabetes; **D2NG**, Diab2-Néphrogène; **retino**, case-control study for retinopathy risk; **nephro**, case-control study for nephropathy risk. (DOCX)

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Author Contributions

Conceived and designed the experiments: AB SH SV-S. Performed the experiments: AB P-JS MGS NG NCN AD SH. Analyzed the data: AB SH SV-S. Contributed reagents/materials/analysis tools: RR MAN OL SH TL BB TH OP PF GC MM SH SV-S JSE-SM. Wrote the paper: AB.

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Associations of vascular endothelial growth factor (VEGF) with adhesion and inflammation molecules in a healthy population

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ABSTRACT

Vascular endothelial growth factor (VEGF) is implicated in numerous pathologies through complex relationships with cellular adhesion molecules (CAMs) and inflammation markers. These have not been assessed in non-pathological conditions. Our aim was the evaluation of associations between VEGF and CAM/inflammation molecules in a healthy population, and of possible genomic interplays in order to better apprehend the underlying mechanisms leading to the pathology. We examined the associations between VEGF and ICAM-1, VCAM-1, E-, L-, P-selectins, TNF- α , CRP and IL-6 plasma levels in 403 healthy individuals. Gene expression of CAM/inflammation molecules and VEGF isoforms (121, 145, 165, and 189) were quantified in peripheral blood mononuclear cells (PBMCs). The effect of four genetic variants (explaining ~50% of the heritability of circulating VEGF levels) and of their interactions on plasma and mRNA levels of CAM/inflammation molecules was examined. VEGF was associated with ICAM-1 and E-selectin in plasma. In PBMCs, VEGF₁₄₅ mRNA was associated with ICAM-1, L-selectin and TNF- α expression. Interactions of the genetic variants were shown to affect ICAM-1, E-selectin, IL-6 and TNF- α plasma levels, while rs4416670 was associated with L-selectin expression. These findings propose a biological connection between VEGF and CAM/inflammation markers. Common genetic and transcriptional mechanisms may link these molecules and control their effect in healthy conditions.

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1. Introduction

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that shows distinguished functions in angiogenesis, lymphangiogenesis, vascular permeability, and hematopoiesis [1]. VEGF has been linked with a number of vascular pathologies including cardiovascular diseases (ischemic heart disease, heart failure, stroke). VEGF is a highly conserved, disulfide-bonded dimeric glycoprotein of 34–45 kDa and it is produced by several cell types including fibroblasts, neutrophils, endothelial cells and peripheral blood mononuclear cells (PBMCs) and macrophages [2]. Six isoforms of human VEGF, which range from 121 to 206 amino acid residues, have been identified. VEGF₁₄₅ and VEGF₂₀₆ have a key role during the angiogenesis of the human ovary as well as in bone and wound healing [1,3,4]. VEGF₁₈₃ also has a broad tissue expression and may have not been easily detected earlier due to confusion with VEGF₁₈₉ [5].

Elevated circulating VEGF levels have been also observed in several types of cancer and in various other disorders, for example: ischemic heart disease, diabetes, reproductive, immune-inflammatory disorders [6,7].

The expression of the VEGF isoforms regulate the gene expression of cellular adhesion molecules (CAMs) and inflammation markers. Cellular adhesion molecules (CAMs) are cell membrane receptors that mediate several interactions known to play a key role in a variety of physiological and pathological conditions related to traffic and interactions between cells, cell-matrix contact and in determining the specificity of cell-cell binding [8]. Their participation in the development of the atherogenic plaque is well described [9]. Furthermore, atherosclerosis is characterized by chronic inflammation and inflammation markers have been associated with cardiovascular pathologies [10].

Indeed, complex relationships have been observed between VEGF, CAM and inflammation markers in many diseases and especially in cardiovascular-related phenotypes. For instance, some studies reported the involvement of E-, P- and L-selectin, vascular cell adhesion molecule 1 (VCAM), intercellular adhesion molecule 1 (ICAM-1) and interleukin 6 (IL-6) in angiogenesis [11]. Also, hypoglycemia has been shown to increase circulating levels of

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VEGF, IL-6, E-selectin and ICAM-1 in healthy individuals and in patients with type 1 diabetes [12]. Moreover, it is noteworthy that several reports linked angiogenesis and inflammation, highlighting a key role of TNF- α (tumor necrosis factor- α) and CRP (C-reactive protein) [13–15]. Other important inflammation marker is interleukin 6 (IL-6). This anti- and pro-inflammatory cytokine is a primary determinant of the hepatic production of CRP [16]. Recent studies indicated the simultaneous increasing of IL-6 and VEGF circulating levels in some pathological conditions such as ovarian hyperstimulation syndrome induced ascites, diabetes, visceral obesity, cancer and hypertension [17–21]. Furthermore, IL-6 seems to induce VEGF expression in diabetic nephropathy and rheumatoid arthritis [22,23]. Serum levels of soluble adhesion molecules have also been correlated with serum levels of some acute phase proteins such as CRP in individuals with breast cancer [24]. In addition, in patients with hepatic cancer, the levels of soluble P-selectin have been associated with plasma levels of VEGF₁₆₅ [25].

The assessment of these relationships in different pathologies is progressing; however, due to the complexity of the pathophysiological mechanisms implicated in a disease, the origin of these biological connexions is difficult to be interpreted. Therefore, their investigation in the healthy state, where there is no implication of evident pathology, could offer important information concerning the physiological connexions between these molecules before the development of a specific disease. This could also be the key for the better understanding of the VEGF biological roles. However, to our knowledge, there is no published study of the link between VEGF and both CAM and inflammation markers in a healthy population.

Therefore, the aim of this study was to examine the associations between VEGF and ICAM-1, VCAM-1, E-, L- and P-selectins, TNF- α , IL-6 and CRP in plasma of supposed healthy individuals. Furthermore, we seek to investigate whether there is a relationship in expression profile of these molecules in peripheral blood mononuclear cells (PBMCs). Finally, we thought to assess possible genetic links between these molecules. We have recently identified four single nucleotide polymorphisms (SNPs) (rs6921438, rs4416670, rs6993770 and rs10738760) explaining ~50% of VEGF circulating levels heritability [7]. Thus, we aimed to determine possible associations of these SNPs with plasma levels of CAM/inflammation markers, as well as their effect on gene expression in PBMCs.

2. Materials and methods

2.1. Study population

The STANISLAS Family Study (SFS) is a 10-year longitudinal survey involving 1006 volunteer families from Vandœuvre-lès-Nancy, France between 1993 and 1995 [26]. Individuals with chronic disorders (cardiovascular diseases or cancer) or having a personal history of cardiovascular disease were not included, as the aim of the study was the assessment of genetic susceptibility factors on the variability of intermediate phenotypes in physiological conditions without the influence of any long term medication and disease. The study protocol was approved by the Local Ethics Committee of Nancy and all subjects gave written informed consent for their participation in the study. Four hundred and three unrelated adults collected during the second examination of the SFS were involved in the present study for which all data were available.

2.2. Laboratory measurements

Blood samples were collected after overnight fasting. Sodium EDTA-plasma was separated by centrifugation at 2000g for

15 min at 4 °C and stored at –196 °C in liquid nitrogen until analysis. Circulating plasma levels quantification was performed by Randox Ltd. (Crumlin, UK) using a biochip array analyzer (Evidence[®]) [27].

Plasma IL-6, TNF- α , ICAM-1, E-, L- and P-selectins were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, UK) according to the manufacturer's instruction. Concentrations of plasma CRP were determined using the BN II nephelometer (Germany).

Before the quantification of CAM and inflammation molecules, we tested the effect of the procedures of storage and sample collection on their concentrations. Fresh and frozen state had no significant effect on the concentration of the different molecules except for VCAM-1, which was less stable. Thus it was not included in the analyses of the present study.

2.3. Gene expression analysis

PBMCs were isolated by centrifugation on a density gradient of Ficoll [28] (Ficoll-Paque[™] PLUS; Amersham BioSciences). Total RNAs were extracted from PBMCs with a MagNaPure automate, using the MagNa Pure LC RNA HP isolation kit and RNA HP Blood External lysis protocol (Roche Diagnostics, France) in short period of time after cell isolation. Reverse transcription of total RNAs were performed using 200 units of M-MuLV Reverse Transcriptase with 0.25 μ g of oligos (dT) (Promega, France) according to a previously described protocol [29]. Quantification of the transcripts coding for the VEGF isoforms (VEGF121 [antisense: 5'-CTCggCTTgTgCACATTTTC-3' and probe: 5'-TgCAgACCAAAgAAAATgCAgACA], VEGF145 [antisense: 5'-CTTgTCAgCATAgCTCCAggAC-3' and probe: 5'AAACgAAAAGgCAAgAAATCCgTA-3'], VEGF165 [antisense: 5'-GCCTTCTCCgCTCgTgCA-3' and probe: 5'-AgCAAgACAAGAAAATCCCTgTggCC-3'], VEGF189 [antisense: 5'-CCACAaggAACgCTCCAggAC-3' and probe: 5'-AgCAAgACAAGAAAATCAGTTCgAggAAA-3']), ICAM-1, E-, L-, P-selectins, TNF- α , IL-6 and the beta 2 microglobulin (β 2M) control gene were performed using TaqMan[®] and LightCycler technologies (LC TaqMan Master Kit, Roche Diagnostics, France). It should be mentioned that a common VEGF forward primer (5'-gAgCTTCCTACgCA CAACAAA-3') was designed based on the fact that all VEGF isoforms share exons 1–5. All experiments were performed in duplicate. RT-PCR optimization and specificity of Real Time-PCR products were conducted using SYBR[®] Green technology (LC FastStart DNA Master^{PLUS} SYBR Green I kit, Roche Diagnostic, France), melting curves analysis and agarose gel electrophoresis of the PCR amplicons, as previously described [29]. VEGF₁₈₃, VEGF₂₀₆, E-selectin and CRP expression in PBMCs were not quantified due to their insufficient expression in this cell type. In total, available mRNA data existed for a subsample of 65 subjects. All mRNA levels were normalized to the mRNA levels of β 2M gene. Total VEGF mRNA was calculated as the sum of the ratio of the four isoforms present in PBMCs.

2.4. Genotyping

SNPs rs6921438, rs4416670, rs6993770, and rs10738760 were genotyped by Genoscreen (<http://genoscreen.fr>), using a Sequenom iPLEX Gold assay-Medium Throughput Genotyping Technology [30]. 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 $\geq 80\%$ were considered in linkage disequilibrium (LD).

2.5. Statistical analysis

Continuous variables are presented as mean value \pm standard deviation and categorical variables are given in percentages. Hardy-Weinberg equilibrium was tested using the chi-square test.

All molecules blood concentrations were log-transformed to normalize their distribution. Similar transformation was performed for mRNA values for all variables with exception of VEGF₁₆₅ and VEGF₁₈₉ whose values were normally distributed and IL-6 whose values were ln-transformed to achieve normal distribution.

Linear regression models adjusted for age, gender and body mass index (BMI) were used to test possible associations between VEGF related SNPs and plasma levels of the adhesion/inflammation molecules. Similar linear regression models adjusted for age and gender and BMI were applied for the assessment of associations between VEGF mRNA levels and mRNA levels of these molecules and between the four SNPs and blood levels and mRNA levels of the assessed markers.

For the SNPs associations analyses an additive model was used. Results are presented using the minor allele as reference allele.

The assessment of gene * gene epistatic interactions was tested using all possible 2 × 2 combinations for the four SNPs. In the regression models adjusted for age, gender and BMI, two SNPs and their interactions term were added in order to test the association of these interactions with adhesion/inflammation molecules plasma levels only. Epistatic interactions were not assessed for associations with mRNA levels due to the small sample size with available transcriptomic data and the low statistical power to assess this type of interactions.

All analyses were performed using PLINK 1.07 (<http://pngu.mgh.harvard.edu/purcell/plink/>) [31] and the SPSS 16.0 (SPSS, Inc, Chicago, Illinois) statistical software. Significance was determined at a two-tailed $P = 0.05$ level.

3. Results

The general characteristics of the studied population and of the four VEGF-related SNPs are presented in Tables 1–3 respectively.

3.1. Associations of VEGF plasma levels with plasma levels of CAM and inflammation molecules

VEGF plasma levels were associated with ICAM-1 levels in regression models adjusted for age, gender and BMI ($\beta = 0.043$, $P = 0.023$) and with E-selectin ($\beta = 0.085$, $P = 0.039$) (Table 4).

3.2. Associations of VEGF isoforms mRNA and gene expression of CAM and inflammation molecules

We then assessed the association between total and four VEGF isoforms and gene expression of ICAM-1, P- and L-selectin, TNF- α , and IL-6 in PBMCs. VEGF₁₆₅ mRNA was the most abundant expressed isoform by PBMCs. In regression models adjusted for age, gender and BMI, total VEGF mRNA was associated with TNF- α mRNA levels ($\beta = 0.412$, $P = 0.015$), while VEGF₁₄₅ isoform was

significantly associated with ICAM-1 mRNA ($\beta = 0.353$, $P = 0.021$), L-selectin mRNA ($\beta = 0.611$, $P = 0.001$) and TNF- α mRNA ($\beta = 0.616$, $P = 0.001$) (Table 4). The other VEGF isoforms were not significantly associated with gene expression of the assessed molecules.

3.3. Associations of rs6921438, rs4416670, rs6993770, rs10738760 and their epistatic interactions with plasma levels and transcripts of CAM and inflammation molecules

Significant epistatic interactions were identified for plasma VEGF, E-selectin, TNF- α , ICAM-1 and IL-6 levels (Table 5, Supplementary Table 1). In particular, an interaction between rs6993770 (TA genotype and T allele) and rs10738760 (G allele) was associated with increased levels of ICAM-1 and E-selectin. The T allele of rs6993770 interacts with the C allele of rs4416670 and the A allele of rs6921438 to increase IL-6 levels, while a decrease in IL-6 levels were observed due to an interaction between rs10738760 (A allele) and T allele of rs4416670 and the G allele of rs6921438. Moreover, the A allele of rs6921438 was associated with increased VEGF levels when combined with the A allele of rs10738760 and increased levels of TNF- α in the presence of the C allele of rs4416670.

A significant association was observed between rs4416670 and mRNA levels of L-selectin ($\beta = 0.196$, $P = 0.039$). No other significant association of the genetic variants with mRNA levels was observed.

4. Discussion

In the present study, we assessed the relationship between VEGF and CAM and inflammation molecules in healthy state conditions and by a multi-dimensional approach: associations in plasma, in gene expression in PBMCs, common genetic determinants and possible functional effects of these determinants on gene expression. In all these levels, significant results that may explain the physiological relationships between these molecules were observed.

VEGF plasma levels were positively associated with ICAM-1 and E-selectin. To the best of our knowledge, this is the first study to report a relationship between VEGF, the main angiogenic factor, ICAM-1, the mediator of leukocytes firm-adhesion to endothelial cells and E-selectin, the mediator of leukocytes rolling on vascular endothelium [31]. CAMs have a pivotal role in angiogenesis [8]. They regulate angiogenesis both indirectly and directly [32]. Among the soluble adhesion molecules, E-selectin and ICAM-1, have been shown to be directly angiogenic [33]. Therefore, the observed association of these adhesion molecules with VEGF could be explained by common angiogenic activities present already in healthy conditions.

In contrary, no association was found with L- and P-selectins and with any of the inflammatory markers and VEGF. Unlike E-selectin which is transcriptionally regulated in endothelial cells, P-selectin is translocated within minutes after the inflammation stimuli from intracellular granules and promotes the immediate reattachment and quick rolling of leukocytes over vascular surfaces [34]. Also, L-selectin is cleaved rapidly from the surface of activated leukocytes [35]. Therefore, in a healthy state, where inflammation process is not evident, these two molecules may not have an important role in the physiological interconnections, with VEGF. The absence of associations of VEGF with inflammation markers, further support this hypothesis. CRP has been previously shown not to be associated with VEGF levels in a healthy population [36]. In the current study we confirmed this finding and extent it to other inflammation molecules as well. These results could be

Table 1
Characteristics of study population (n = 403).

Variable	Mean ^a	SD
Age (years)	44.460	4.885
Gender (%) male	50.4	
Body mass index (kg/m ²)	24.9	3.9
Vascular endothelial growth factor (pg/ml)	42.748	43.330
Interleukin 6 (pg/ml)	1.376	1.695
Tumor necrosis factor α (pg/ml)	2.320	4.535
E-selectin (mg/l)	53.159	25.882
L-selectin (mg/l)	1060.693	407.889
P-selectin (mg/l)	138.917	42.891
C-reactive protein (mg/l)	1.794	3.426
Intercellular adhesion molecule-1 (mg/l)	263.023	69.486

^a Mean values for continuous variables and percentages for categorical variables.

Table 2
Gene expression levels of study population (n = 65).

Variable	Mean ^a	SD	Range
Vascular endothelial growth factor isoform 121	50.0	21.9	18.9–120.0
Vascular endothelial growth factor isoform 145	49.4	27.1	5.99–105.7
Vascular endothelial growth factor isoform 165	258.9	135.3	41.4–615.3
Vascular endothelial growth factor isoform 189	15.0	7.16	3.4–32.3
Total vascular endothelial growth factor	2547.9	1641.6	739.3–5821.5
Interleukin 6	5.7	7.3	0.8–28.1
Tumor necrosis factor α	849.4	942.6	165.3–1905.7
L-selectin	11,338.5	10,317.2	2635.5–36,065.2
P-selectin	2990.4	3737.5	316.7–9686.1
Intercellular adhesion molecule-1	153.5	183.1	59.8–410.0

^a Mean values are presented as normalized ratio with the beta 2 microglobulin ($\beta 2M$) control gene.

Table 3
SNPs characteristics.

Chromosome	SNP	Function	Closed to/on gene	Minor allele	Common allele	Minor allele frequency
6	rs6921438	Intergenic	MCC45491 and MRPL14 (near VEGF)	A	G	0.445
6	rs4416670	Intergenic	MCC45491 and MRPL14 (near VEGF)	C	T	0.476
8	rs693770	Intronic	ZFPM2	T	A	0.283
9	rs10738760	Intergenic	VLDLR and KCNV2	A	G	0.491

SNP: single nucleotide polymorphism, MCC: Ancient (alternative) acronym of C6orf223 (chromosome 6 open reading frame 223), MRPL14: mitochondrial ribosomal protein L14, ZFPM: zinc finger protein, multiple 2, VLDLR: very low density lipoprotein receptor, KCNV2: potassium channel, subfamily V, member 2.

Table 4
Association between plasma VEGF and its isoforms transcripts and adhesion and inflammation molecules, adjusted for age, gender, and BMI.

VEGF	ICAM-1		L-selectin		E-selectin		TNF- α	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
mRNA								
Total VEGF							0.412 ($P=0.015$)	
VEGF ₁₂₁								
VEGF ₁₄₅	0.353 ($P=0.018$)		0.611 ($P=0.001$)				0.616 ($P=0.001$)	
VEGF ₁₆₅								
VEGF ₁₈₉								
Plasma levels								
VEGF		0.043*				0.085*		

The values corresponding to beta coefficient (P -value)

Table 5
Epistatic interactions of SNPs on blood levels of VEGF, E-selectin, TNF- α , ICAM-1 and IL6 (regression models adjusted for age, gender and BMI).

SNPs	rs6921438	rs 4416670	rs 693770	rs 10738760
rs 6921438				VEGF ($P=0.01$)
rs 4416670	TNF- α ($P=0.035$)			
rs 693770	IL-6 ($P=0.004$)	IL-6 ($P=0.0008$)		E-selectin ($P=0.041$)
rs 10738760	IL-6 ($P=0.050$)	IL-6 ($P=0.004$)	ICAM-1 ($P=0.021$)	

explained by the fact that the study population is healthy, thus, the relationship between VEGF and inflammation markers could be consequence of the pathology, such as vascular dysfunction (e.g. in individuals with diabetes mellitus) [37].

Apart from the associations in the level of plasma concentrations, we examined possible similarities in the expression profile of CAM/inflammation molecules in PBMCs with VEGF. This cell type is easily accessible and contain cell types (lymphocytes and monocytes) that interact with different tissues, thereby probably acting as 'sensors' differentiating individuals with CVDs risk, especially for the pro-inflammatory state [38,39]. Our results confirmed this fact that VEGF₁₆₅ is the most abundant VEGF isoform [1,3] as we found that VEGF₁₆₅ mRNA is one that prevails in PBMCs. We demonstrated that VEGF₁₄₅ isoform mRNA is positively associated with ICAM-1, L-selectin and TNF- α expression while total VEGF mRNA is associated with TNF- α . Nowak et al. [40] have reported

that TNF- α induces a significant up regulation of circulating total VEGF. Therefore, we could hypothesize that TNF has an effect on VEGF splicing.

These associations could indicate the existence of common transcriptional factors or other molecular mechanisms that regulate the expression of these molecules in PBMCs in non-pathological situations.

The regulation of VEGF levels occurs at the gene transcriptional, translational, and post-translation levels. Upon binding of VEGF to its corresponding receptor (VEGFR2) in endothelial cells, three main pathways promote cell proliferation, migration, survival and nitric oxide production [31]. These pathways included the PI 3'-kinase (phosphoinositide)/Akt (protein kinase b) cascade, the tyrosine phosphorylation of PLC gamma (phospholipase c), and the MAPK/ERK (mitogen-activated protein kinase/extracellular-regulated kinase) cascade. Among these regulatory pathways the stimulation

of PLC gamma activates the protein kinase C (PKC) [29] and then leads to activation of NF- κ B transcription factor [41]. A growing body of evidence indicates that the activation of NF- κ B induces expression of ICAM-1 transcripts in endothelial cells [42–44]. In agreement with this concept, Kim and colleagues [31] have examined the signal transduction mechanisms by which VEGF induces adhesion molecules in human umbilical vein endothelial cells (HUVECs). Indeed, they have shown that VEGF-induced expression of ICAM-1 is mediated mainly through NF- κ B activation. Interestingly, Kiriakidis and colleagues [2] have documented the involvement of the transcriptional factor NF- κ B in the up-regulation of VEGF in human macrophages to lipopolysaccharide-induced response. Also, it regulates many of the genes involved in immune and inflammatory responses [45]. Concerning TNF- α , it has been shown to induce VEGF production via the stimulation of its corresponding transcription factor, HIF1- α [46]. Westra and colleagues [47] have shown that blocking of TNF- α reduces TNF- α induced VEGF production. Further studies have documented that TNF- α up regulates VEGF production by synovial membrane cells and PBMCs of patients with rheumatoid arthritis [48,49]. It has also been shown that NF- κ B is partly responsible for the up-regulation of VEGF mRNA expression in response to TNF- α [50]. Our study revealed that VEGF₁₄₅ isoform mRNA is positively associated with L-selectin.

Concerning IL-6, Hao and colleagues [51] have illustrated that VEGF, via vascular endothelial growth factor receptor 2-protein kinase D (VEGFR2-PKD) axis induces the production of some proinflammatory cytokines such as IL-6 in endothelial cells but not in leukocytes. This could probably explain the lack of association between VEGF and IL-6 expression in PBMCs. Also, as mentioned before, P-selectin's reaction in inflammation is not transcriptionally regulated [34]. Thus, the absence of common expression profile between VEGF and P-selectin could be explained in non-pathological state.

It is important also to mention that the expression of a gene in one cell type does not reflect the total concentration in plasma. Therefore, the associations between VEGF, L-selectin and TNF- α mRNA levels are not expected to be necessarily present in plasma. Our transcriptomic approach showed the utility of this epidemiological model in identifying unsuspected until now relationships of these molecules in healthy conditions.

Furthermore, very interestingly, only VEGF₁₄₅ isoform mRNA showed associations with the CAM/inflammation molecules expression. VEGF₁₄₅ binds to heparin but behaves like VEGF₁₂₁ with regard to its receptor recognition ability. It also has angiogenic effects similar to VEGF₁₂₁ and VEGF₁₆₅. Also, among VEGF isoforms, VEGF₁₄₅ is predominantly expressed in reproductive tissues and in several tumor cell lines derived from female reproductive organs as well as in PBMCs of acute myeloid leukemia patients [52,53]. These findings highlight the importance of VEGF₁₄₅ in both vascular and malignant diseases and in combination with our results in healthy population, may give new directions for further research concerning the implication of this isoform in the development of cancer pathologies.

Finally, as we have previously identified four SNPs that explain a large percentage of VEGF plasma levels heritability in healthy individuals, we sought to examine whether these SNPs could have an effect on CAM/inflammation traits and whether they could be associated with their gene expression in PBMCs. Significant epistatic interactions were revealed. In particular, an interaction between rs6993770 (TA genotype and T allele) and rs10738760 (G allele) was associated with increased levels of ICAM-1 and E-selectin. This result further supports the associations of VEGF with these molecules (especially ICAM-1) in plasma and expression levels. We also demonstrated multiple epistatic interactions between the VEGF-related SNPs and IL-6 plasma levels. Specifically, the T allele of rs6993770 interacts with the C allele of rs4416670 and the A allele of rs6921438 to increase IL-6 levels.

These two latter SNPs show a similar positive interaction with rs10738760. A complex but common genetic regulation between VEGF and IL-6 is thus speculated. However, as no association was found for IL-6 and VEGF in plasma concentrations and in expression levels, the effect of SNPs on IL-6 is likely to be independent from VEGF. Moreover, a significant interaction was detected between rs4416670 and rs6921438 with a positive effect on TNF- α plasma levels.

It should be mentioned that these SNPs have not been previously associated with any of the CAM/inflammation assessed traits. Therefore, these interactions may indicate novel genetic markers for these molecules. However, replication of these findings is needed before the establishment of a conclusion.

Finally, we examined whether these genetic polymorphisms had a functional effect on the traits in terms of gene expression modification. A positive association was identified only for the C allele of rs4416670 with L-selectin mRNA. Nevertheless, this SNP was not associated with L-selectin levels; therefore a direct effect of the SNP cannot be speculated. rs4416670 could have a functionality in L-selectin gene expression modification, indirectly, possibly through a transcriptional factor or a more complex mechanism. As VEGF₁₄₅ mRNA was positively associated with L-selectin expression, this mechanism could be the common link between the two molecules and rs4416670.

The multi-dimensional approach used in this study can be considered as a strong point of this work in the assessment, validation and explanation of the relations between VEGF and adhesion/inflammation molecules in healthy individuals, thus giving new perspectives and hypotheses for further studies in order to identify the exact molecular mechanisms of VEGF implication in the development of specific diseases.

In conclusion, we have addressed in this study for the first time the associations of VEGF with adhesion molecules and a number of inflammation cytokines in a healthy population. We have found significant associations of VEGF with ICAM-1 and E-selectin in plasma concentrations and common expression profiles for VEGF and ICAM-1, L-selectin, and TNF- α in PBMCs. Finally, epistatic interactions between VEGF-related SNPs were shown to affect the levels of ICAM-1, E-selectin, IL-6 and TNF- α and the expression of L-selectin. These findings support the existence of complex relationships between angiogenic, adhesion and inflammation molecules that exist even in non-pathological situations, which should be taken into account in future studies concerning the implication of VEGF in related pathologies. Their understanding may promote the knowledge of the molecular mechanisms and the understanding of the processes that mediate complex pathologies such as cardiovascular diseases and cancer.

Disclosures

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cyt.2012.10.024>.

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